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Phytochemicals in Human Health

Edited by Venketeshwer Rao, Dennis Mans and Leticia Rao

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Meet the editors

Dr. Venketeshwer Rao, Professor Emeritus, Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, has established a major focus in the area of diet and health. His research focuses on the role of phytochemicals in the prevention and management of human diseases including cancer, cardiovascular disease, and osteoporosis. The main area of his research is oxidative stress and antioxidant phytochemicals with particular emphasis on the role of lycopene. He is credited for bringing international awareness to the role of lycopene in human health. In addition to carotenoids, his research interests also include plant polyphenols and the role of prebiotics and probiotics in human health. He has published extensively including research papers, reviews, and books. He has a distinguished academic career spanning over 52 years. He is popularly sought by the international media to express his opinions on the subjects of nutrition and health.

Dennis R.A. Mans (October 16, 1955, Paramaribo, Suriname) graduated as a Medical Biologist in 1984 at the Utrecht University, The Netherlands. He received his PhD diploma in 1991 from the Free University of Amsterdam, The Netherlands, with a thesis on the antineoplastic agent etoposide. Between 1992 and 2000, he worked in Brazil on plants with anticancer properties. He returned to Suriname in 2000 to hold an academic position at the Anton de Kom University. In 2001, he became Head of the Department of Pharmacology, in 2006 he was appointed Associate Professor, and in 2008 Full Professor with a chair in Pharmacognosy. His scientific production amounts to more than 100 full papers and more than 200 scientific abstracts.

Dr. Leticia Rao is an Emerita Associate Professor of Medicine and former Director of the Calcium Research Laboratory, Department of Medicine, and St Michael's Hospital, Toronto, Ontario. Her degrees include BSc Chemistry, University of the Philippines; MSc Food Science, Oregon State University; and Ph.D. Biochemistry, University of Toronto. Her area of expertise is bone cell biology and osteoporosis with an emphasis on effects of drugs and nutritional supplements. She has studied the effect of gravity on bone formation in osteoblast cells in an experiment sent to space with the Space Shuttle Columbia STS 107. Her research has been presented to national and international symposia and published in peer-reviewed scientific journals. She co-authored a book entitled "Bone Building Solution" and co-edited a book on phytochemicals.

Contents

Preface

In recent years there has been a great deal of interest in naturally occurring bioactive compounds referred to as 'Phytochemicals' and their role in the prevention, management, and treatment of many human health disorders. This heightened interest in phytochemicals can be attributed to the global dietary recommendations to increase the consumption of plant-based foods, which are the main source of phytochemicals in our diet. The consumption of phytochemicals is now looked upon as being an important complementary approach to the use of traditional pharmaceutical compounds in the treatment and management of human diseases. Phytochemicals, also referred to as 'Phytonutrients', vary significantly in terms of their occurrence, chemistry, and mode of action. They must first be isolated, purified, and their physico-chemical properties established. Once identified, they can be studied for their mechanisms of action leading to the evaluation of their biological properties and their role in human health using in vitro, animal, and human clinical studies. Recognizing the importance of these compounds in humans, we undertook to edit and publish a book with chapters authored by internationally recognized scientists who are experts in their respective fields of research in this important area of the role of phytochemicals in human health. The chapters of the book include original research articles as well as up-to-date reviews. The book is intended to benefit researchers, health professionals, industrial scientists as well as government regulatory agencies. It is our hope that with a better understanding and knowledge of phytochemicals, we will be able to formulate guidelines as to their safety and their use in the management of human diseases and improvement of the quality of life.

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Section 1

Phytochemical Composition

Chapter 1

Anthocyanins and Proanthocyanidins in Natural Pigmented Rice and Their Bioactivities

Pornngarm Limtrakul (Dejkriengkraikul), Warathit Semmarath and Sariya Mapoung

Abstract

Natural pigmented rice is mainly black, red, and dark purple and contains a variety of flavones, tannins, phenolic, sterols, oryzanols, and essential oils. Anthocyanins and proanthocyanidins belonging to plant flavonoids are thought of as the major functional components found in black, red, and purple rice and contribute to the intense color of many fruits, vegetables, and pigmented cereals such as blueberries, grapes, red cabbages, and purple sweet potatoes. Recent data have indicated the potential for isolating and characterizing the nutrition and non-nutritive components in colored fruits, vegetables, and cereals for their potential chemopreventive and pharmaceutical agents. This chapter provides up-to-date coverage of pigmented rice in terms of the bioactive constituents, isolation, extraction and analytical methods, and related bioactivities. Special focus has been placed on the anti-inflammation, anticancer, and antiaging processes of the major components found in pigmented rice, especially with regard to germ and bran extracts.

Keywords: anthocyanins, proanthocyanidins, pigmented rice, nonpigmented rice, bioactivities of black rice, bioactivities of red rice

1. Introduction

Although white rice is consumed as a major staple food worldwide, quite a few countries in Southeast Asia (SEA) also consume pigmented cultivars such as red, black, purple, and brown rice. Rice cultivars that originated in Southeast Asia (SEA) have been classified in the species of *Oryza sativa* L., which differs from the *Oryza glaberrima* Steud. species that is cultivated in West Africa. In Thailand, the total area of cultivation has been recorded at 56.3 million Rai (22.3 million acres) with the majority being comprised of white rice cultivars (90%), while pigmented rice is only 0.1% or makes up approximately 62,000 Rai (24,506 acres) [1]. The largest cultivated area is located in the northeast of Thailand (63.10%) followed by the northern region of Thailand (21.93%), the central area (14.5%), and the south (0.47%). India and Indonesia have more cultivated area of pigmented rice than any other SEA countries, although they report a smaller proportion than that of the white rice cultivar. The total cultivated area in India has been recorded at

43.77 million acers (29.4% of the global rice area) with a production of 90 million tons [2]. The world production of rice is estimated at around 680 million tons, which is equivalent to that of wheat [3]. The color intensities of pigmented rice are obtained from the value of lightness, redness, and yellowness and seem to be correlated to the indicators of its bioactive compounds [4–6].

Recently, pigmented rice varieties have received increased amounts of attention from consumers for their high bioactive compounds that present potential nutraceutical benefits to health. It is also well known that these compounds are primarily located in the outer layer of the rice grain, which is regarded as a rice by-product. The by-products of rice processing are rice germ and bran, along with the rice hulls which protect the rice seeds during growth. These account for 20% of the rice crop. These by-products are frequently used as animal feed in developing countries. However, recently, significant amounts of data have revealed the beneficial nutritional impacts of these by-products on human health. The major bioactive compounds that are found in red, black, purple, and brown rice include gallic, protocatechuic, hydroxybenzoic, and vanillic acid, cyanidin 3-O-glucoside, peonidin-3-O-glucoside, proanthocyanidin, flavanol, catechin and epicatechin, carotenoids, and γ-oryzanol content. Several research findings have reported on the biological modulating effects of pigmented rice seeds and bran phytochemicals, including anti-inflammatory activities [7, 8], anticancer activities that have suppressed tumor growth in mice and several human cancer cell lines [9–13], the anti-metastasis properties of cancer cell invasion [14–16], antiaging effects with the reduction of oxidative stress in both in vitro and in vivo models [17, 18], the modulation of serum lipid profiles and the enhancement of mRNA expression levels of fatty acid metabolism-related genes [19], a reduction of platelet hyperactivity and hypertriglyceridemia in dyslipidemic rats [20], and skin antiaging treatments [21–24].

In this current review, we have focused on the health benefits of pigmented rice and the relevant bioactive compounds. We have tried to present the information in this chapter in a way that is easy to understand, even for readers who are not experts in this field of research. The bioactive compounds found in pigmented rice display significant immersion potential with regard to a range of beneficial health effects and also provide significant informative data that could lead to the expansion in the growing of pigmented cultivated areas in Thailand and other Southeast Asian countries. There is also the prospect of additional practical implications, not only for agriculture expansion but in the food industry as well. Several pigmented rice varieties have been used to create new nutraceuticals, and these seem to hold a promise in terms of potential cosmeceutical utilization in the new global business era.

2. Pigmented rice and bioactive compounds

The rice processing industry is well-developed and produces a number of products from rice kernels or grains (70%) along with a large quantity of rice by-products. These by-products include rice bran (8–9%), rice germ (1–2%), and rice husks (20%). Figures on rice paddy composition are presented in **Figure 1**. These by-products are frequently used as animal feed in developing countries [25], but the demand for these by-products in terms of their human nutritional impacts has increased due to their potential health benefits. Rice kernels are primarily a good source for the energy intake of carbohydrates and proteins in humans. Rice bran makes up the outer layer of the rice kernel and is mainly comprised of a pericarp,

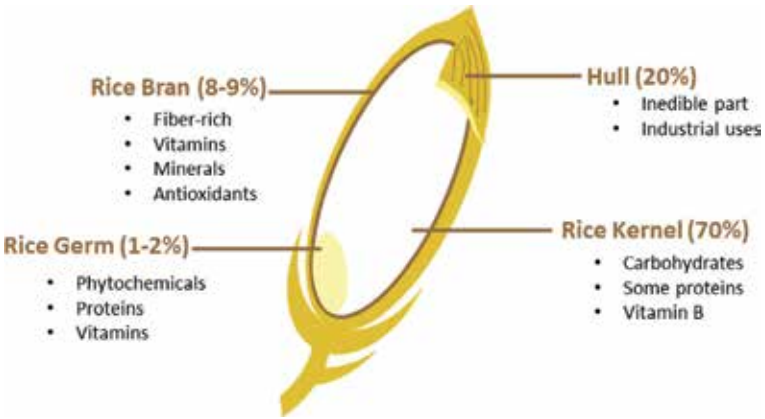

Figure 1.
Rice paddy composition.

aleurone, sub-aleurone layer, and germ. Rice bran and germ contain appreciable quantities of fiber, vitamins, minerals, unsaturated fatty acids, tocopherols, γ-oryzanol, and tocotrienols, which offer potent antioxidant content along with a range of other potential health benefits [26, 27].

2.1 Extraction methods for bioactive compounds in pigmented rice

Several common extraction techniques that are used in the process of rice extraction include the method of solvent extraction, which is a conventional technique used to extract bioactive compounds from pigmented rice, supercritical fluid extraction, and subcritical water extraction. With regard to the conventional technique, a number of organic solvents are commonly used such as acetone, methanol, ethanol, butanol, and water in certain proportions as the extraction solvent [28–30]. In our study, 50% ethanol was used as an extraction solvent at a proportion of 1:5 grain or bran liquid, and extraction was carried out at room temperature for 3–12 h. The extracts were then concentrated with a rotary evaporator until all ethanol residues were removed and then further partitioned against saturated butanol to obtain the medium polar bioactive compounds of the black rice extract [31] or red rice extract [7, 15, 16]. The bioactive compounds present in these fractions shall be described in a later section. In another study, 60% ethanol containing 0.1% HCL was used as an extraction solvent with a 1:10 feed to liquid proportion, and extraction was carried out for 3–12 h. The extracts were then concentrated and further partitioned against petroleum ether [8]. In another study, the rice bran was extracted with 70% ethanol for 30 min repeated three times and was then further partitioned with ethyl acetate at pH 2–3 [32]. The same method was used to extract soluble phenolic compounds in white rice, brown rice, and germinated brown rice [33].

Supercritical fluid extraction has been widely used for the extraction of functional active compounds from medicinal plants including rice and cereals. This was in common with the use of supercritical carbon dioxide as an extraction solvent in other successful experiments. Kim et al. [34] used the method of supercritical fluid extraction of rice bran oil from pigmented rice, which provided higher yields of polyunsaturated fatty acids than the conventional use of organic solvent extraction. In yet another study, supercritical carbon dioxide extraction was used, and yields of 17.5% oil were achieved from powdered rice bran, and a yield of 37% of γ-oryzanols was also obtained, which was characterized as 85% of the extraction efficiency [35].

Another extraction technique is the subcritical water extraction method that has been developed for the extraction of bioactive compounds from pigmented rice through the use of hot water at temperatures between 100 and 374°C under high pressure to maintain a liquid status. This technique is considered to be very friendly to the environment because no organic solvents are used, and this can potentially alleviate some of the problems associated with the conventional methods [36, 37].

There were differences in the extraction procedure and the varieties of the rice cultivars that were used to detect the amounts of bioactive compounds in different portions of rice such as in the whole grains, kernels, endosperm, husks, rice, and bran. More than 1000 published studies have been reviewed to make up the cited data based on this information. Some data on rice composition have been selectively recorded elsewhere [27].

2.2 Various bioactive compounds present in black rice

Phytochemical profiles of black rice are characterized by the presence of anthocyanins, which are a group of reddish to purple flavonoids that exist in black rice and other pigmented cereal grains. The main anthocyanins in black rice were found to be present in quantities more than 95% and were cyanidin 3-O-glucoside (2.8 mg/g) and peonidin-3-O-glucoside (0.5 mg/g) followed by flavones and flavonols (0.5 mg/g) and flavan-3-ols (0.3 mg/g) [38]. The concentrations of total anthocyanins in black rice cultivars significantly varied from one report to another, while much higher concentrations of anthocyanins were detected in Chinese black-purple rice that contained cyanidin 3-O-glucoside (6.3 mg/g) and peonidin 3-O-glucoside (3.6 mg/g) [39]. The variations of the anthocyanin content in the reports on black rice might be due to the use of different cultivars and the variety of differing growing conditions. The anthocyanidins or aglycons, the basic structure of anthocyanins, consist of an aromatic ring (A) that is bonded to a heterocyclic ring (C) that contains oxygen, which is bonded by a carbon–carbon bond to a third aromatic ring (B). When the anthocyanidins are bonded to a sugar moiety in the glycosidic linkage, they are known as anthocyanins. More than 500 different anthocyanins and 23 anthocyanidins have been reported. Anthocyanins exist as mono-, di-, or tri-O-linked glycosides and acyl glycosides of anthocyanidins in plants. The sugar moiety may be substituted by aliphatic, hydroxybenzoic, or hydroxycinnamic acids. The structural characteristics of anthocyanins make them highly reactive toward the reactive oxygen species (ROS) [27]. The basic structure of this is shown in **Figure 2**. Major flavone and flavonol glycosides present in black rice are taxifolin, quercetin, apigenin, and luteolin, which are comprised of monomeric and oligomeric constituents. The concentrations of the flavone and flavonol contents were

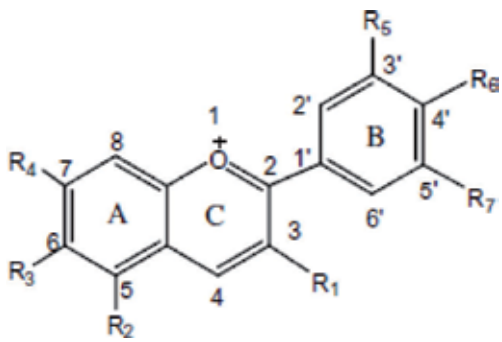

Figure 2.
General structure of anthocyanins.

significantly higher in black rice than in red, brown, or white rice. This was especially true with regard to taxifolin O-hexoside, quercetin 3-O-glucoside, and quercetin 3-O-rutinoside, which were detected only in black rice [38]. Abdel-Aal et al. [40] also reported that the mean anthocyanin content in black rice (3.276 mg/g) was about 35-fold higher than that of red rice (0.094 mg/g). Additionally, the contents of anthocyanin present in Northern Thai black rice cultivar obtained from Doi Saket, Chiang Mai, were 8.1 mg/g extract, which was considered very high when compared to the anthocyanin content found to be present in the Northern Thai red rice cultivar obtained from Dok Khamtai [31].

The total procyanidin content in black rice has been found to be present in high variations depending on the grain cultivar; however, it is noteworthy to mention that procyanidins are typically observed in red rice but not in black rice varieties [41–45]. Interestingly, some black cultivars have shown the presence of oligomeric procyanidins with a 2–10 degree of polymerization [38]. Furthermore, black and red rice were found to contain only one flavan-3-ol monomer, catechin. Additionally, a Canadian black rice variety also contained catechins at levels four times higher than epicatechin. Furthermore, the concentration of catechin was much higher in red rice (92 μg/g) than in black rice (20 μg/g) [46]. Other phytochemicals have been detected in black rice including all four derivatives of γ-oryzanol, such as 24-methylenecycloartenol, campesterol, cycloartenol, and β-sitosterol ferulates, along with lower levels of carotenoids. The main carotenoids detected in black rice were xanthophylls, lutein, and zeaxanthin, while lycopene and β-carotene could be detected but were found to be present as a minor component [38]. The value of the carotenoid content in black rice kernels is lower than the carotenoids found to be present in black rice bran. It was reported that values in a range of 33–41 μg/g of carotenoids were found in the bran extracts of four varieties of Thai black rice [47]. A range of phenolic compounds including vanillic acid, protocatechuic acid, chlorogenic acid, ferulic acid, and coumaric acid has been detected in black rice with the dominant phenolic acids being present in red and black rice bran [7, 31]. The contents of phenolic compounds, flavonoids, catechins, anthocyanins, and proanthocyanidins, are summarized in **Table 1** as examples of the phytochemicals that were detected in Doi Saket Thai black rice cultivar. The germ and bran extracts of the black and red rice varieties were found to have the greatest phytochemical content with decreasing amounts occurring in the rice hull and even less in the seeds or kernels. Additionally, the expected low levels of these phytochemicals were found in white rice as a consequence of the milling process.

2.3 Various bioactive compounds present in red rice

Red rice was characterized by a high quantity of oligomeric procyanidins (0.2 mg/g) with more than 60% of total phytochemicals found in the rice seeds. Proanthocyanidins are high molecular weight polymers or complex flavan-3-ol polymers that consist mainly of catechin, epicatechin, gallocatechin, and epigallocatechin units that can also be found in rice germ and bran, particularly in pigmented rice. The degree of polymerization varied, and the reddish colored test was associated with the presence of a class of polymeric compounds of the proanthocyanidins. These could be in the sum class of the oligomer and polymer contents of the total proanthocyanidins present in the red rice bran extract fraction. The degree of polymerization and galloylation can affect their bioactivity and proanthocyanidin profiles differently depending on the food sources [27, 48]. Proanthocyanidins can be classified into several classes depending on the degree of hydroxylation of the constitutive units and the linkages between them. Our research group has reported on the type of proanthocyanidins found in the red rice that was collected from Dok Khamtai

Compound	(mg/g extract)
Total phenolic content	117.6 ± 14.6
Vanillic acid	4.2 ± 0.4
Protocatechuic acid	2.3 ± 0.1
Gallic acid	ND
Coumaric acid	0.5 ± 0.2
Ferulic acid	1.4 ± 0.0
Chlorogenic acid	1.7 ± 0.3
Total flavonoid content	42.9 ± 2.1
Anthocyanin	8.1 ± 1.9
Catechin	ND
Proanthocyanidin	ND

Values are mean ± S.D., ND = not detectable.

Table 1.
Phytochemical content of black rice extract (polar fraction).

cultivar, Northern Thailand, as a type B proanthocyanidin. The monomeric units of proanthocyanidin in the acid hydrolysis of the red rice extract fraction were found to be catechins, epicatechins, gallocatechins, and epigallocatechins [16]. The results revealed that the proanthocyanidin types were procyanidin (catechin and/or epicatechin) and prodelphinidin (gallocatechin and/or epigallocatechin), while the degree of polymerization was recorded at approximately 4. Interestingly, the majority of proanthocyanidins in our red rice extract were of the oligomer with the same degree of polymerization that was found in grape seed extracts [49]. As has been mentioned previously, red rice has a high content of catechins and proanthocyanidins, but some of the black rice cultivars found in France and Canada have revealed the presence of catechins in their black rice cultivars (four times less than the red rice cultivars). It is worth mentioning that many other records have shown that procyanidins have been typically observed in red but not black rice varieties, including in the Northern Thai black rice cultivar obtained from Doi Saket, Chiang Mai [31]. The general structure of proanthocyanidins is shown in (**Figure 3**).

The other active compounds were γ-oryzanol and carotenoids at 27%, whereas flavones, flavonols, and anthocyanins were present in a much less quantity at less than 9% [38]. The main carotenoid detected in red rice bran was lutein, while xanthophylls and zeaxanthin were the carotenoids that were found to be present in lesser quantities. A range of phenolic acids including gallic, protocatechuic, hydroxybenzoic, vanillic, and ferulic acids in red, black, and brown rice have been detected as the dominant phenolic acids present in red and black rice bran [50, 51]. The contents of the phenolic compounds, flavonoids, catechins, anthocyanins, and proanthocyanidins, are summarized in **Table 2** as an example of the phytochemicals that were detected in Dok Khamtai Thai red jasmine rice cultivar. The contents of these bioactive compounds can be used to determine the antioxidant activities that may then provide health benefits.

2.4 Various bioactive compounds present in brown and white rice

The rice bran of whole grain brown rice (unpolished) has been acknowledged as a potential source of edible oil. Although rice bran oil is not very popular worldwide, its demand is increasing due to numerous reports on its health benefits.

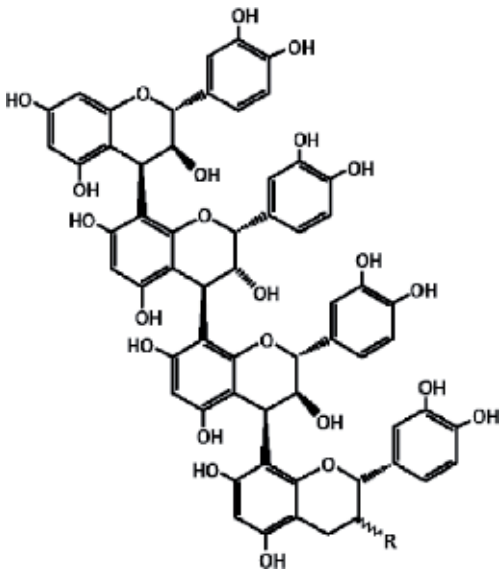

Figure 3.
General structure of proanthocyanidins [16].

Previously, rice bran obtained from brown rice has received a significant amount of attention from the nutraceutical industry as brown rice bran is recognized as the primary source of oil extraction. On this issue, agro-industrial by-products are gaining special attention from the food processing industry because rice bran oil presents a positive fatty acid profile along with the presence of other phytochemicals like ϒ-oryzanol, tocopherols, and tocotrienols. Basically, rice bran is rich in carbohydrates (34–62%), lipids (15–20%), proteins (11–15%), and dietary crude fiber (7–11%) [52]. The health benefits of rice bran include the strong antioxidant potential of rice bran oil. This is not only a consequence of the presence of significant quantities of linolenic acid (34%), oleic acid (38.4%), and other unsaturated

Compounds	**(mg/g extract)**
Total phenolic content	237.78 ± 17.26
Vanillic acid	1.53 ± 0.19
Protocatechuic acid	0.35 ± 0.03
Gallic acid	ND
Coumaric acid	0.2 ± 0.01
Ferulic acid	0.56 ± 0.04
Chlorogenic acid	ND
ϒ-Tocotrienol	ND
ϒ-Oryzanol	1.75 ± 0.23
Anthocyanin	ND
Catechin	6.65 ± 0.57
Proanthocyanidin	53.45 ± 3.23

Values are mean ± S.D., ND = not detectable.

Table 2.
Phytochemical content of red rice extract (polar fraction).

fatty acids but also occurs as a result of the high contents of γ-oryzanol, tocopherols, and tocotrienols that reveal strong oxidative stability along with a range of other health benefits [53, 54].

The protein content present in rice bran of brown rice is characterized as a good source of protein that is nutritionally superior and hypoallergenic in nature. Rice bran is a rich source of essential amino acids such as lysine, which seems to be present in minute quantities in other cereal grains [55, 56]. The proteins in rice bran are highly digestible and can be utilized as an effective food ingredient. Rice bran is rich in dietary fiber, and, consequently, the rice bran by-products of rice processing are now often present in food commodities and functional foods that have been marketed for the ability to add dietary fiber to the diets of consumers and to offer health benefits in terms of daily nutrition. Additionally, brown rice possesses high contents of a variety of nutrients, such as fiber, vitamins, and minerals that are lost during the process of refining and milling in the production of white rice within the rice agro-industry. Notably, brown rice possesses four times as much dietary fiber as white rice [57].

White rice is a major source of energy nourishment for the world's population, especially in Asian countries. However, the carbohydrate content in white rice accounts for 80% of its makeup, which is considered a higher amount than wheat. Wheat is a popular grain among European countries and contains a lessor proportion of carbohydrates (approximately 50–70%) [58]. For this reason, there are concerns that white rice possesses a high glycemic content and that it may not be a suitable source of carbohydrates for people who have weight problems. It is interesting to note that white rice does not contain anthocyanins and proanthocyanidins, which are the important phytochemicals that are found in black rice and red rice, respectively, particularly in portions of rice germ and bran extracts. While total flavonols and phenolic compounds are observed to be significantly high in pigmented rice, nonpigmented rice such as white rice possesses a minute quantity of flavone/flavanol content [50].

3. Pigmented rice and bioactivities that benefit health

Phytochemicals found in pigmented rice (brown, black, purple, and red rice) are not present in white rice because many valuable phytochemicals, fiber, vitamins, and nutrients are lost during the processes of refining and milling [57]. Since brown rice contains higher dietary fiber and nutrients, previous studies have revealed that when compared to a white rice diet, a brown rice diet was found to significantly reduce weight, body mass index (BMI), and the circumference of the waist and hips, as well as to lower diastole blood pressure and inflammatory biomarkers such as C-reactive proteins (CRP). Arabinoxylan and β-glucan, prebiotics that are found in brown rice, are beneficial for human gut microbiota such as *Bifidobacterium* and *Lactobacillus*. They are considered as contributing factors in producing an anti-obesity effect [57, 59]. Moreover, in terms of their antidiabetic effects, brown rice was used as an intervention for preventing type 2 diabetes. This is likely because one of their components, ϒ-oryzanol, plays an important role in controlling high-fat diet-induced ER stress in the hypothalamus, which helps in reducing the preference for fatty foods [60]. ϒ-Oryzanol in brown rice has also been found to prevent the apoptosis of pancreatic β cells and to reduce levels of blood cholesterol [61]. Dietary rice brans that give brown rice its brown color also reveal potent anticancer activities through their antioxidant activity, as well as offering antiproliferation, immune modulation, and mucosal protection [62, 63].

Natural pigmented rice, such as black and red rice, may even offer more health benefits than brown rice. Not only is natural pigmented rice higher in the beneficial antioxidant activities of black and red rice, but it also displays strong anti-inflammatory activities as well as anticancer and anti-metastasis activities. The antiaging properties of the major components found in pigmented rice may be anthocyanins and proanthocyanidins, which have been found to be especially rich in content in the germ and bran extracts of black and red rice, respectively. The details of which will be described in greater detail in the following section.

3.1 Antioxidant activities

The antioxidant activities of black and red rice and their crude extracts have been studied, and the results demonstrated that the addition of the pigmented rice could increase antioxidant capacity, both in vivo and in vitro [64–66]. In a study involving the supplementation of diets with black rice pigment fractions, the diets that attenuated atherosclerotic plaque formation in apolipoprotein E-deficient mice [66] and the anthocyanin-rich extract of the black rice might play an important role in the enhancement of atherosclerotic plaque stabilization [8]. In another study, a mixture of brown and black rice improved the lipid profiles and antioxidant status in rats [67]. Another animal study also demonstrated that black rice bran pigment effectively escalated hepatic antioxidant enzyme activities including superoxide dismutase and glutathione peroxidase in high-cholesterol-fed rats [68]. In addition to the in vivo studies, in a cell culture experiment, superoxide anions and reactive oxygen species were significantly suppressed after black rice extract exposure in HepG2 hepatocellular carcinoma [17]. When the antioxidant activities of pigmented rice were compared with those of nonpigmented rice in several studies [30, 41], the results demonstrated that the extracts from pigmented rice displayed higher antioxidant activity than did the nonpigmented rice. In another study, the radical scavenging activities of the extracts from white, black, and red rice were tested. The highest activity was observed in red rice (2.77 μmol of Trolox or vitamin E equivalents/ml), followed by black (0.92 μmol) and white (0.26 μmol) [41, 42]. Polymeric proanthocyanidins play an important role as radical-scavenging components in red rice. The relationships between the antioxidant activities and the components of pigmented rice were explored [41, 69, 70]. The antioxidant activities correlated well with the content of polyphenols and phytochemicals that contribute to the intense color of the pigmented rice. Interestingly, some studies have shown that the antioxidant activity of black rice may be reduced by up to 53% during cooking [71–73].

3.2 Anti-inflammatory properties

Inflammation is an important mechanism of immune pathogenesis, which is our body's response to tissue injury, infection, and stress. Importantly, the prolonged production of inflammatory mediators by macrophage can cause damage to the host and can contribute to the pathology of many diseases including inflamm-aging, arthritis, asthma, cancer, diabetes, and atherosclerosis. Macrophage plays a key role in response to an immediate defensive mechanism of our body against attacking foreign agents, especially with a microbial lipopolysaccharide (LPS) [74]. Macrophage is activated and produces many kinds of inflammatory mediators including nitric oxide (NO), prostaglandins, and many cytokines such as interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor (TNF)-α [75]. Many researchers have studied in vitro and in vivo models to elucidate that natural products are able to ameliorate the inflammatory response in LPS-stimulated macrophage.

During the last decade, it has been shown that anthocyanins reduce the risks of cardiovascular diseases and cancers with inflammatory, antioxidant, and chemoprotective properties [15, 76, 77]. Some reports have demonstrated that lipophilic phytochemicals contained in pigmented rice germ and bran, such as γ-oryzanol and vitamin E derivatives, exert anti-inflammatory activities [78, 79]. On the other hand, pigmented rice contains high amounts of medium polar or hydrophilic compounds such as phenolics, bioflavonoids, anthocyanin, and proanthocyanidins that have been reported for their anti-inflammatory properties, in both in vitro and in vivo models [80–82].

Pigmented rice contains a variety of bioactive compounds with anti-inflammatory properties; however, there have been quite a few reports employing experimental designs that provide direct evidence to support using the extracts of pigmented rice. For the first time, our research group has demonstrated the molecular mechanisms underlying the anti-inflammatory effects. The anthocyanin-rich fraction of black rice extract significantly inhibited LPS that induced many pro-inflammatory mediators in RAW 264.7 macrophage white blood cells [31]. The pro-inflammatory mediators in this study were NO, TNF-α, and IL-6, and they effectively reduced the expression of two important inflammatory enzymes, the inducible NO synthase (iNOS) and the inducible cyclooxygenase-2 (COX-2). These results were regulated by an inhibition of the mitogen-activated protein kinase signaling pathway (MAPK pathway), leading to a decreased nuclear translocation of NF-κB and AP-1, two major transcription factors involved in the inflammation process. In testing the anti-inflammatory properties of anthocyanin and hydroxybenzoic acid, the major components were detected in the black rice extracts based on our extraction protocol, and similar results were obtained. A schematic diagram of the proposed mechanism of the anti-inflammatory properties of black rice anthocyanin is presented in **Figure 4**. In a study on cyanidin-3-glucoside and protocatechuic acid, no beneficial effects were found against inflammation induced by LPS [73]. Therefore, the anti-inflammatory properties of black rice might require the synergistic action of many phytochemicals, which are rich in anthocyanin and other phenolic compounds that play a role in this process. Interestingly, the same study has demonstrated that the cooking process did not alter the anti-inflammatory potential of black rice. In another study, other researchers reported that cyanidin-3-glucoside displays anti-inflammatory effects [8]. Our group also conducted a study on the anti-inflammatory effects of proanthocyanidin-rich red rice extract via the suppression of the MAPK, AP-1, and NF-κB pathways in RAW 264.7 macrophages that induced inflammation by LPS [7]. It was found that the red rice medium polar fraction that was enriched with polyphenols and proanthocyanidins exerted potent anti-inflammatory activities by inhibiting the production of TNF-α, IL-6, and NO in LPS-activated macrophage, whereas the red rice nonpolar fractions displayed no anti-inflammatory properties. All of the above results indicate that black rice that is rich in anthocyanins and red rice that is rich in proanthocyanidins exhibit therapeutic potential for the treatment of inflammatory diseases.

3.3 Anticancer properties

Cancer is one of the leading causes of morbidity and mortality worldwide. Notably, only 10% at the most of all cancers are due to genetic factors, while 90% are directly or indirectly correlated with an individual's lifestyle and dietary habits [83]. Many scientific reports have shown that a healthy lifestyle, including a diet rich in natural products, such as herbs, cereals, fruits, and vegetables, can help reduce the risk of cancer [84, 85]. Some of the phytochemicals found in these natural products

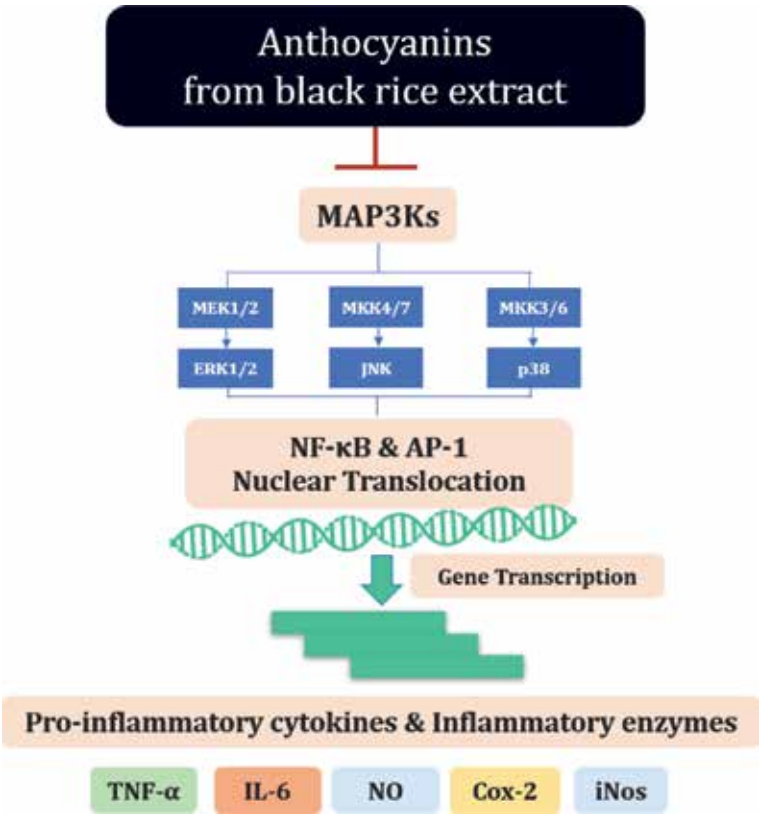

Figure 4.
Schematic diagram of anti-inflammatory properties of black rice anthocyanin.

are secondary metabolites, including phenolic compounds, bioflavonoids, terpenoids, and alkaloids. In this chapter we shall focus more on the presence of phenolic compounds and flavonoids, including anthocyanins and proanthocyanidins, as the major compounds found in pigmented rice, especially in rice germ and bran.

Active components of pigmented rice bran have demonstrated anticancer properties in in vitro cancer cell models, including those involving leukemia, colon, breast, liver, and stomach cancer cells. In a study on the anticancer potential of rice bran against the proliferation of leukemic cell lines, the antioxidant activities of the active compounds found in rice bran were noted for this beneficial effect [10]. Another investigation on the tumor suppression activities of rice bran from different pigmented and nonpigmented rice varieties reported that 70% ethanolic extract of the pigmented rice bran inhibited phorbol ester-induced tumor promotion in a better manner when compared to the nonpigmented rice bran variety [11]. In yet another study, the growth inhibitory effect of rice bran polyphenols, mainly γ-oryzanol and its derivatives, has been reported in human colorectal adenocarcinoma [86]. The anticancer activity of rice bran could be varied considerably in different rice cultivars or varieties in accordance with the different chemical profiles of the active compounds. In addition, the second study had analyzed seven varieties of rice bran for their growth inhibition potential against human colorectal cancer cells and reported on variations in the degree of growth inhibition depending upon the rice bran variety [9]. Some evidences have indicated that cyanidin-3-glucoside and peonidin-3-glucoside obtained from black rice anthocyanin can be combined with doxorubicin to inhibit cancer cell growth, while both anthocyanin compounds could inhibit cancer invasion into other tissues through the downregulation of

the degradative enzymes MMP-2 and MMP-9 [14]. Interestingly, Chen et al. [87] compared the relationship of the bioactive compounds with the growth inhibitory effects of pigmented rice bran extracts. The results revealed that the light brown bran had no effect, the purple bran exhibited a minor effect on leukemia and cervical cancer cells, and the red bran exhibited strong inhibitory effects on leukemic, cervical, and stomach cancer cells. High concentrations of protocatechuic acid and anthocyanins in purple bran and proanthocyanidins in red rice bran have been singled out for their growth inhibitory effects against human cancer cells.

Many studies on anticancer properties have been reported in Thai rice cultivars. In an important study, Kum Phayao black rice cultivar was found to be highly cytotoxic to human HepG2 cells when compared with other Northern Thai purple rice cultivars [12]. In yet another study, the alcoholic extracts of black-purple rice grain cultivar Kum Doi Saket demonstrated an antimutagenic activity against aflatoxin B1 in Ames tests [88]. The therapeutic potential of black rice anthocyanin for treating inflammatory diseases that are associated with cancer has been proposed for its mechanism via the inhibition of the MAPK signaling pathway [31]. A very recent study conducted by our research group revealed that the proanthocyanidin-rich fraction isolated from the red rice germ and bran of the Kum Doi Saket cultivar grown in the northern part of Thailand significantly reduced the cell viability of HepG2 cells (IC_{50} value at 20 μg/ml) [13]. The proanthocyanidin-rich fraction could inhibit cell proliferation and induce cell apoptosis by increasing the apoptotic proteins, such as cleaved PARP-1, cleaved caspase 8, and cleaved caspase-3, and decreasing the anti-apoptotic protein survivin without p53 protein changes. A schematic diagram of this mechanism is presented in **Figure 5**. In addition, our previous studies have demonstrated that red rice grain extracts with high proanthocyanidin content displayed an anti-metastasis effect on invasive human breast carcinoma cells MDA-MB231 [16] and human fibrosarcoma HT1080 cell lines [15]. In addition, proanthocyanidins in other colored plants, such as grapes and blackberries, have demonstrated anticancer, anti-inflammatory, and antioxidant activities to a similar extent as the proanthocyanidins that are found in red rice germ and bran [7–9, 13].

3.4 Anti-inflamm-aging properties

Inflamm-aging, a state of chronic, low-level systemic inflammation, is a widespread feature of human aging and a major risk factor for disabilities and mortality in aging individuals [89, 90]. Inflamm-aging is characterized by an overall increase in plasma levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, and

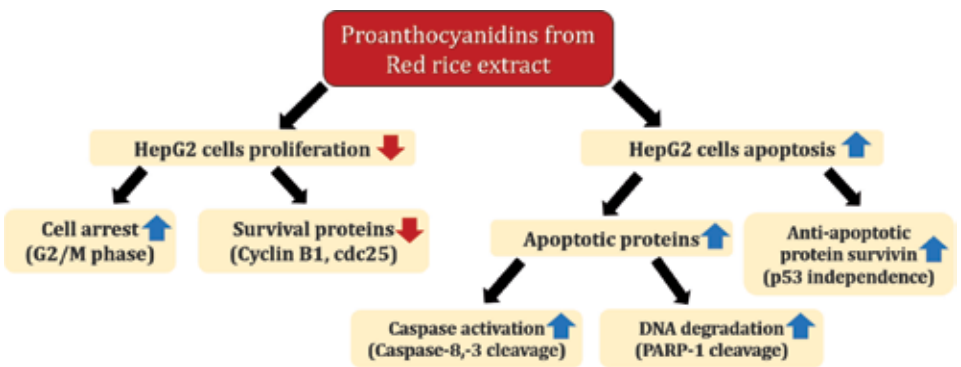

Figure 5.
Schematic diagram of anticancer properties of red rice proanthocyanidins.

subsequently can increase major inflammatory markers such as C-reactive protein (CRP) and serum amyloid A. This generalized pro-inflammatory status potentially triggers the onset of the most important age-related diseases such as cardiovascular diseases, atherosclerosis, metabolic syndrome, type 2 diabetes, obesity, neurodegeneration, sarcopenia, frailty, and cancer [91, 92].

Since the anti-inflammatory effects of phytochemical components in black rice and red rice (anthocyanins and proanthocyanidins) are able to target many inflammatory signaling pathways, such as the MAP kinase and AMP-activated protein kinase (AMPK) and mTOR pathway, the result can also decrease free radical production by their antioxidant activity, inhibiting NF-κB activation and reducing the expression of inflammatory mediators (NO, iNOS, and pro-inflammatory cytokines) [7, 31, 93, 94]. Therefore, this has made natural pigmented rice a promising candidate as an anti-inflamm-aging agent. Some relevant studies have found that a Mediterranean diet (a diet involving high consumption of vegetables, fruits, and whole grains such as pigmented rice, olive oil, and fish, but low in the intake of saturated fats and other animal fats) can modulate the multi-interconnected processes that are involved in inflammatory responses such as free radical production, NF-κB activation, and the expression of inflammatory mediators by balancing between pro- and anti-inflamm-aging activities as well as maintaining healthy gut microbiota homeostasis and epigenetic modulation of oncogenesis through specific microRNAs [95, 96].

Several studies have identified a number of actions of anthocyanins in a phytochemical diet in the context of neuroinflammation and neurodegeneration in aging individuals. It was also recently reported in an experimental model of multiple sclerosis that anthocyanins (100 mg/kg) could effectively suppress the secretion of pro-inflammatory mediators and protect cellular components against oxidative damages that were induced by demyelination [97]. Anthocyanins also protect neuronal cells from prooxidant and pro-inflammatory damage via the modulation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and the inhibition of NF-κB pathways [98]. Moreover, anthocyanins also exhibited a similar degree of anti-inflammatory effects, and these compounds suppressed the expression and secretion of pro-inflammatory mediators in macrophages by inhibiting the nuclear translocation of NF-κB [99].

Red rice extracts that contain high levels of proanthocyanidins were also found to have neuroprotective effects and anti-inflamm-aging effects that are similar to those of anthocyanins. Previous studies have found that in primary hippocampal neuronal cells that had been treated with proanthocyanidins (14 μg/ml) and exposed to LPS, the major neuroprotective effects of proanthocyanidins were involved with a reduction of NF-κB, p38, and JNK [100]. In brief, the consumption of foods rich in polyphenols has been associated with the prevention of chronic diseases. In particular, anthocyanins, proanthocyanidins that act through various mechanisms that modulate the inflammatory signaling pathways, result in a reduction of inflammation that is often seen in aging individuals. A schematic diagram of the proposed mechanism of anti-inflamm-aging properties of black rice and red rice is presented in **Figure 6**. From the aforementioned results, it has been determined that black rice and red rice with their anti-inflamm-aging properties have a therapeutic potential that would likely need to be further investigated in geriatrics and gerontology fields.

3.5 Skin anti-aging properties

Many studies have shown that bioactive compounds found in pigmented rice, such as proanthocyanidin, catechin, vanillic acid, and oryzanol, may be useful in the cosmetic and nutraceutical industries as skin antiaging agents. As mentioned

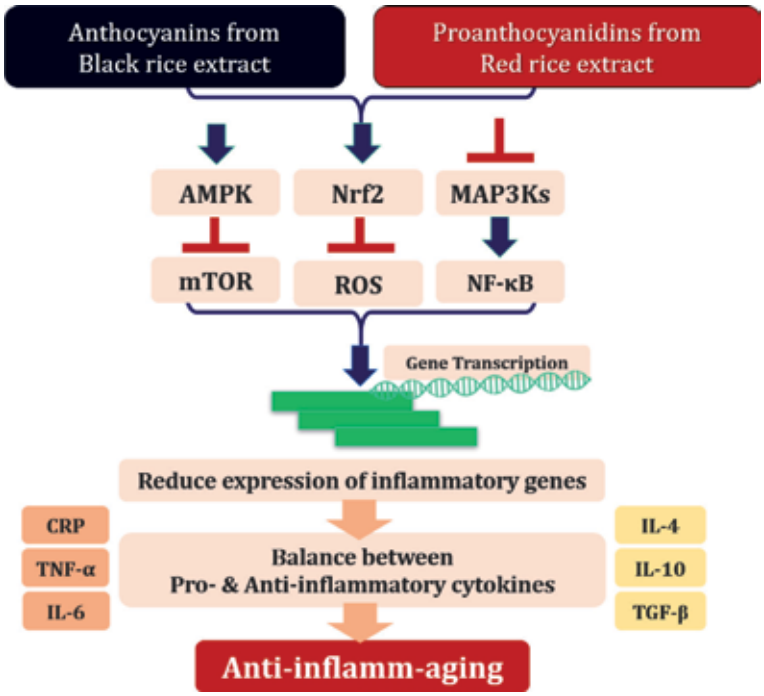

Figure 6.
Schematic diagram of anti-inflamm-aging properties of pigment rice.

in the previous section in this chapter, these bioactive compounds demonstrated antioxidant and anti-inflammatory properties. For the enhancement of the knowledge of skin antiaging properties, the bioactive compounds in the pigmented rice extract have been elucidated in a number of research laboratories. Skin aging is a process characterized by progressive physiological and structural changes in the skin. These changes could be considered as individually intrinsic and extrinsic factors, such as those associated with age, lifestyle, diet, and sunlight. Additionally, certain environmental factors can contribute to skin aging [101]. In the skin aging process, the level of degradative enzymes, such as elastase and collagenase, in skin fibroblasts are elevated, and this can lead to a loss of skin firmness and the appearance of wrinkles [102]. Mature skin in the elderly or those with sun-exposed skin can cause dark spot formations on the skin or result in the over-synthesis of melanin [103]. Hence, natural or herbal products that can exert skin benefits, including scavenging reactive oxygen species (ROS), the suppression of extracellular matrix degradation enzymes, and the inhibition of melanin synthesis, can be applied in skincare products for their beneficial skin anti-aging properties.

As pigmented rice has been reported to possess antioxidant properties, the extracts could be used for skin-anti-aging purposes. In a study by our research group, red rice extract showed anti-photoaging activity by protecting UV-induced collagen and hyaluronic acid degradation in human skin fibroblasts [21]. The red rice extract also inhibited collagenase and MMP-2 activity. In another study our group [22] has elucidated the skin antiaging properties of the main bioactive compounds in red rice extract including proanthocyanidin, catechin, hydroxybenzoic acid, vanillic acid, and oryzanol. The results showed that collagenase and MMP-2 activity were strongly inhibited by proanthocyanidin and catechin, whereas hydroxybenzoic acid, vanillic acid, and oryzanol had no effect. Both proanthocyanidin and catechin significantly induced the synthesis of collagen and hyaluronic acid, which is an important biological target for skin antiaging agents. Proanthocyanidins

and γ-oryzanol could reduce the melanin content in B16-F10 melanoma cells. Some studies have proposed the use of red rice callus or stem cells as a source of materials for replenishing the aging body in a series of experiments. The results demonstrated the efficacy of red rice callus in cosmetic products on 28 volunteer subjects aged 30–55 years and proved to promote skin lightening, hydration, and elasticity. On the other hand, a study performed involving five different varieties of Thai pigmented rice demonstrated that all rice crude extracts with 50% ethanol exhibited a weak level of activity on tyrosinase inhibition [23]. This result is similar to our findings which demonstrated that proanthocyanidin and oryzanol could reduce melanin content but had no effect on mushroom tyrosinase activity [22]. However, our results have produced experimental data to support that proanthocyanidin decreased cellular tyrosinase activity leading to a decrease in melanin content. As has been mentioned previously, proanthocyanidin is highly present in red rice germ and bran and is very similar in chemical structure to the oligomers of catechin and epicatechin that are found in grape seeds and red wine. It is noteworthy to cite the findings of a study that found that the oral administration of grape seed extract was effective in lightening UV-induced pigmentation of guinea pig skin by a reduction in the number of 3,4-dihydroxyphenylalanine (DOPA)-positive melanocytes, Ki-67 positive, proliferating cell nuclear antigen (PCNA)-positive melanin-containing cells in the basal epidermal layer of the UV-irradiated skin in grape seed extract-fed guinea pigs. In addition, this study has demonstrated that grape seed extract effectively inhibited mushroom tyrosinase activity and inhibited melanogenesis without inhibiting the growth of culture B16-F10 mouse melanoma cells.

4. Conclusion

In this chapter, the by-products of rice processing, such as germ and bran, contain a wide range of biologically active compounds that can be recovered and used in a variety of approaches in nutraceuticals. This is in correlation with an increasingly deeper understanding of the predominant bioactive compounds found in pigmented rice, particularly anthocyanin and proanthocyanidin found in black and red rice, respectively. The dietary intervention and other high-value applications in functional food and cosmetic products have been attracting ever-growing attention in recent decades. The need for scientific evidence of pigmented rice bioactive compounds in different cultivars is encouraging for future perspectives within the new global business era of nutraceutical and agriculture expansion.

Most of the studies on the biological properties of black or red rice bioactive compounds have been conducted through an in vitro approach; however, only a few reports have been applied in preclinical or in animal studies. Further investigations will be needed to produce evidence on the efficacy of pigmented rice in terms of the anticancer activities and anti-inflammation properties in sub-chronic cases, especially among the aging members of the society in which sub-chronic inflammation commonly leads to noncommunicable diseases in later life. In addition, scientific studies have determined that the skin antiaging properties of pigmented rice should be useful and available in clinical studies for their efficacy and their further development in skincare products.

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Conflict of interest

The authors declare no conflict of interest.

Notes/thanks/other declarations

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Chapter 2

Calophyllum inophyllum: Beneficial Phytochemicals, Their Uses, and Identification

David Febrilliant Susanto, Hakun Wirawasista Aparamarta, Arief Widjaja, Firdaus and Setiyo Gunawan

Abstract

Calophyllum inophyllum Linn. is one type of mangrove plant. This plant is commonly called nyamplung. This plant is abundant in Indonesia and has many properties that can be exploited from the roots, stems, and leaves to the seeds. All parts of this plant can be useful for human needs. Its oil is generally only used as biodiesel feedstock. The aim of this chapter is to discuss the identification and the uses of phytochemicals contained in *C. inophyllum* leaves. There are various kinds of phytochemicals contained in *C. inophyllum* leaves, such as triterpenoids, steroids, flavonoids, coumarins, xanthones, fatty acids, esters, alkenes, ethers, and alicyclic compounds. They have benefits to health, such as anticancer, anti-HIV, antiviral, antitumor, anti-inflammatory, antimicrobial, antineoplastic, antiplatelet, antipsychotics, antioxidant, antiaging, antileukemic, antimalarial, anticoagulant, antifeedant, analgesic, photoprotective, molluscicidal, and piscicidal agents. Extraction is a famous method for isolating phytochemicals in *C. inophyllum* leaves, based on the solvent polarity index.

Keywords: *C. inophyllum*, human health, identification, isolation, phytochemicals

1. Introduction

The name of Calophyllum inophyllum is Kallos that is taken from the Greek word, which means beautiful and meaningful Phullon leaves. *C. inophyllum* has many name designations that vary by region country. In the UK, the tree is known as a beautiful leaf (translation from Greek), Indian laurel (because it comes from India), Alexandrian laurel, and beach *Calophyllum* (because the trees usually grow on the waterfront). Moreover, the tree is also called as tamanu (Tahiti), fetau (Samoa), damanu (Fiji Island), te itai (Kiribati Island), nyamplung (Indonesia), Penaga Laut (Malaysia), kamani (Hawaii), foraha (Madagascar), and puna (island of Lakshadweep) [1].

According to Ong [2], the distribution map of *C. inophyllum* in the world is quite extensive. This species is commonly found in areas with a tropical climate. In the world, this species is found in countries such as Australia, Cambodia, the Cook Islands, Fiji, French Polynesia, India, Indonesia, Japan, Kiribati, Laos, Madagascar, Malaysia, the Marshall Islands, Myanmar, New Caledonia, Norfolk Island, Papua New Guinea, the Philippines, Reunion, Samoa, Solomon Islands, Sri Lanka, Taiwan, Province of China, Thailand, Tonga, Vanuatu, and Vietnam.

As for exotic species (endemic to a region), it can be found in the state of Djibouti, Eritrea, Ethiopia, Kenya, Nigeria, Somalia, Tanzania, Uganda, and the USA.

C. inophyllum plant spreads almost evenly throughout Indonesia, such as in the island of Sumatra (West Sumatra, Riau, Kepulauan Riau, Lampung, and Bangka Belitung), Java (Banten, West Java, Central Java, Yogyakarta, East Java), Bali Island, East Nusa Tenggara and West Nusa Tenggara, Kalimantan (West Kalimantan, Central Kalimantan, and South Kalimantan), Sulawesi (North Sulawesi, Gorontalo, Central Sulawesi, South Sulawesi, and Southeast Sulawesi), Maluku and North Maluku Islands, and Papua [3]. *C. inophyllum* plant has a taxonomy as follows [4]:

Kingdom: Plantae
Subkingdom: Tracheobionta
Super division: Spermatophyta
Division: Magnoliophyta
Class: Magnoliopsida
Subclass: *Dilleniidae*
Order: *Theales*
Family: Clusiaceae

C. inophyllum is a plant that is grown in the earthy sand and coastal areas with a hot weather [5]. It can also grow well at an altitude of 0–800 meters above sea level such as in forests, mountains, and swamps [6]. *C. inophyllum* is a versatile crop; all parts of this plant, such as leaves, root, and fruit (**Figure 1**), can be useful for humans. The benefit of its tree, bark, and seed is as plant conservation, source of timber and non-timber forest products (NTFPs), and vegetable oil, respectively [7]. In pharmaceuticals, it is known to function as an antibacterial, anticancer, antineoplastic, anti-inflammatory, antiplatelet, antipsychotics, antiviral, photoprotective, molluscicidal, and piscicidal agent [1]. **Table 1** shows the benefits of *C. inophyllum* crops obtained from previous works.

Because all parts of this plant can be useful in treating various diseases, some researchers have conducted further research on the phytochemical content of this plant. According to Ling et al. [1], the compounds which are contained in these plants include inophynone; canophyllol; canophyllic acid; calophyllolide;

Figure 1.
Parts of C. inophyllum crop.

Part of crops	Medicinal function		
	Iskandari and Anna [8]	**Su et al. [9]**	**Ling et al. [1]**
Leaves	Inhibit the growth of larvae of *Culex quinquefasciatus* and *Aedes aegypti*, an inhibitor of the HIV virus	Treat skin rashes, swelling of the legs, caring for burns, eye irritation, dysentery, migraine, and vertigo	Treat skin diseases, arthritis, sciatica, eye irritation
Root	Antibacterial	Treat dysentery, gonorrhea, indigestion, wounds, ulcers, and others	Treating internal hemorrhage
Fruit/ seed	Inhibit the growth of larvae of *Culex quinquefasciatus*, antimicrobial compounds, and toxic agents	Treating stomach pain, itching, arthritis, burns, gonorrhea, arthritis, ulcers, and ringworm	Treat wounds, leprosy, neurological diseases, burns

Table 1.
Benefits and uses of C. inophyllum crops.

inophyllolide; inophyllum B, C, P, and E; jacareubin; (+)-calanolide A; inocalophyllins A and B; calophynone; calophyllumin C; inophyllin A; and others. Su et al. [9] mentioned that according to Filho et al. [10], in various parts of the tree, C. inophyllum contains phytochemicals, including xanthones, coumarins, chromanones (flavonoids, biflavonoids), triterpenes, tripenoids, and steroids. Coumarins in *C. inophyllum* contain two components, namely, calanolides A and B. From these studies it was found that coumarin compounds in *C. inophyllum* may be effective in treating cancer and inhibiting the HIV virus.

According to Lim [11], at least nine components have been isolated from the leaves of *C. inophyllum*, including 2-hydroxyxanthone; 4-hydroxyxanthone; 1,5-dihydroxyxanthone; 1,7-dihydroxyxanthone; 1,3,5-trihydroxy-2-methoxyxanthone; 6-6-deoxyjacaerubin; flavonoids, amentoflavone; kaempferol-3-O-α-L-rhamnoside; and quercetin-3-O-α-L-rhamnoside.

Of the three studies on the leaves above, there are some differences as well as questions obtained from the leaves of *C. inophyllum* content analysis. Some of the same compounds that have been isolated from *C. inophyllum* plants are quite diverse, including derivatives of xanthones [12, 13], coumarins [9], flavonoids [13], benzodipyranones [14], triterpenoids [12, 15], and steroids [9].

2. Identification of phytochemicals in *C. inophyllum* leaves

2.1 Xanthones

Xanthones are polyphenol components in nature with molecular formula $C_{13}H_8O_2$. They consist of bonding of two benzene rings connected by a carbonyl group and one oxygen. These conjugated ring systems inhibit the free rotation carbon bond. Xanthones have a basic framework consisting of 13 carbon atoms that make up the composition of C6-C1-C6 (**Figure 2**).

Xanthones are compounds with the basic framework of two phenyls connected by bridges carbonyl and oxygen (ether). Their biosynthesis is not known clearly but allegedly still in close contact with the biosynthesis of flavonoids and stilbenoid. It can be seen from the type of oxygenation and two types of aromatic rings which are derived from the shikimate (shikimic acid) and the acetate-malonate pathways.

Xanthones compound that was isolated from *C. inophyllum* plants, there are prenylated and some are not prenylated. Most xanthone compounds isolated from these plants showed a characteristic, one of which is a hydroxy group at C1. The possible oxygenation position is shown in **Figure 2**.

Xanthones are known to have a variety of bioactive properties, notably the ability of antioxidants as can be seen in **Figure 3**. Mangosteen xanthones were isolated from *Garcinia mangostana* found against free radicals and prevent oxidative damage of low-density lipoprotein [16]. Moreover, isolated xanthones from mangosteen also can inhibit HL60 leukemia cells [17]. Also, α-mangosteen extracted from *G. mangostana* L. has antibacterial activity against vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) [18].

Various xanthone compounds can be isolated from *C. inophyllum* leaves, such as caloxanthone A, caloxanthone B, caloxanthone C, maclura xanthones, inoxanthone, calophynic acid, 3,4-dihydroxy xanthones [4, 12, 19], brasilixanthone B, buchanaxanthone [20], inophyxanthone A, pancixanthone A, gerontoxanthone B, jacareubin, pyranojacaereubin, 2-hydroxy xanthone, 4-hydroxyxanthone, 1,3,5-trihydroxy-2-methoxyxanthone, and xanthones [21, 22].

2.2 Coumarins

Coumarin (benzopyrones) compound is one of the members of benzopyrone components. In the coumarin structure, there is a benzene ring which is tied with pyrone ring [23] as can be seen in **Figure 4**. They can be divided into four main types: simple coumarins, pyranocoumarins, furanocoumarins, and

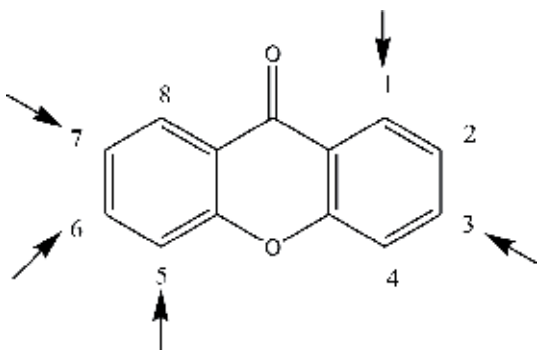

Figure 2.
Possible position oxygenation xanthone compound.

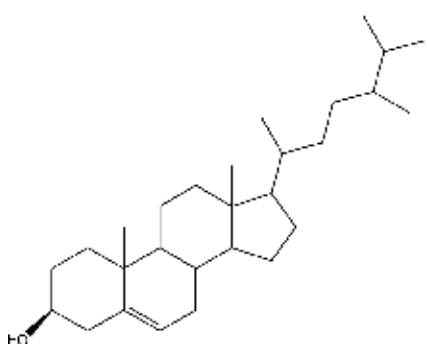

Figure 3.
Molecular structure of xanthones.

pyrone-substituted coumarins. All the reactions of coumarins focus on activation of C3,4—the double bond of the α,β-unsaturated lactone—and form a heterocyclic system [24].

Coumarins are commonly used in the agrochemical, perfume, and medical industries. They have high antitumor and antibacterial activities. Antitumor activity of 7-hydroxycoumarins against several tumor cell lines has been identified. Coumarins and their derivatives have activity as barrier against cellular proliferation in various carcinoma cell lines [25]. Besides that, they also have anticoagulant, antioxidant, antimicrobial, antiviral, anti-inflammatory, antimalarial, and analgesic activities [26].

The biosynthesis of coumarin compounds is derived from the shikimic acid pathway or still in line with the phenyl group propanoid. The skeleton benzopyran-2-on of coumarin is originating from the acid-cinnamic acid via ortho-hydrolysis. Ortho-coumaric acid produced after undergoing *cis-trans* isomerization undergoes condensation [27]. Characteristic of these compounds is their lactone group formed from the acid on the tip of propane with a hydroxy group on the phenyl group. Oxygenation coumarin compounds in the aromatic ring are also typical and are intermittent. The structure of the coumarin derivatives can be divided into four categories based on the group bound to the C_4 : 4-metilcoumarin, 4-fenilcoumarin, and 4-(n-propyl)coumarin.

2.3 Benzodipyranones

Benzodipyranones are derivative of chromone. These compounds have a skeleton similar to stilbene with two additional prenyl groups. Some benzodipyranone compounds have been isolated from the *C. inophyllum* leaves, such as (2S, 3R) and (2R, 3R)-2,3-dihydro-5-hydroxy-2,3,8,8-tetramethyl-6-(1-phenylethenyl)-4H, 8H-benzo [1,2-b: 3,4-b ‘] dipyran-4-one [14], inophynone, and isoinophynone [20, 28].

2.4 Terpenes and terpenoids

Terpenes are naturally derived component in the biosynthesis of isoprene C5 with molecular formula C_5H_8 (CH_2═C (CH_3)-CH═CH_2) (**Figure 5**). They commonly expressed in the formula $(C_5H_8)_n$ with n states the amount of isoprene which are there, so the amount of carbon is a multiple of 5. They are classified in hemiterpenes, monoterpenes (consisting of 2 units of C5 or 10 carbon atoms), sesquiterpenes (consisting of 3 units of C5 or 15 carbon atoms), diterpenes (consisting of

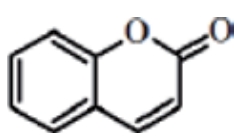

Figure 4.
Molecular structure of coumarins.

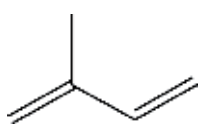

Figure 5.
Molecular structure of isoprene.

4 units of C5 or 20 carbon atoms), sesterterpenes, triterpenes (consisting of 6 units of C5 or 30 carbon atoms), tetraterpenes (consisting of 8 units of C5 or 40 carbon atoms), and polyterpenes.

Moreover, terpenoids are isoprenoid structural components which contain oxygen in its structure and can react with ketone, aldehyde, or alcohol. Chemically, they are generally soluble in fat and contained within the plant cell cytoplasm. Usually, they can be extracted with petroleum ether, ether, or chloroform and can be separated by chromatography on silica gel [29].

Terpenes are widely used as a medicine and flavor enhancers. They are commonly used in the rubber industry. They have a low molecular weight, such as essential oils that are used as natural food additives and fragrances in the perfume industry. They are also used in anticancer drug Taxol which is a diterpene. Taxol is used in the treatment of breast, ovarian, and lung cancer. One example is imberbic acid, a triterpenoid that has activity against *Mycobacterium fortuitum* and *S. aureus* [30].

Triterpenoids are a class of terpenoid compounds which consist of 30 carbon atoms or 6 units of isoprene. In plant tissue, they can be found in their native form but are also often found in the form glycoside. They are divided into cyclic and acyclic structures. The important acyclic triterpenoid is only the squalene that is considered only as an intermediate in the biosynthesis of steroids. The most widespread of triterpenoids are the pentacyclic triterpenoids. The frameworks most often found on a class of compound triterpenoids are ursam, lupan, oleanan, and friedelin [31].

Friedelin has the molecular formula $C_{30}H_{50}O$ and a molecular weight of 426,7174 g/mol (**Figure 6**). Friedelin has a melting point of 259–260°C. The structure mass spectrometry of friedelin is 426 (M^+), 411, 302, 273, 246, 231, 218, 205, 191, 179, 163, 149, 137, 125, 123, 109, 95, 81, 69, and 55. The IR spectra of friedelin in KBr was obtained using v_{max} at 1720 cm^{-1}. The form of friedelin is white crystalline-amorphous solid. Friedelin has an anti-fungal activity and has antinociceptive effects in rodents [32]. Friedelin was developed on a TLC plate by using a solvent system of 10% ethyl acetate and 90% hexane. Friedelin gave a dark spot on a TLC when exposed under UV light and iodine vapor chamber. Friedelin gave an Rf value of 0.75 with the use of a relatively nonpolar solvent system [33].

Several studies have been conducted on the benefits of friedelin. Friedelin has hepatoprotective activity [34]. It has an activity against Bacillus Calmette-Guerin (BCG) that causes tuberculosis [35]. It and some types of friedelin compound are widely used for the treatment of cancer of the bladder [36], convulsion, inflammation [37], topical ulcers, rheumatic inflammation, fever, and dysentery [38]. It is also found to have antifeedant activity in some insects [39].

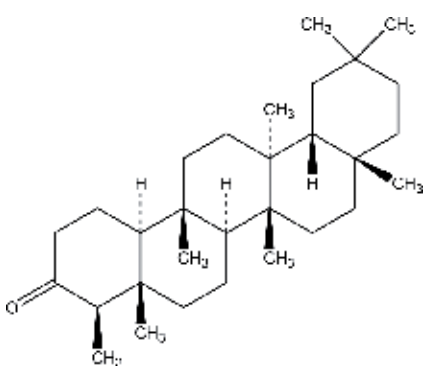

Figure 6.
Molecular structure of friedelin.

Moreover, some compound triterpenoids have been isolated from the *C. inophyllum* leaves, such as 3β, 23-epoxy-friedelane-28-OIC acid, 3-oxofriedelin-28-OIC acid, epifriedelanol, oleanolic acid [40], 3,4-secofriedelane-3,28-dioic [41], β-amyrin [20], friedelin, canophyllal, canophyllol, and canophyllic acid [4, 20, 41].

2.5 Steroid

Sterols are steroids which have a hydroxy group at C3 position as can be seen in **Figure 7**. They are found in free form or in association with glucose to form glycosides (sterolin) or as fatty acid esters (FASE). They are the natural compound that is generally composed of 27 carbon atoms [31]. They are terpenoids in which their basic framework consists of the system perhydrophenanthrene cyclopentane ring. They are a class of secondary metabolic compounds which are widely used as a drug. Steroid hormones are generally derived from natural steroid compounds, especially in plants [42]. Some steroid compounds have been isolated from the *C. inophyllum* leaves such as campesterol [20]. Campesterol also has analgesic activity.

2.6 Flavonoids

Flavonoids are the largest group of phenolic compounds found in nature, especially in tissues of higher crops. They are the product of secondary metabolites that occur from the cells and accumulate on the body crop as a toxic substance [43]. They are commonly known as flavonoids, which are water-soluble polyphenol component. They have a basic framework consisting of 15 carbon atoms where a chain of benzene (C6) is bound to a chain of propane (C3), thus forming a bond arrangement C6-C3-C6 which is particularly called phenylbenzopyran (**Figure 8**). This arrangement can produce three structures, namely, 1,3-diarilpropana (flavonoids), 1,2-diarilpropana (isoflavonoids), and 2,2-diarilpropana (neoflavonoid) [44]. Moreover, flavonoids are classified into various categories based on differences in molecular structure, such as chalcones, flavanols, catechins, flavonoes, isoflavone, dihydroflavonol, and anthocyanidins [45, 46].

According to Markham [47], flavonoids are polar compounds because they have a hydroxyl group which does not bind to sugar, so the flavonoid is quite soluble in polar solvents such as ethanol, methanol, butanol, or water. Because of the presence of sugar bound, flavonoids become more soluble in water. Conversely, the less polar aglycone, such as isoflavones, flavanones, flavones, and flavonols, which is methoxylated tends to be more soluble in solvents, such as ether and chloroform.

The largest group of flavonoids is flavones. Flavonoids have a 2-phenyl Croman order in which the ortho-position of the A ring and the carbon atom attached to the ring B of 1.3 diarilpropana is connected by bridging oxygen to form a new heterocyclic ring [47].

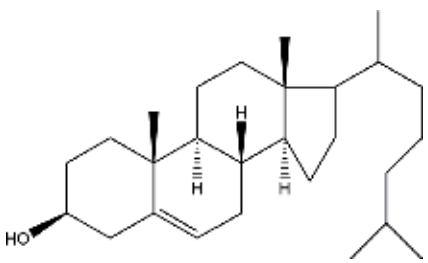

Figure 7.
Molecular structure of cholesterol.

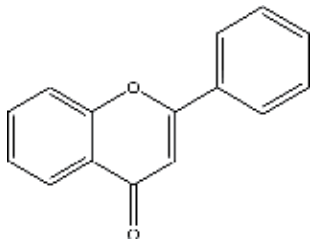

Figure 8.
Molecular structure of flavone.

Flavonoids have a variety of biological functions including pharmaceutical use and their function in plants. Examples of pigments in flowers, they provide color and attract insects for pollination. Flavonoids which are contained in the leaves have to prevent fungal infections and protect leaves from UV radiation [45]. In the aspect of pharmacology, flavonoids interact with cytochrome P450 and are used to treat heart disease. They are also known to have antioxidant activity and anti-free radicals that are useful in anticancer and antiaging. Furthermore, they also have antileukemic activity, vitamin C, 5-lipoxygenase, cyclooxygenase inhibitors, protein kinase C, tyrosine kinase, and genetic toxicity [27].

Several flavonoid compounds that have been isolated from the *C. inophyllum* leaves are bioflavonoids, neoflavonoid [48], amentoflavone [20, 40], and quercetin-3-O-α-L-rhamnoside [8, 48].

2.7 Oxygenated hydrocarbon (fatty acids)

Some of the compounds of fatty acid that has been found in the *C. inophyllum* leaves are tetradecanoic acid (myristic acid, $C_{14}H_{28}O_2$), n-hexadecanoic acid (palmitic acid, $C_{16}H_{32}O_2$), oleic acid ($C_{18}H_{34}O_2$), and octadecanoic acid (stearic acid, $C_{18}H_{36}O_2$) [49].

2.8 Esters

Some ester compounds that have been found in the *C. inophyllum* leaves are 1,2-benzenedicarboxylic acid (diisooctyl ester/phthalic acid, bis(6-methylheptyl) ester), 9,12-octadecenoic acid methyl ester, 16-octadecanoic acid methyl ester, heptadecanoic acid, and 16-methyl ester [49, 50].

2.9 Tannins

In chemistry, there are two types of tannins, namely, (1) condensed tannins or flavolan and (2) hydrolyzed tannins.

2.9.1 Condensed tannins

The condensed tannins are widespread in angiosperm plants, especially in woody plants. Another name of condensed tannins is proanthocyanidin because when they reacted with hot acid, some of the carbon-carbon connecting bond units disconnect and free monomer anthocyanidins. Most proanthocyanidin is procyanidin because when reacted with acids will produce cyanidin. Proanthocyanidin can be detected directly by dipping the plant tissue into 2 M HCl boil for half an hour that will produce a red color which can be extracted with amyl or butyl alcohol.

No.	Phytochemicals	Chemical structure	References
1.	Triterpenoids		
	3β, 23-Epoxy-friedelan-28-oic acid	$C_{30}H_{48}O_3$	[41]
	Friedelin	$C_{30}H_{50}O$	[4, 20, 28, 32, 41]
	3-Oxofriedelin-28-oic acid		[40, 41]
	Canophyllal	$C_{30}H_{48}O_2$	[20, 41]
	Canophyllol	$C_{30}H_{50}O_2$	[20, 41]
	Canophyllic acid (27-hydroxyacetate canophyllic acid)	$C_{30}H_{50}O_3$	[4, 20, 41]
	3,4-Secofriedelane-3,28-dioic acid	$C_{30}H_{50}O_4$	[19]
	Inophynone	$C_{24}H_{24}O_4$	[20, 28]
	Isoinophynone	$C_{24}H_{24}O_4$	[20, 28]
	β-Amyrin	$C_{30}H_{50}O$	[20]
	Epifriedelanol	$C_{30}H_{52}O$	[41]
	3-Oxo-27-hydroxyacetate friedelan-28-oic acid		[19]
	Oleanolic acid	$C_{30}H_{48}O_3$	[41]
	Squalene	$C_{30}H_{50}$	[50]
2.	Steroids		
	Cholesterol	$C_{27}H_{46}O$	[28]
	Campesterol	$C_{28}H_{48}O$	[20]
3.	Flavonoids		
	Biflavonoids	$C_{30}H_{20}O_{10}$	[49]
	Neoflavonoids	$C_{20}H_{18}O_8$	[49]
	Quercetin-3-O-α-L-rhamnoside (4H-1-benzopyran-4-one,2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy)	$C_{15}H_{10}O_7$	[22, 49]
	Amentoflavone	$C_{30}H_{18}O_{10}$	[20, 22, 40]
4.	Coumarins		
	Inophyllum C	$C_{25}H_{23}O_5$	[12, 40, 42]
	Inophyllum E	$C_{25}H_{22}O_5$	[12, 40]
	Inophyllum B	$C_{25}H_{24}O_5$	[4, 42]
	Inophyllum P	$C_{25}H_{24}O_5$	[4, 42]
	Calophyllic acid	$C_{25}H_{24}O_6$	[4, 20, 40]
	Isocalophyllic acid	$C_{25}H_{24}O_6$	[20, 40]
	Inophyllum G-1	$C_{25}H_{24}O_5$	[4, 42]
	Inophyllum G-2	$C_{25}H_{24}O_5$	[4, 42]
	Calocoumarin-A		[20]
	Calocoumarin-B		[20]
	Calocoumarin-C		[20]
	Apetalolide	$C_{26}H_{24}O_5$	[20]
	4-Phenylcoumarins		[20]
	Pyranocoumarins	$C_{20}H_{18}O_4$	[42]

No.	Phytochemicals	Chemical structure	References
	Calophyllolides (calophyllolide 2a, 3a, 3b, 6)	$C_{26}H_{24}O_5$	[4, 12, 42]
5.	Xanthones		
	Caloxanthone A	$C_{23}H_{22}O_6$	[4, 12]
	Caloxanthone B		[4, 12]
	Caloxanthone C		[4]
	Brasilixanthone-B	$C_{23}H_{20}O_6$	[20]
	Buchanaxanthone	$C_{14}H_{10}O_5$	[20]
	Inoxanthone	$C_{23}H_{22}O_5$	[12]
	Maclura xanthone	$C_{23}H_{22}O_6$	[12]
	Calophynic acid	$C_{35}H_{44}O_6$	[12]
	3,4-Dihydroxyxanthone	$C_{13}H_8O_4$	[12, 19]
	Inophyxanthone A		[21]
	Pancixanthone A	$C_{18}H_{16}O_5$	[21]
	Gerontoxanthone B	$C_{23}H_{22}O_6$	[21]
	Jacareubin (6-deoxyjacareubin)	$C_{18}H_{14}O_6$	[21, 22]
	Pyranojacaereubin	$C_{23}H_{20}O_6$	[21]
	2-Hydroxyxanthone	$C_{13}H_8O_3$	[22]
	4-Hydroxyxanthone	$C_{13}H_8O_3$	[22]
	1,3,5-Trihidroxy-2-methoxyxanthone		[22]
	Xanthone	$C_{13}H_8O_2$	[21]
6.	Oxygenated hydrocarbons (fatty acids)		
	Tetradecanoic acid (myristic acid)	$C_{14}H_{28}O_2$	[50]
	n-Hexadecanoic acid (palmitic acid)	$C_{16}H_{32}O_2$	[50]
	Oleic acid	$C_{18}H_{34}O_2$	[50]
	Octadecanoic acid (stearic acid)	$C_{18}H_{36}O_2$	[50]
7.	Esters		
	1,2-Benzenedicarboxylic acid (diisooctyl ester) (phthalic acid, bis(6-methylheptyl) ester) (diisooctyl phthalate)	$C_{24}H_{38}O_4$	[50]
	Methyl linoleic (9,12-octadecanoic acid methyl ester)	$C_{19}H_{34}O_2$	[50, 51]
	Methyl oleate (16-octadecanoic acid methyl ester)	$C_{19}H_{36}O_2$	[51]
	Methyl isostearate (heptadecanoic acid, 16-methyl, methyl ester)	$C_{19}H_{38}O_2$	[51]
8.	Alkenes (unsaturated compounds):		
	Azulene, 1,4-dimethyl-7-(1-methylethyl)-	$C_{15}H_{18}$	[50]
9.	Ethers		
	3-Trifluoroacetoxypentadecane (pentadecyl trifluoroacetate) (trifluoroacetic, pentadecyl ester)	$C_{17}H_{31}F_3O_2$	[50]
	1-Monolinoleoglycerol trimethylsilyl ether	$C_{27}H_{54}O_4Si_2$	[50]
10.	Alicyclic compounds		
	Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene-,[S-(R*,S*)]	$C_{15}H_{24}$	[50]

No.	Phytochemicals	Chemical structure	References
11.	Aromatic hydrocarbon:		
	Benzene (1-methyldodecyl)	$C_{19}H_{32}$	[50]
12.	Androstan-1α-ol-17-one,23 isopropylidenedioxy-4β-methyl-	$C_{23}H_{36}O_4$	[50]
13.	Proanthocyanidin (condensed tannin)	$C_{31}H_{28}O_{12}$	[20, 49]
14.	Benzodipyranone (chromone) derivatives:		
	a. (2S,3R)-2,3-Dihydro-5-hidroxy-2,3,8,8-tetramethyl-6-(1-phenylethenyl)-4H,8H-benzo [1,2-b:3,4-b'] dipyran-4-one		[14]
	b. (2R,3R)-2,3-Dihydro-5-hidroxy-2,3,8,8-tetramethyl-6-(1-phenylethenyl)-4H,8H-benzo [1,2-b:3,4-b'] dipyran-4-one		[14]
15.	Asam inophylloidic	$C_{32}H_{46}O_6$	[12, 20]
16.	Calaustralin	$C_{25}H_{25}O_5$	[12]
17.	Shikimic acid	$C_7H_{10}O_5$	[40]
18.	Brasiliensic acid	$C_{32}H_{46}O_6$	[12]
19.	Adenanthin (7,8,12-tri-0-acetyl-3-desoxy-ingol3-one)	$C_{26}H_{34}O_9$	[51]
20.	Carbazole	$C_{12}H_9N$	[51]
21.	Diphenyl methane (1'-biphenyl, 2-methyl)	$C_{13}H_{12}$	[51]
22.	2-Phenazinamine (1,1'-biphenyl, 4-azido)	$C_{12}H_9N_3$	[51]
23.	5-Aminomethyl-dibenzosuberane (2-naphtalenecarbonitrile, 6-pentyl-)	$C_{16}H_{17}N$	[51]
24.	Phytol	$C_{20}H_{40}O$	[50, 51]
25	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	[50]
26.	Phenol (2,4-bis(1-phenylethyl)-phenol)	$C_{22}H_{22}O$	[50]

Table 2.
Phytochemicals contained in the C. inophyllum leaves.

When dry tissues are used, the result of tannins somewhat diminished because of the occurrence of sticking tannins in place within the cell.

2.9.2 Hydrolyzed tannins

The hydrolyzed tannins are contained in dicotyledonous plants. They mainly consist of two classes; the simplest is galloylglucose. In this compound, glucose is surrounded by five or more galloyl ester groups. The second type is the core molecules of a compound gallic acid dimer, namely, hexahydroxidifenate acid that binds to glucose. Hydrolyzed tannins can be detected by determining the gallic acid or ellagic acid in ether or ethyl acetate extracts.

2.10 Other components

Some chemical compounds that have been found in the *C. inophyllum* leaves are azulene ($C_{15}H_{18}$), squalene ($C_{30}H_{50}$), 3-trifluoroacetyl pentadecane (pentadecyl trifluoroacetate), 1-monolinolein glycerol trimethylsilyl ether, cyclohexane, benzene, androstane [49], inophylloidic acid [12, 20, 52], shikimic acid [40], calaustralin, brasiliensic acid [12], adenanthin, carbazole, diphenyl methane, 2-phenazinamine, 5-aminomethyl-dibenzosuberane [50], phytol, phenol, and 3,7,11,15-tetramethyl-2-hexadecene-1-ol [49, 50]. The summary of phytochemicals in *C. inophyllum* leaves is presented in **Table 2**.

3. Isolation method of phytochemicals in *C. inophyllum* leaves

Polarity is one of the characteristics of chemical bonding, where two different atoms within the same molecule have a different electronegativity. As a result, the electrons in the bond are not shared equally by the two atoms. This causes the electric field (pole) to be asymmetric. Covalent bonding of molecules can be described as polar or nonpolar.

The polar compound is a compound formed by a single atom which has electronegativity substantially greater than the other. The more electronegative the atom, the pull of the bonding electrons is greater. The result is a bond with an uneven electron dense distribution. The nonpolar compound is a compound formed by atoms with the same or nearly the same electronegativity and forms covalent bonds, where both atoms apply traction which equals or nearly equals to the bonding electrons. Generally, the carbon-carbon and carbon-hydrogen bonds are the most common types of nonpolar bond [53].

To identify polar and nonpolar compounds from the *C. inophyllum* leaves, the first idea is separating their compounds based on the solvent used (solvent polarity index). Methanol and water are polar solvent with a polarity index of 5.1 and 9, respectively. For n-hexane or petroleum ether is nonpolar solvent with a polarity index of 0 [54]. It can be expected that polar compounds which are contained in the *C. inophyllum* leaves can be dissolved in a polar solvent and vice versa. Relative polarities of several solvents can be seen in **Table 3.**

Extraction is the separation process of material from a solid or some material from liquid with the help of the solvent. Extraction can be defined as a method of separating components of a mixture by using a suitable solvent. Solutes (dissolved substances) are separated in a manner distributed between two layers of solvents based on their solubility. Extraction is a separation of the compounds contained in the liquid material/solid using certain solvents at any given temperature.

In general, extraction techniques can be classified into two general categories:

1. Short-term extraction is extraction techniques typically used to separate a substance (liquid form), on the basis of differences in solubility of the two immiscible solvents.

2. Long-term extraction is an extraction technique normally used to separate the natural material (solid form) contained in plants or animals. It is a classic procedure to obtain the organic matter content of dry plant tissue by soaking with certain solvents (polar or nonpolar solvents) [29].

Percolation is an extraction technique that done repeatedly and performed at a room temperature. This is similar to maceration, but after soaking for a certain time, the solvent is removed and replaced with a new solvent. After filtration, the filtrate obtained is called percolate [55].

According to Mulyono [55], in terms of the extraction mechanism, known to some type of extraction, namely:

1. Single-stage extraction

Single-stage extraction is the extraction method using a single type of solvent, and extraction is only done once with a solvent.

Relative polarity	Formula	Group	Solvents
Nonpolar	R-H	Alkanes	Petroleum ethers, hexanes, ligroin
	Ar-H	Aromatics	Toluene
	R-O-R	Ethers	Diethyl ether
	R-X	Alkyl halides	Trichloromethane, chloroform
	R-COOR	Esters	Ethyl acetate
	R-CO-R	Aldehydes, ketones	Acetone, MEK
	R-NH2	Amines	Pyridine, triethylamine
	R-OH	Alcohols	MeOH, EtOH, IPA, butanol
	R-COHN2	Amides	Dimethylformamide
	R-COOH	Carboxylic acid	Ethanoic acid
Polar	H-O-H	Water	

Table 3.
Relative polarity of solvents [54].

2. Repeated extraction

Repeated extraction is the extraction method using a solvent, but the process is repeated with a number of solvents.

3. Stage extraction

Stage extraction is the extraction method using some type of solvent extraction, such as after extraction with the first solvent, followed by using other solvents, and so on.

Solvents are not or only partially soluble solids or liquids with continuous contact; the active agents move from a mixture of solids/liquid (raffinate) to the solvent (extract). After mixing the two phases, the separation process is done on the principle of gravity or centrifugal force [56].

Yunitasari [57] describes the effect of solvent on the various types of tray number from 6 to 10 for taking *C. inophyllum* oil with column extraction. From the experimental results, the authors explain that the more the number of trays, the less time is required for a solvent to extract the oil. The solvent used are between n-petroleum and n-hexane. From the experimental results, the authors explain that the maximum condition extraction was achieved by n-petroleum in the seventh tray. The amount of oil was decreasing by increasing number of tray. In the other hand, the amount of oil was increasing with number of tray while n-hexane was used.

4. Conclusions

The identification and uses of beneficial phytochemicals contained in *C. inophyllum* leaves were presented in this book chapter. It was found that all parts of *C. inophyllum* plant can be used for human needs. The information is limited to extraction and identification of mixture of phytochemical compounds that are obtained from plant extracts. The separation of individual phytochemical compounds still remains unknown. Therefore, further research on the determining of phytochemicals content in this plant is necessary.

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Conflict of interest

We declare that we have no conflict of interest.

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Chapter 3

Analytical Methods of Isolation and Identification

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Abstract

The chemical constituents of plants are complicated, and monomeric compounds must be obtained via extraction and isolation before structure identification, bioactivity screening, and so on. In recent years, the new technologies and methods of the extraction, isolation, and structural identification have come forth, which promote the speed of extraction and analysis of phytochemicals. The chemical structures of compounds from plants must be identified or elucidated, which may provide the necessary basis for further study on the bioactivities, structure-activity relationships, metabolisms *in vivo*, structural modification, and synthesis of the active compounds. The amount of chemical constituents isolated from plants is often minor, so the structural studies are often difficult to carry out with classical methods. Therefore, spectral analysis is mainly used. This chapter describes the isolation and identification methods during the study of phytochemicals.

Keywords: extraction, isolation, structure elucidation, spectroscopic methods

1. Introduction

The phytochemicals rich in plants have shown to be beneficial for prevention of diseases as well as long-term health. Plants are generally consumed as sources of essential compounds such as saccharides, coumarins, lignans, flavonoids, terpenoids, and steroids. The health benefits and the composition from plant have been described more and more in the literature. Because of the complexity of plant chemical constituents, pure phytochemicals must to be obtained via extraction and isolation before structure identification, bioactivity screening, and so on. In recent years, new technologies and methods of extraction occurred, which accelerate the extraction and analysis of phytochemicals.

Extraction is the first step of phytochemistry research, which is also the necessary work before the isolation of effective constituents. The purpose of extraction is to get the objective chemical constituents to the utmost extent and avoid or reduce the solution of unwanted constituents.

The separation of phytochemicals is a process of isolating the constituents of plant extracts or effective parts one by one and purifying them into monomer compounds by physical and chemical methods. Classical isolation methods, including solvent extraction, precipitation, crystallization, fractional distillation, salting-out, and dialysis, are still used commonly at present. On the other hand, modern separation technologies such as column chromatography, high performance liquid chromatography, ultrafiltration, and high performance liquid drop countercurrent chromatography also play an important role in the separation of phytochemicals [1–3].

The chemical structures of plant compounds must be identified, which may provide the necessary basis for further study on the bioactivities, structure-activity relationships, metabolisms *in vivo*, structural modification, and synthesis of the active phytochemicals.

The structural studies are often difficult to carry out with classical chemical methods, such as chemical degradation and derivative synthesis, because of the minute amount of compound isolated from plants. Therefore, spectral analysis is mainly used. That is, consuming sample as little as possible to obtained structural information as much as possible by measuring and analyzing various spectra [4].

2. Extraction of phytochemicals

2.1 Solvent extraction methods

2.1.1 Principles

Solvent extraction is the commonest method to extract plant material. The main purpose is to select the suitable solvent to extract target plant materials efficiently. During the extraction, the solvent has to diffuse into the cell membrane in the first instance, in the following step it has to dissolve the solutes, then intracellular and extracellular concentration difference is formed, and finally it has to diffuse out of the cells enriched in the extracted solutes [5].

Selecting suitable solvents is the key of the solvent extraction method. Using a solvent of an appropriate polarity according to the principle of "like dissolves like" is the main point to select solvent. Thus, hydrophilic solvents are used to solubilize hydrophilic chemical constituents and vice versa. The hydrophilicity or lipophilicity of solvents and chemical constituents could be predicted by polarity. The plant compounds, such as terpenoids and steroids, possess low polarity, and could be dissolved into lipophilic solvents such as chloroform and ether, while chemical constituents, such as carbohydrates and amino acids, possess rather high polarity and could be dissolved into water and aqueous ethanol.

Solvents commonly used for extracting chemical constituents of plants are in the order of weak to strong polarity as follows: petroleum ether < carbon tetrachloride < benzene < dichloromethane < chloroform < ether < ethyl acetate < n-butanol < acetone < ethanol < methanol < water.

Water is a cheap, easy to get, and nontoxic solvent with strong polarity. It could be used to extract phytochemicals with strong polarity, such as inorganic salts, saccharides, amino acids, tannins, proteins, organic acid salts, alkaloid salts, and glycosides. Acid or alkaline water is applied sometimes to increase the solubility of certain specific components. Acid water could extract alkaline materials, such as alkaloids, via the formation of salts. Similarly, organic acids, anthraquinoids, flavonoids, coumarinoids, phenols, and other acidic materials could be extracted via the formation of salts. The disadvantage to extract chemical constituents with water is that the aqueous extract is easy to go moldy, so difficult to preserve. Additionally, water possesses high boiling point, and the water extract needs to be concentrated for a rather long time. Furthermore, the water extract contains many impurities such as proteins, pectins, tannins, mucilages, and inorganic salts, which make the extraction of target components difficult.

Hydrophilic organic solvents are strong-polarity and water miscible, such as methanol, ethanol, and acetone. Ethanol is the most commonly used hydrophilic organic solvent. Chemical constituents could be extracted by ethanol of different concentrations according to their properties. Furthermore, ethanol is inexpensive,

safe, and concentrated easily. Additionally, ethanol extract is not readily moldy and glycosides are hard to be hydrolyzed in ethanol extract. Thus, ethanol is one of the most commonly used solvents in laboratories and industrial production. Methanol possesses similar property to ethanol and lower boiling point. However, methanol has rather strong toxicity, so we have to pay attention to safety when it has to be used. Acetone is a good solvent to extract lipid-soluble chemical constituents. However, acetone is easy to volatilize and flame, and it possesses certain toxicity.

Petroleum ether, benzene, chloroform, ether, ethyl acetate, dichloroform, and so on are lipophilic organic solvents and are not miscible with water. They could be applied to extract lipophilic components, such as volatile oils, fats, chlorophyll, lactones, phytosterols, some alkaloids and some aglycones (aglycones of flavonoids, anthraquinoids, steroids, and so on). These solvents possess low boiling points and are easy to concentrate. However, strong-volatility, large loss, flammability, toxicity, and high price are their disadvantages. Additionally, they are difficult to permeate into plant cell tissues.

Solvent extraction methods could be classified as cold extraction and hot extraction roughly by whether heating or not.

2.1.2 Immersion method

It is a method to dissolve out phytochemicals with appropriate solvents at room or low temperatures (<80°C). It is suitable to extract phytochemicals easily to be destroyed at high temperature. The plants with abundant starches, pectins, gums, or mucilages could also be extracted with this method. Firstly, plant powder or pieces should be loaded in the adequate container, and then the suitable solvents (water, ethanol, aqueous ethanol, and so on) are added into it to immerse the material for the given length of time. Discontinuous stirring or shaking during the process could accelerate dissolution rate. The immersion method is simple but inefficient, and the extraction ratio is also low. Furthermore, aqueous extract is easy to go moldy, so addition of appropriate preservatives is necessary.

2.1.3 Percolation method

The coarse particles of plants should be loaded in percolation apparatus and immersed with suitable solvent for 24–48 h, then collect the percolates at the bottom of percolation apparatus. New solvent should be added at the top of percolation apparatus constantly during the percolation process. It possesses higher efficiency than the immersion method because of the sustained concentration difference during the process. However, this procedure is complex and consumes rather much solvent and long time.

2.1.4 Decoction method

Load short segments, thin pieces, or coarse powder into an appropriate container, add water, and heat it to boiling; the components are then extracted. It is easy to operate; most of the constituents could be extracted in various degrees. Nevertheless, rather much nontargeted components could also be extracted, and it is not suitable to the extraction of volatile compounds and thermal unstable compounds. Furthermore, it is not suitable to extract plants with lots of starches [6].

2.1.5 Refluxing method

It is a method to extract plant chemical constituents by organic solvent using heating and refluxing. Refluxing apparatus is necessary so as not to waste solvents,

and the toxicity to operators or ruin the environment is deduced. It is applicable to extraction of lipophilic phytochemicals, such as steroids, anthraquinoids, and terpenoids. It is an extraction method of high efficiency but complex, and consumes much more solvent. This method is not applicable to extract thermal unstable chemical constituents because of long time heating.

2.1.6 Constant refluxing method

It is a method developed based on the refluxing method. Soxhlet extractor is the most frequently used constant refluxing apparatus. This method avoids disadvantages of consuming too much solvent and complex operation. However, as a refluxing method, constant refluxing method is not applicable to extract thermally unstable compound either because of long time heating.

2.1.7 Supercritical fluid extraction method

In the supercritical state, the supercritical fluid is contacted with the plant tissues. By controlling different temperatures, pressures and different kinds and contents of entrainers, the supercritical fluid can selectively extract the components of different polarities, boiling points, and molecular weights successively. This method is called the supercritical fluid extraction (SFE) method [7].

The critical point of a pure substance is defined as the highest temperature and pressure at which the substance can exist in vapor-liquid equilibrium. At temperatures and pressures above this point, a single homogeneous fluid is formed, which is known as supercritical fluid (SF). SF is heavy like liquid and has low viscosity like gas meanwhile. SF possesses rather large diffusion coefficient and could dissolve many compounds well. A number of materials could be used as SFs, such as ammonia, ethane, difluoro-dichloromethane, heptane, and so on, while the most widely used SF is CO_2. The critical temperature of CO_2 (T_c = 31.26°C) is close to room temperature, and the critical pressure (P_c = 7.2 MPa) is not too high. CO_2 also has a series of other advantages, such as nontoxicity, odorless, nonflammable, chemical stability, and low cost, which allowed it to be the most commonly used solvent in SFE. CO_2 is a nonpolar substance and applicable to extract lipophilic compounds. However, its dissolvability is weak compared to strong polar substances. Hence, entrainers are always added to improve the solubility of SF CO_2 during the extraction of polar compounds. Entrainers, which are added into SF little, could enhance solubility of SF significantly. The commonly used entrainers are methanol, ethanol, water, acetone, ethyl acetate, acetonitrile, and so on.

The extraction of nonpolar and medium-polar components by SFE can avoid the sample loss and environmental pollution caused by solvent recovery in traditional extraction methods, especially for the extraction of volatile compounds with thermal instability.

The biggest advantage of SFE is that it can be performed at near-room temperature, and almost all the active ingredients in the product can be retained. There is no residual organic solvent in the process. The product has high purity and high yield. Additionally, the operation is simple and energy saving.

Compared with other conventional separation methods, SFE possesses the following advantages: (1) No residual organic solvents, fast extraction speed, simple process, high yield, and easy operation; (2) no flammable and explosive dangers, no environmental pollution. Low extraction temperature, suitable for the extraction of thermal unstable components; (3) the dissolution properties

of SF are easy to improve, only the pressure needs to be changed at a certain temperature; (4) entrainers can be added to change the polarity of the extraction medium to extract polar substances; extraction medium can be recycled with low cost; (5) it could be applied combined with other chromatographic techniques, such as GC, IR, GC–MS, and HPLC, to extract, separate, and determine phytochemicals efficiently and quickly, so as to achieve the integration of extraction and quality analysis. However, supercritical extraction has some limitations: strong solubility of fat-soluble components, weak solubility of water-soluble components, high cost of equipment, resulting in higher product costs, and cleaning equipment is difficult.

Supercritical fluid extraction (SFE) technology has achieved gratifying results in the fields of medicine, chemical, food, light industry, and environmental protection. Especially, it has been widely used in phytochemical extraction field, such as the extraction of alkaloids, volatile oils, phenylpropanoids, flavonoids, organic acids, glycosides, terpenoids, and so on.

2.1.8 Ultrasonic extraction method

It is a method of solvent extraction assisted by ultrasound. Ultrasonic wave is a kind of elastic mechanical vibration wave. The vibration frequency is as high as 20 KHz in elastic medium. The ultrasonic wave could vibrate the liquid medium. When the vibration is sparse, many small holes are formed in the medium. The instantaneous closure of these small holes can cause a pressure of up to thousands of atmospheric pressures. At the same time, the local temperature can rise to 1000°C. It can cause instantaneous rupture of the cell wall of plants and the whole organism, and make the solvent permeate into the cells of plants. This accelerates the dissolution of active ingredients in plants into solvents. Ultrasonic wave extraction could shorten the extraction time and improve the extraction efficiency, but could not change the structures of chemical constituents meanwhile.

Ultrasonic extraction technology has been widely used in the extraction of natural products in recent years, for example, extraction of soy isoflavones; see [8].

2.1.9 Microwave-assisted extraction method

Microwave refers to the electromagnetic wave whose wavelength is in the range of 0.1–100 cm (the corresponding frequency is 300–300,000 MHz), which is between infrared and radio waves. Polar molecules can absorb microwave energy, then release energy in the form of thermal energy, which makes the temperature inside the medium rise rapidly, causes the rather high pressure inside, and then the components flow out and dissolve in the solvent. On the other hand, the electromagnetic field produced by microwave can make some components diffuse to the interface of the extraction solvent, accelerating their thermal movement, which not only improves the extraction efficiency but also reduces the extraction temperature [9].

Microwave-assisted extraction has the advantages of less decomposition of chemical constituents, shorter time, lower energy consumption and less environmental pollution. Microwave-assisted extraction has been widely used in a series of fields of perfume, condiments, natural pigments, herbal medicine, cosmetics, soil and environmental analysis, and so on. In China, microwave-assisted extraction technology has been used in hundreds of Chinese herbal medicine extraction, such as *Pueraria lobata*, Panax notoginseng, Ginkgo, and so on, for example, the extraction of tea polyphenols and tea caffeine from green tea leaves; see [10].

2.2 Steam distillation method

Steam distillation is suitable for the extraction of volatile components which can be distilled with steam without being destroyed and are insoluble in water. These compounds' boiling points of are mostly higher than 100°C, and they possess certain vapor pressures at about 100°C. The principle of steam distillation is that the vapor pressure of each component is equal to that of their pure state, while the existence of another liquid does not affect their vapor pressure. The total vapor pressure of the mixing system is equal to the sum of the vapor pressures of the two components. Because the total vapor pressure of the system is higher than that of any single component, so the boiling point of the mixture is lower than that of any component. It is mainly used to extract volatile oils, some alkaloids, and phenolic substances of small molecules from plants.

2.3 Sublimation method

The process that solid material converts into steam directly without melting after heating is called sublimation. The phenomenon that steam condenses into solid after cooling is called deposition. Some natural chemicals have sublimation properties, which can be extracted directly with the sublimation method, for example, the extraction of camphor from camphor wood and caffeine from tea. In addition, some small molecular alkaloids, coumarins, organic acids, and other components also have sublimation properties, such as aesculetin and benzoic acid. However, it is easy to carbonize natural products because of long heating time. The volatile tar-like substances often adhere to sublimates, which are difficult to remove and often accompanied with thermal decomposition. The yield of this method is often low, and it is not suitable for large-scale production.

2.4 Pressing method

When the content of active ingredients is relatively high and exists in the juice of plants, the juice can be extracted directly from fresh raw materials. Volatile oils can also be extracted from plant tissues by mechanical pressing, such as orange peel oil and lemon oil. It is performed at room temperature, so its components will not be decomposed by heat. However, the products obtained are impure and often contain impurities such as water, mucoid substances, and cell tissues, so they are often turbid, and it is not easy to press the volatile oil in plants entirely. Therefore, the crushed residue is often distilled by steam to extract volatile oils completely. For example, the black soybean oil from black soybean is often extracted with the low-temperature pressing method.

3. Isolation and purification of phytochemicals

The separation of phytochemicals is a process of isolating the constituents of plant extracts or effective parts one by one and purifying them into monomer compounds by physical and chemical methods. Classical isolation methods, including solvent extraction, precipitation, crystallization, fractional distillation, salting-out, and dialysis, are still used commonly at present. On the other hand, modern separation technologies such as column chromatography, high performance liquid chromatography, ultrafiltration, and high performance liquid drop countercurrent chromatography also play an important role in the separation of phytochemicals. This section describes the common methods and their specific applications in isolation of phytochemicals.

3.1 Solvent method

3.1.1 Acid and basic solvent method

It is carried out according to the different acidity and alkalinity of each component in the mixture. Water-insoluble alkaline organic components, such as alkaloids, could react with inorganic acids and form salts, which can be separated from nonalkaline and water-insoluble components. Acid components with carboxyl or phenolic hydroxyl groups can be salted by bases and dissolved in water. Components with lactone or lactam substructures can be saponified and dissolved in water and then isolated from other water-insoluble components. The total extract can be dissolved in lipophilic organic solvents (ethyl acetate is commonly used) and extracted respectively with acid water and alkali water, and then the total extract would be divided into acidic, alkaline, and neutral parts. Of course, the total extract can also be dissolved in water and extracted with organic solvents after adjusting the pH value. The alkalinity or acidity of the fractions are different and can be separated further by pH gradient extraction.

When using the acid and basic solvent method, attention should be paid to the strength of acidity or alkalinity, the contact time with the separated components, heating temperature, and time, so as to avoid the structural changes of some compounds under severe conditions or the chemical structures cannot be restored to the original states.

3.1.2 Polarity gradient extraction method

This method is to achieve the separation aim based on the different polarity of each component in plant extracts and the different partition coefficients in two-phase solvents. Generally, different two-phase solvent systems are selected according to the polarity of components in plant extracts. For example, the components with strong polarity can be separated by n-butanol-water system, the components with medium polarity can be separated by ethyl acetate-water system, and the components with weak polarity can be separated by chloroform (or ether)-water system. During the operation, the plant extract should be dissolved by water firstly, and then the solution or suspension is extracted in a separating funnel with different organic solvent which is not miscible with water based on the polarity difference. Usually, the extract was extracted with petroleum ether (or cyclohexane) firstly, then ethyl acetate (or chloroform), and finally with water saturated n-butanol, as shown in **Figure 1**. Petroleum ether layer contains lipid-soluble compounds with low polarity. Ethyl acetate layer contains medium polar compounds such as monoglycosides, flavonoids, and compounds with more polar functional groups. N-butanol layer contains compounds with strong polarity, such as oligoglycosides and other water-soluble components. Compounds in water layer possess strongest polarity, such as glycosides with more glycosyl groups, carbohydrates, amino acids, proteins, and other water-soluble compounds.

3.2 Precipitation method

It is a method based on the formation of precipitation of some phytochemicals by reaction with specific reagents, or the precipitation of some components from the solution by adding specific reagents, which can reduce the solubility of some components in the solution. The precipitation reaction must be reversible if the target components are required to form precipitation. While if the components are nontarget, the precipitation generated will be removed, so the precipitation reaction can be irreversible. According the addition of reagents or solvents, this method could be classified as follows [11].

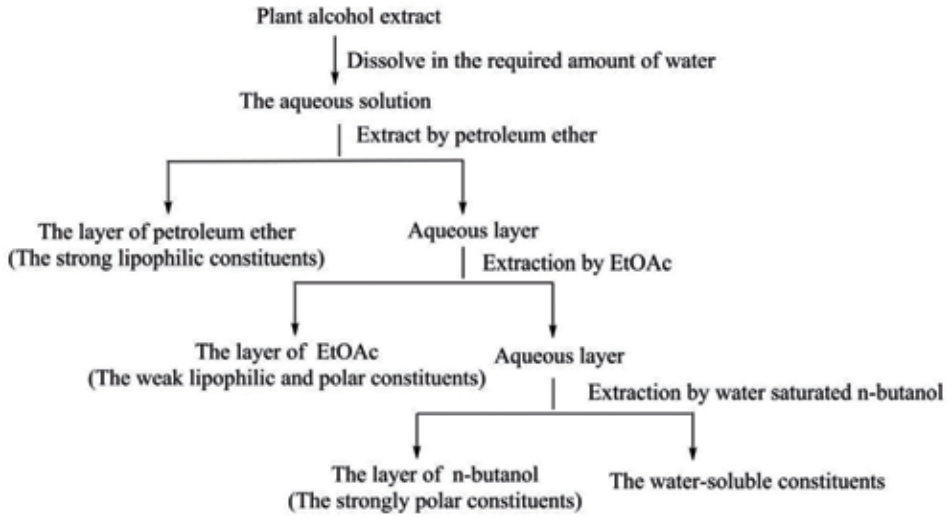

Figure 1.
Flow charts of common polarity gradient extraction method.

3.2.1 Solvent precipitation method

The solubility of some components in the mixed component solution can be changed by adding a specific solvent that can be mutually soluble with the solution, so it can be precipitated from the solution. The gradual precipitation by changing the polarity or amount of solvent added is called fractional precipitation. For example, using water as an extracting solvent to extract phytochemicals, ethanol is added to the water extracting concentrate to make its alcohol content more than 80%, and then polysaccharides, proteins, starch, gum, and so on will be precipitated and removed after filtration. The preceding procedure is called water extraction and ethanol precipitation. Crude polysaccharides from plants are often separated with this method. For example, see [12].

3.2.2 Exclusive reagent precipitation method

Some reagents could react selectively with certain chemical constituents to produce reversible precipitation, and the separation aims are achieved, which is called the exclusive reagent precipitation method. For example, alkaloid precipitation reagents such as Reynolds ammonium salt can precipitate after reacting with alkaloids, which can be used to separate alkaloids and nonalkaloids, or water-soluble alkaloids and other alkaloids. As another example, reactions of cholesterol and sterol saponins could form precipitation, which can separate them from triterpene saponins. Additionally, gelatin can precipitate tannins, which can be used to separate or remove tannins. In practical application, appropriate precipitation reagents should be selected according to the properties of target constituents and impurities in plants.

3.2.3 Salting out method

Adding inorganic salts to a certain concentration or saturated state in the water extract of plants can reduce the solubility of some components in water, thus they could be separated from water-soluble compounds. The inorganic salts commonly used for salting out are sodium chloride, sodium sulfate, magnesium sulfate, ferric

sulfate, etc. For example, extractions of tetrandrine from *Daemonorops margaritae* and berberine from *Berberis poiretii* could be achieved by salting out with sodium chloride or ammonium sulfate. Some water-soluble substances, such as proto-anemone, ephedrine, and matrine, are often extracted with organic solvents after adding a certain amount of salt to the water extract. For example, see [13].

3.3 Dialysis method

It is a method to let substances selectively penetrate through natural or synthetic semi-permeable membranes (or dialysis bags) under the action of concentration difference, pressure difference, or potential difference, so as to achieve the purpose of separation, classification, purification, or concentration. For example, when saponins, proteins, polypeptides, polysaccharides, and other substances in plants are separated and purified, dialysis can be used to remove inorganic salts, monosaccharides, and other impurities. On the contrary, large molecular impurities can also be left in the semi-permeable membrane, while small molecular substances can be separated and purified through the semi-permeable membrane into the solution outside the membrane [14].

3.4 Fractional distillation method

Fractional distillation is a method of separating components in liquid mixtures based on their different boiling points. It is usually categorized into atmospheric, vacuum, molecular distillation, and so on. It is mainly used for the separation of volatile oils and some liquid alkaloids in plants. For example, the boiling points of the two alkaloids in total alkaloids of *Cicuta virosa,* coniine, and conhydrine are 166–167°C for the former and 226°C for the latter, which are quite different from each other, and then they can be separated by the fractional distillation method. Generally, if the boiling point difference of compounds in liquid mixtures is above 100°C, the separation can be achieved by repeated distillation of the solution. If the boiling point difference of compounds is below 25°C, the fractionation column is needed. The smaller the boiling point difference is, the finer the fractionation device is needed [15].

3.5 Crystallization method

Crystallization is the process of solute precipitation from mother liquor with complex components, and it is an effective method to prepare pure substances. The initial crystallization is often impure and needs to crystallize again, which is called recrystallization. It is a method to separate compounds from the mixture by using the difference of solubility of each component in the solvent. Crystallization is one of the important technologies for plant chemists to prepare pure compounds.

When the content of a phytochemical is very high in one plant, crystals can be obtained by cooling or slightly concentrating the extract after extraction with appropriate solvent. For example, see [16].

Selecting suitable crystallization solvent is the key of the crystallization method. The ideal solvents for crystallization should possess the following characteristics: high solubility for the components to be purified at high temperature, low solubility at low temperature, insoluble for the impurities at high and low temperature, or soluble for the impurities at high and low temperature, moderate boiling point, no chemical reaction with the components to be crystallized, safe, low price, easy to obtain, and so on. Solvents commonly used for crystallization are methanol, ethanol, acetone, ethyl acetate, acetic acid, pyridine, etc. When crystals cannot be obtained with a single solvent, the crystallization operation can be carried out with a mixture of two or more solvents. Mixed solvents generally consist of two miscible

solvents, one of which has high solubility for the component to be crystallized, and the other has low solubility. Firstly, the sample to be crystallized is heated and dissolved in as few solvents as possible with high solubility. Then the second solvent with low solubility is added to the hot solution to make it turbid. Then the first solvent is added to dissolve the sample. The solution reaches saturation at this point and crystallizes when it is cooled. The purity of crystallization can be preliminarily identified by the crystal form, color, melting point, melting range, thin layer chromatography, paper chromatography, etc.

3.6 Classical chromatographic methods

Chromatography is the most commonly used method for the separation of chemical constituents of natural products. It possesses advantages of high separation efficiency, rapidity, and simplicity. By choosing different separation principles, different operation modes, different chromatographic packings, or applying various chromatographic methods jointly, the separation and purification of various types of phytochemicals could be achieved. It can also be used for the identification of compounds.

3.6.1 Adsorption chromatography

It is a kind of chromatography based on the difference of adsorptive capacity of adsorbents to different compounds. The commonly used adsorbents include silica gel, alumina, activated carbon, polyamide, and so on. Silica gel adsorption chromatography is widely used, and it is suitable to the separation of most of the plant chemical constituents. Alumina adsorption chromatography is mainly used for the separation of alkaline or neutral lipophilic components, such as alkaloids, steroids, and terpenoids. Activated carbon is mainly used for the separation of water-soluble substances, such as amino acids, carbohydrates and some kinds of glycosides. Polyamide, which allows the separation to take place based on the formation of kinds of hydrogen bonds, is mainly used for the separation of phenols, quinones, flavonoids, anthraquinones, tannins, etc. [17].

3.6.2 Gel chromatography (exclusion chromatography, molecular sieve chromatography)

Molecular sieve is the main principle of gel chromatography, which can separate mixture compounds according to the pore size of the gel and the molecular size of the compounds. Gel is a kind of solid material with a porous network structure. The molecules of the separated substances are different in size, so their ability to enter the gel is different. When the mixture solution passes through the gel column, the molecules smaller than the gel pores can enter the gel interior freely, while the molecules with larger size than the gel pores cannot enter the gel, and only pass through the gel particle gaps. Therefore, different movement rates are emerged. The molecules with large sizes are not excluded, and the retention time is shorter. The molecules with small sizes are detained because of its diffusion into the pores, thus the retention time is longer. There are many kinds of commercial gels, dextran gel and hydroxypropyl dextran gel are used most commonly [18].

3.6.3 Ion exchange chromatography

It is to separate chemical constituents according to the difference of dissociation degrees. In this method, ion exchange resin is applied as stationary phase and water or solvent mixed with water as mobile phase. The ionic components existing in the

mobile phase are absorbed by ion exchange resin after ion exchange reaction. Ion exchange chromatography is suitable for the separation of ionic compounds, such as alkaloids, amino acids, organic acids, peptides, and flavonoids. The ability of ion exchange reaction between compounds and ion exchange resins mainly depends on the compounds' dissociation degree and the amount of electric charges. If the dissociation degree of a compound is high (acidic or alkaline), it is easily exchanged on resins and difficult to elute. Therefore, when the compounds with different degree of dissociation are exchanged on the resin, the compounds with lower degree of dissociation are eluted before those with higher degree of dissociation [19].

3.6.4 Macroporous adsorption resin chromatography

It is a chromatographic method which combines the principle of adsorption and molecular sieve. Its chromatographic behavior possesses reversed-phase properties. Macroporous resin is a kind of solid macromolecule material with no dissociable group and porous structure and is insoluble in water. It is widely used in the separation and enrichment of natural compounds because of its stable physical and chemical properties (insoluble in acids, bases, and organic solvents).

In practical work, the water solution of the mixture to be separated is usually washed by water, water-containing alcohol solution with low to high concentration. The mixture can be separated into several components. The regeneration of macroporous adsorbent resin is convenient. It is often washed by 1 mol/L hydrochloric acid and 1 mol/L sodium hydroxide solution, respectively, first, then washed by distilled water to neutral, and stored in methanol or ethanol. The alcohol should be washed out with distilled water before using.

3.6.5 Partition chromatography

It is a kind of chromatography method to separate components by using different partition coefficients between stationary phase and mobile phase, which are immiscible liquids. Partition chromatography could be divided into normal phase chromatography and reverse phase chromatography. The polarity of stationary phase is stronger than that of mobile phase in normal phase partition chromatography, which is mainly used to separate polar and moderately polar molecular compounds. Carriers commonly used in normal phase distribution chromatography include silica gel, diatomite, cellulose powder, etc. Silica gel with water content of more than 17% can be used as a carrier for partition chromatography because of its loss of adsorption. It is the most widely used carrier for partition chromatography. In reverse phase partition chromatography, the polarity of mobile phase is stronger than that of stationary phase. The commonly used stationary phase is octadecylsilylated silica (ODS). The mobile phase is usually methanol-water or acetonitrile-water system, which is mainly used for the separation of nonpolar and moderately polar molecular compounds.

3.7 New technologies and methods

3.7.1 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is a rapid separation and analysis technology developed on the basis of conventional column chromatography. Its separation principle is the same as regular column chromatography, including adsorption chromatography, gel chromatography, partition chromatography, ion exchange chromatography, and other methods. HPLC columns are produced

with particle fillers (particle diameter 5–20 μm) and high pressure homogenate column loading technology. The eluents are pressed into the column by a high pressure infusion pump and equipped with high sensitive detectors and automatic recording and collection devices. As a result, it is far superior to conventional column chromatography in separation speed and efficiency. It has the characteristics of high efficiency, high speed, and automation. Preparative HPLC can be used to prepare a large amount of samples of high purity. HPLC has played an increasingly important role in the separation, qualitative identification, and quantitative analysis of plant chemical constituents. During the separation of many plant chemical constituents, it is necessary to separate trace constituents from a large amount of crude extracts. Usually, in the final stage of separation, samples with high purity are prepared by high or medium pressure liquid chromatography. Constant concentration eluents are mostly used in preparative HPLC. However, gradient elution is sometimes applied for samples that are difficult to be separated. Moreover, HPLC retains the advantages of liquid chromatography, such as a wide range of application and flexibility of mobile phase change. It can be applied to chemical constituents of difficult gasification, high molecular weight, or thermal instability.

The detectors commonly used in HPLC are ultraviolet detectors and differential refractive index detectors, but both have limitations. Differential refractive index detectors are sensitive to temperature change, the detection of a small amount of substances is often not ideal, and gradient elution cannot be used. As for ultraviolet detectors, they cannot detect samples without ultraviolet absorption. In recent years, a kind of mass detector, called evaporative light scattering detector (ELSD), has been applied in HPLC. It can not only detect samples without ultraviolet absorption, but also use gradient elution. It is suitable for most nonvolatile components [20].

3.7.2 Droplet counter-current chromatography (DCCC)

DCCC is an improved liquid-liquid partition chromatography based on the counter-current partition method. The formation of droplets is required when the mobile phase passes through a liquid stationary phase column. Droplets of mobile phase contact with stationary phase effectively, and form new surfaces in thin partition extraction tubes constantly, which promote the partition of solutes in two-phase solvents, and the chemical components of mixtures are isolated in immiscible two-phase droplets due to different partition coefficients. This method is suitable for the separation of phytochemicals with strong polarity. The separation effect is usually better than counter-current partition chromatography, and there is no emulsification phenomenon. Furthermore, nitrogen is used to drive the mobile phase, so the separated substance will not be oxidized by oxygen in the atmosphere. However, the solvent system which can generate droplets must be selected in this method, the amount of sample treated is small, and special equipment is needed.

DCCC possesses good reproducibility, and can handle crude extract samples of milligram to gram grade. It can be used in either acidic or basic conditions. Because no solid separation carriers are used, the phenomenon of irreversible adsorption and band broadening of chromatographic peaks can be avoided. Compared with preparative HPLC, DCCC consumes less solvent, but the separation time is longer and the resolution is lower. For example, see [21].

3.7.3 High speed counter-current chromatography (HSCCC)

HSCCC is also a liquid-liquid partition chromatography. It is another mild form of chromatography with no solid support and hence no chance of loss of substrate by binding to the column. The only media encountered by the sample are solvent

and Teflon tubing. The former is common to all forms of chromatography and the latter to most. The chemical constituents with higher partition coefficient in mobile phase are eluted first, whereas those with higher partition coefficient in stationary phase are eluted later.

HSCCC chromatography could avoid the shortcomings of irreversible adsorption and abnormal tailing of chromatographic peaks caused by solid carriers in liquid chromatography because it does not need solid carriers. The sample recovery is near 100% from a chromatography. It also has advantages of good reproducibility, high purity of separated compounds, and fast speed. It is suitable for the isolation and purification of wide kinds of phytochemicals, such as saponins, alkaloids, flavonoids, anthraquinoids, lignans, triterpenes, proteins, and carbohydrates. For example, see [22].

3.7.4 High performance capillary electrophoresis (HPCE)

It is an instrumental analysis method developed in the late 1980s combining classical electrophoresis with modern microcolumn separation technologies. In pharmaceutical analysis, the most commonly used separation modes are capillary zone electrophoresis, micellar electrokinetic capillary chromatography, and capillary gel electrophoresis. It is an efficient separation technology of large and small molecules in a hollow and thin inner diameter capillary (10–200 μm). The two ends of the capillary are immersed in a buffer solution and electrodes connected with a high voltage power supply are inserted separately. The voltage makes samples migrate along the capillary. According to the charge and volume of the separated substances, various molecules are separated under high voltage. In zone capillary electrophoresis, separation could be achieved by the movement of electrophoresis and electroosmotic flow. The strength of electroosmotic flow depends on the strength of electric field, PH value of electrolyte, composition of buffer solution, ionic strength, internal friction, and so on. Sample injection could be accomplished by pressing the sample into a capillary tube by atmospheric pressure or voltage.

HPCE has the advantages of high efficiency, microamount, economy, high automation, and wide application. However, it has the disadvantages of poor preparation ability, low sensitivity, and poor separation reproducibility. For example, see [23].

3.7.5 Affinity chromatography (AC)

Affinity chromatography is a unique chromatographic separation method based on the principle of reversible combination of high affinity and specificity between molecules. By simulating the reversible and specific interaction between biological molecules, affinity chromatography uses the adsorption medium coupled with affinity ligands as the stationary phase to adsorb target compounds. It is a development of adsorption chromatography. This method can selectively separate and analyze specific chemical constituents from complex samples. Firstly, ligands that can specifically bind to the target compounds are fixed on the filler carrier to make the chromatographic column. Then the mixture containing the target compounds is passed through the column. Only the target compounds which show affinity with the ligands can bind to the ligands and remain in the column. Finally, the adsorbed target compounds are eluted by changing the composition of the mobile phase and are separated from other chemical constituents. AC is mainly used for the separation and purification of proteins, especially enzymes, antigens, and antibodies. Its application range has been expanding along with the continuous development of technology in recent years. For example, see [24].

4. Structural identification of phytochemicals

The chemical structures of plant compounds must be identified or elucidated, which may provide the necessary basis for further study on the bioactivities, structure-activity relationships, metabolisms in vivo, structural modification, and synthesis of the active phytochemicals.

The quality of physiological active substances isolated from plants is often small, sometimes only a few milligrams, and the structural studies are often difficult to carry out with classical chemical methods, such as chemical degradation, derivative synthesis, etc. Therefore, spectral analysis is mainly used, that is, consuming sample as little as possible to obtain structural information as much as possible by measuring various spectra. Then comprehensive analysis is carried out with the assistance of literature data. If necessary, chemical means would be integrated into the former methods to determine the planar- and even the stereo-structures of the compounds.

4.1 Determination of the purity of the compounds

Before the structural investigation of an active compound, the purity must be determined, which is a prerequisite for the structural identification.

4.1.1 Measurement of physical properties

The crystals of each compound have certain shape, color, and melting point, which can be used as the basis for the preliminary determination of the purity. Generally, the crystal shape of a specific compound under the same solvent is consistent, the color is pure, and has a short melting range (generally at 1~2°C). But for compounds with double melting points or amorphous substances, the purity cannot be determined by this method.

4.1.2 Thin layer chromatography (TLC)

TLC, such as silica gel and paper chromatography, is the most commonly used method to determine the purity of compounds. Generally, a specific sample, showing an only spot (Rf value at 0.2~0.8) in three different developing agents, could be considered as a pure compound. In some cases, both normal and reverse phase chromatographic methods are needed.

4.1.3 Gas chromatography (GC) and high performance liquid chromatography (HPLC)

GC and HPLC are important methods in the purity determination of phytochemicals. GC is widely used in the analysis of volatile compounds. Both volatile and nonvolatile substances could be analyzed with HPLC, which possesses various advantages of high speed, high efficiency, sensitivity, and accuracy.

4.2 Major procedures of structural determination

The general procedures of structural determination of phytochemicals are shown roughly in **Figure 2**.

The structural identification of phytochemicals can be greatly simplified according to the researchers' habits, experiences, and skill levels of different technologies. However, the literature search almost runs through the whole process of structural

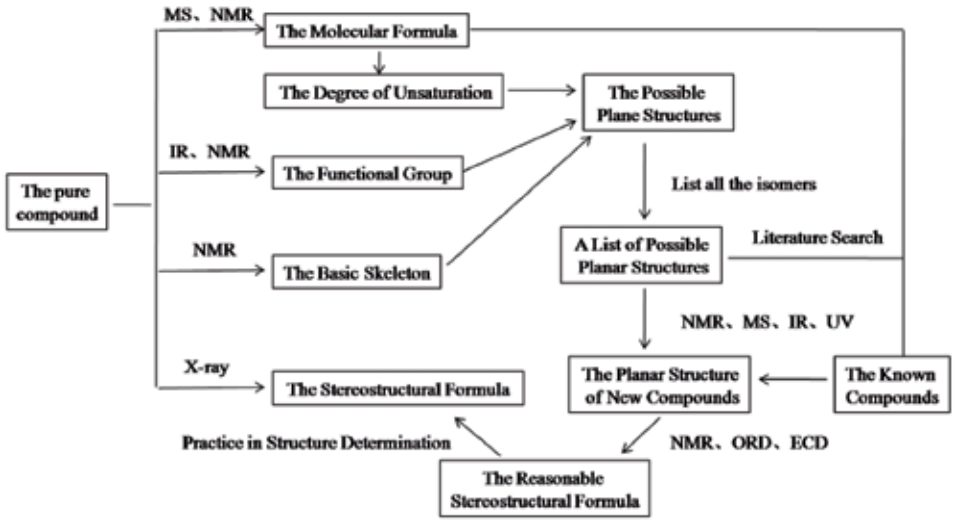

Figure 2.
The main procedures for studying the structures of phytochemicals.

research, no matter for known or new compounds. A large number of facts have been proved that taxonomically related plants, that is to say, plants of same or similar genus often contain chemical constituents of similar or even same chemical structures. Therefore, it is necessary to investigate literatures of chemical studies of the study object and the plants of its same and similar genera. It is necessary to understand not only the components from different plants of similar genera, but also their extraction methods, physicochemical properties, spectral data, and biosynthesis pathways before the extraction and separation of one specific plant. The SciFinder Scholar database is used most widely to quickly determine whether the compound was "known" or "unknown".

4.3 Spectral technologies

At present, spectrum analyses have become the main means to determine the chemical structures of plant chemicals. Particularly, with the developing of the superconducting nuclear magnetic resonance (NMR) and mass spectroscopic (MS) technologies, the speed of structural determination is greatly accelerated and the accuracy is improved. Here, the applications of infrared (IR), ultraviolet (UV), nuclear magnetic resonance (NMR), and mass (MS) spectra in the structural identification of phytochemicals are introduced briefly.

4.3.1 Ultraviolet-visible spectra (UV-Vis)

UV-vis spectrum is a kind of electron transition spectrum, which is generated after the molecules absorbing the electromagnetic waves with wavelength at the range of 200–800 nm. The valence electrons in the molecules absorb light of certain wavelengths and jump to the excited state from the ground state, and then UV spectra are recorded.

Compounds containing conjugated double bonds, α,β-unsaturated carbonyl groups (aldehydes, ketones, acids, and esters), and aromatic compounds could show strong absorption in UV spectra because of $n \rightarrow \pi^*$ or $\pi \rightarrow \pi^*$ transitions. Therefore, UV spectrum is mainly used to identify the presence of conjugated systems in the structures.

UV spectra could provide the following information: (1) the compounds show no UV absorption at 220–800 nm, indicating the compounds were aliphatic hydrocarbons, aliphatic cyclic hydrocarbons, or their simple derivatives. (2) The compounds show strong absorption at 220–250 nm, indicating that the compounds possess conjugated diene, α,β-unsaturated aldehyde, or ketone substructures. (3) The absorption at 250–290 nm is moderately strong, indicating that the compounds possess benzene rings or aromatic heterocycles. (4) Weak absorption at 250–350 nm indicates the presence of carbonyl or conjugated carbonyl groups. (5) Strong absorptions at above 300 nm indicate that the structures possess long conjugated chains.

Generally, UV spectrum can only provide part of the structural information, rather than the whole structural information of a compound, so it can only be used as an auxiliary method to identify the structures. It possesses practical value to determine the structures of phytochemicals with conjugated substructures.

4.3.2 Infrared spectra (IR)

IR is caused by the vibration-rotational energy level transition of the molecule, ranging from 4000 to 625 cm^{-1}. The region above 1250 cm^{-1} is functional group region, and the absorption of characteristic functional groups such as hydroxyl, amino, carbonyl, and aromatic rings occurs in this region. The region of 1250 to 625 cm^{-1} is fingerprint region, and the peaks appear mainly due to the stretching vibrations of C-X (X = C, O, N) single bonds, and various bending vibrations. IR is mainly used for the determination of functional groups and the types of aromatic ring substitution. In some cases, IR can also be used to determine the configuration of plant chemical constituents. For example, there is a significant difference between 960 and 900 cm^{-1} for 25R and 25S spirostanol saponins.

4.3.3 Mass spectrometry (MS)

In a mass spectrometer, mass and strength information of molecular and fragment ions is recorded after the molecules are ionized and enter into the collector under the action of electric and magnetic fields. The abscissa represents the mass-to-charge ratio (m/z) and the ordinate represents the relative intensity in a MS spectrum. Unlike IR, UV, and NMR spectra, MS is mass spectrum, which characterizes fragment ions, not an absorption spectrum. Its role is to determine weights, formulas, and fragment structures of molecules.

With the rapid development of modern techniques, new ion sources have emerged in recent years, which make MS play more important role in determining the molecular weights, elemental composition, detecting functional groups by cleavage fragments, identifying compound types, and determining carbon skeletons [25]. In the structural analysis, the information of molecular weights could be obtained on the basis of molecular ion peaks, and the molecular formula could be obtained by high-resolution mass spectrometry (HR-MS). Fragment ion peaks, combined with molecular ion peak, could be applied to conjecture chemical structures. Tandem mass spectrometry even can isolate and analyze the mixed ions again. According to the types of ion sources, common mass spectrometry could classified as electron impact mass spectrometry (EI-MS), chemical ionization mass spectrometry (CI-MS), field desorption mass spectrometry (FD-MS), fast atom bombardment mass spectrometry (FAB-MS), matrix-assisted laser desorption mass spectrometry (MALDI-MS), electrospray ionization mass spectrometry (ESI-MS), tandem mass spectrometry (MS–MS), and so on.

4.3.4 Nuclear magnetic resonance (NMR)

With the birth of Fourier transform spectrometer, the great progress of radionuclide research such as ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P, and the advancement of two-dimensional and three-dimensional nuclear magnetic technology, NMR has become the most important spectroscopic method to determine chemical structures. Particularly, hydrogen spectrum and carbon spectrum are most widely used. During the operation of nuclear magnetic resonance spectrometer, compound molecules are irradiated by electromagnetic waves in a magnetic field, energy level transitions occur after the atomic nuclei with magnetic distance absorb a certain amount of energy, and then NMR spectrum is obtained by mapping the absorption strength with the frequencies of the absorption peaks. It can provide structural information about the type and number of hydrogen and carbon atoms in the molecule, the modes they are connected, the surrounding chemical environment, configuration, and conformation [26].

4.3.4.1 Commonly used deuterated reagents

Samples used to measure NMR spectra include solids, liquids, and gases. Liquid high-resolution NMR is most widely used. The solvent used in the measurement of NMR must be deuterated. The commonly used deuterated reagents to dissolve samples and their chemical shifts of their residual proton and carbon signals are shown in **Table 1**.

4.3.4.2 Proton nuclear magnetic resonance spectroscopy (1H-NMR)

Resonance absorption peaks are generated after hydrogen protons absorb electromagnetic waves of different frequencies in an external magnetic field. ^{1}H-NMR possesses high sensitivity, easy measurement, and wide application. ^{1}H-NMR spectrum can provide structural information of chemical shifts (δ), coupling constants (J) that indicate the coupling relationships between different hydrogen nucleus, and the number of protons (the peak area is proportional to the number of protons that cause the absorption).

Because of the different surrounding chemical environment, the ^{1}H nuclei possess different magnetic cloud densities and magnetic shielding effects caused by the rotation around the nucleus, and then different types of ^{1}H nuclear resonance signals appear in different regions. Tetramethylsilane (TMS) is usually used as a reference compound. Compared with the general compounds, the shielding effect of protons and carbons on the methyl groups is stronger in TMS. Therefore, regardless of the hydrogen spectrum

Solvent	δ_C	δ_H
$CDCl_3$	77.0	7.24
CD_2Cl_2	53.8	5.32
CD_3OD	49.0	3.3
Acetone-d_6	29.8, 206.0	2.04
D_2O	—	4.7
DMSO-d_6	39.5	2.49
C_6D_6	128.0	7.16
C_5D_5N	123.6135.6149.9	7.2, 7.6, 8.7

Table 1.
Chemical shifts of common deuterated solvents (TMS is an internal standard).

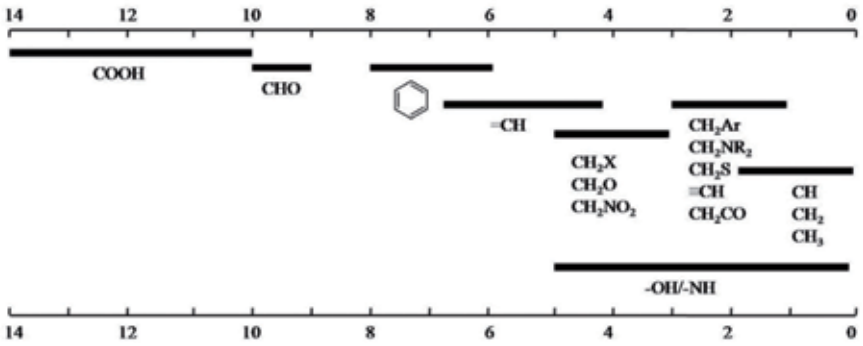

Figure 3.
[1]H-NMR chemical shift range of common hydrogen protons.

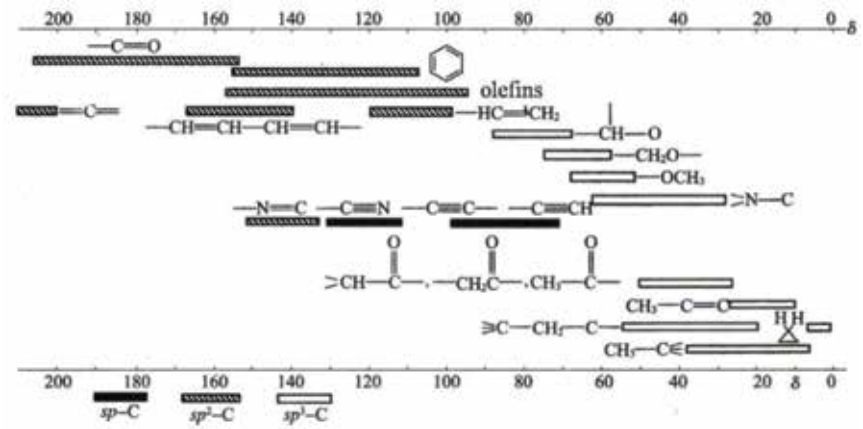

Figure 4.
[13]C-NMR chemical shifts of common carbon signals.

or the carbon spectrum, the absorption peaks generated by the general compounds appear in the lower field than TMS, that is to say, δ values generated by common compounds is positive. The chemical shifts of the ^{1}H-NMR spectrum is mostly in the range of δ0–20. Some typical chemical shifts of ^{1}H nuclei are shown in **Figure 3** [4].

In addition to the normal ^{1}H-NMR spectrum technique, there are some auxiliary techniques that assist in structural analysis, such as selective decoupling, heavy hydrogen exchange, addition of reaction reagents, and dual irradiations.

4.3.4.3 Carbon nuclear magnetic resonance spectroscopy (^{13}C-NMR)

^{13}C-NMR spectra can provide structural information of organic compounds, including the number, types, and chemical environment of carbon atoms [27]. It is one of the important means for the structural identification of organic compounds. Especially, where there are serious signal peak overlaps in the ^{1}H-NMR spectrum, or the molecules contain several quaternary carbon atoms, ^{13}C-NMR spectra will provide crucial information for the structure identification. The chemical shifts of common carbon signals are shown in **Figure 4** [4].

Common ^{13}C-NMR techniques include proton broadband decoupling, off resonance decoupling (OFR), insensitive nuclei enhanced by polarization transfer (INEPT), and distortionless enhancement by polarization transfer (DEPT). Proton broadband decoupling and DEPT spectra are most commonly used at present.

4.3.4.3.1 Proton broadband decoupling

Proton broadband decoupling spectrum is measured after ^{1}H nuclei are saturated with broadband electromagnetic radiation. At this point, the couplings between ^{1}H and ^{13}C are completely eliminated, and all ^{13}C signals are shown as singlets, so it is very convenient to determine the chemical shift of ^{13}C signals. In addition, because of the NOE effect of ^{1}H after irradiation, the signal of ^{13}C signal connected with ^{1}H will be increased, while the quarterly carbon signal will show weak absorption peaks.

4.3.4.3.2 Distortionless enhancement by polarization transfer (DEPT)

It is an improved method of INEPT, in which a *J*-modulation is accompanied by a polarization transfer from the protons to coupled carbons, leading to significant improvement in sensitivity. In DEPT spectrum, by changing the pulse width (θ), which could be designed as 45°, 90°, and 135°, during irradiation of ^{1}H, different carbons could show different strengths and signs. The results are similar with INEPT spectrum. When θ = 45°, all CH, CH_2, and CH_3 groups display positive signals; when θ = 45°, only CH groups show positive signals; when θ = 135°, both CH and CH_3 groups show positive signals, while CH_2 groups show negative signals. Quarterly carbons show no signal peaks in DEPT spectra. An example of DEPT spectra is shown in **Figure 5**.

4.3.4.4 Two-dimensional nuclear magnetic resonance spectroscopy (2D-NMR)

Two-dimensional correlation spectroscopy (2D-COSY) is the most important and widely used in 2D-NMR spectroscopy. 2D-COSY spectra can be divided into homonuclear and heteronuclear correlation spectra. Both abscissa and ordinate represent chemical shifts in 2D-COSY. Common correlation spectrum types are show as follows.

4.3.4.4.1 ^{1}H-^{1}H COSY spectrum

It is a kind of chemical shift correlation spectrum between ^{1}H and ^{1}H. It is the coupling correlation spectrum between protons in the same coupling system. The adjacent hydrogen groups could be determined by their coupling relationships (^{3}J) shown in ^{1}H-^{1}H COSY spectra.

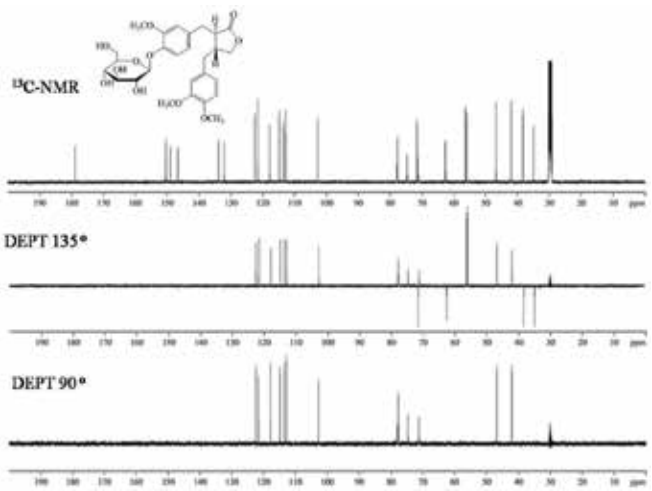

Figure 5.
The DEPT spectrum of Arctiin (CD_3OD).

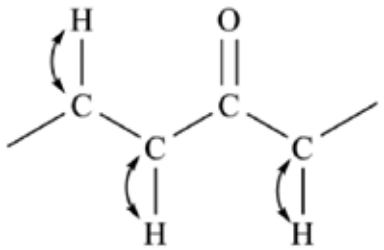

Figure 6.
Schematic diagram of correlations between ^{1}H and ^{13}C in the HSQC or HMQC spectrum.

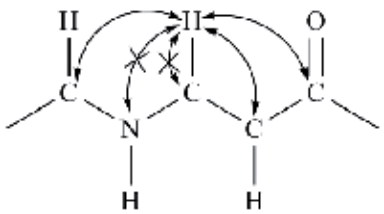

Figure 7.
Schematic diagram of correlations between ^{1}H and ^{13}C in the HMBC spectrum.

In addition, for compounds of aromatic systems, double bond systems, and some particular configuration systems, ^{1}H-^{1}H COSY spectra can show ^{4}J coupling or longer coupling relationships of hydrogen groups. It is very important for the elucidation of an unknown structure.

4.3.4.4.2 HSQC (HMQC) spectrum

^{1}H detected heteronuclear single quantum coherence (HSQC) and ^{1}H detected heteronuclear multiple quantum coherence (HMQC) can display the correlations between ^{1}H and ^{13}C. HSQC possesses higher sensitivity and wider application than HMQC. In the HMQC or HSQC spectrum, the signals occurred at the crosses of chemical shifts generated by corresponding carbons and protons (**Figure 6**).

4.3.4.4.3 HMBC spectrum

HMBC spectrum is short for ^{1}H detected heteronuclear multiple bond correlation, which associates the ^{1}H nucleus with ^{13}C nucleus of long-range coupling. HMBC could detect the long-range coupling of ^{1}H-^{13}C sensitively ($^{n}J_{CH}$, $n \geqq 2$). Moreover, the correlation signal peaks between protons and quaternary carbons that are two or three bonds apart could also be shown in HMBC spectra, as shown in **Figure 7**. From the HBMC spectrum, we can get the connection information of the carbon chain skeletons, the structure information of the quaternary carbons, and the structural information of the coupling systems that are cut off by heteroatoms.

4.3.4.4.4 NOESY spectrum

When two groups of protons are located at rather close spatial distances, irradiation of one group will enhance the signal strength of another, which is known as nuclear Overhauser enhancement (NOE). The NOE spectrum can determine the spatial relative position, stereoscopic configuration, and dominant conformation of some groups in the molecule, which is very important for the study of the stereostructures of organic compounds.

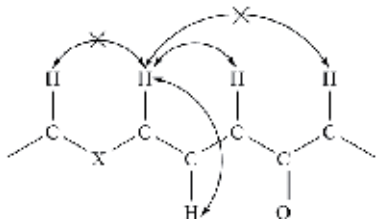

Figure 8.
Schematic diagram of correlations between ^{1}H and ^{13}C in the TOCSY spectrum.

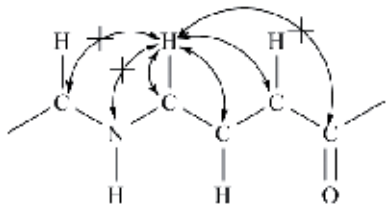

Figure 9.
Schematic diagram of correlations between ^{1}H and ^{13}C in the HSQC-TOCSY spectrum.

2D-NOE (NOESY) spectra could show the NOE correlations of protons. The greatest advantage of NOESY is that all the NOE information between protons of a compound could be shown in one spectrum. However, not all the cross peaks are NOE correlation signals, the residual correlation signals of COSY are often shown in NOESY spectrum as well, which should be paid attention during spectroscopic analysis.

4.3.4.4.5 Total correlation spectroscopy (TOCSY) spectrum

The TOCSY spectrum shows the correlation of the entire spin system, which is different from the ordinary ^{1}H-^{1}H COSY. The relationships between the nuclei that generated the correlation peaks are shown in **Figure 8**. Not only the correlation signals of a proton with protons connected to the adjacent carbons, but also its correlation signals with other protons in a whole spin system could be shown in the TOCSY spectrum, which provides important basis for the connection of structural fragments.

4.3.4.4.6 HSQC-TOCSY spectrum

HSQC-TOCSY is a kind of combined 2D-NMR spectrum. Comprehensive results of HSQC and HMBC are obtained by using a long pulse sequence. The correlation is shown in **Figure 9**. It is very useful for the assignment of carbon and proton signals in complex chemical structures. For example, for saponins with a series of glycosyl groups, the signals generated by glycosyl groups are often overlapped seriously in common NMR spectra, which causes difficulty to assign signals of glycosyls. HSQC-TOCSY spectrum will play an important role in this case. The spectrum includes the information of HSQC, HMBC, and ^{1}H-^{1}H COSY.

4.3.5 Optical rotary dispersion (ORD) and circular dichroism (CD)

Polarimetry is an optical method used widely in the studies of asymmetric structures, which appeared very early. The progress of the sensitive method such as ORD and CD made it possible to study stereostructures of chiral compounds more deeply. Both of them are spectra related to the optical activity of compounds, and

could provide information of absolute configurations, dominant conformations, and reaction mechanisms of chiral compounds, that cannot be replaced by any other spectroscopic methods [28].

4.3.5.1 Optical rotary dispersion (ORD) spectrum

The specific rotation [α] of a chiral compound depends upon the wavelength of the monochromatic light wave. The measurement of specific rotation as a function of wavelength is called optical rotator dispersion (ORD). The common types of ORD curves are as follows.

4.3.5.1.1 Plain curves

The ORD spectrum of an optically active compound with no chromophores is plain without peaks and troughs. An ORD curve of specific rotation increases with decrease of wavelength which is called positive plain curve, while in the case of negative plain curve, negative rotation increases with decrease of wavelength (see **Figure 10**).

4.3.5.1.2 The cotton effect curve

If there is a simple chromophore in the molecule, the ORD curve is very different from plain curve. Near the absorption wavelength region of chromophore, a peak and a trough are exhibited, which is called the Cotton effect, and the spectrum drawn is called the Cotton effect curve. The spectrum with only one peak and one trough is called pure Cotton effect curve, while the spectrum with several peaks and troughs is called complex Cotton effect curve. The Cotton effect is called positive when the trough is observed at a shorter wavelength then peak. Conversely, the Cotton effect is called negative if the trough is observed at a longer wavelength than the peak. Cotton curves of $\triangle^5$-cholestenone are shown in **Figure 11,** which shows A and B possess the same structural formula, while different opposite configurations.

4.3.5.1.3 Complex Cotton effect curve

For compound with two or more different chromophores, its ORD curve may possess multiple peaks and troughs, which is called complex Cotton effect curve. Each ORD curve is the average effect of each chromophore in the molecule, and the contribution of each orientation and conformation of the molecule. Hence the Cotton effect curve is often complex.

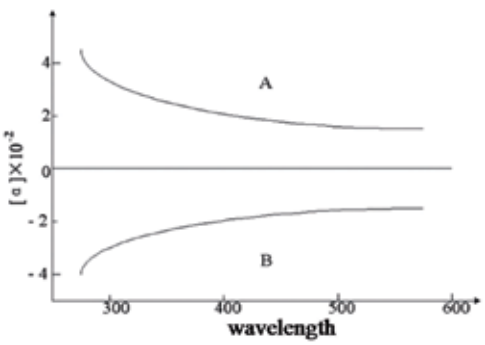

Figure 10.
ORD plain curves (A: Positive plain curve; B: Negative plain curve).

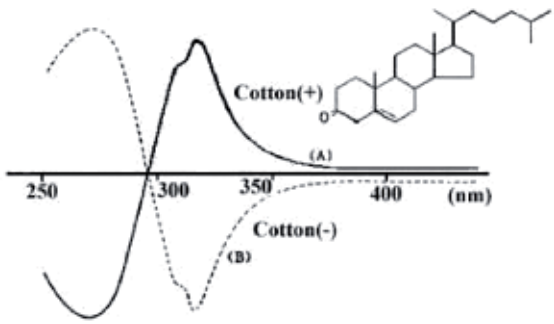

Figure 11.
The Cotton effect curves of △⁵-cholestenone (A) natural cholesterone (+) cotton; (B) Cholesterone in the opposite absolute configuration (−) cotton.

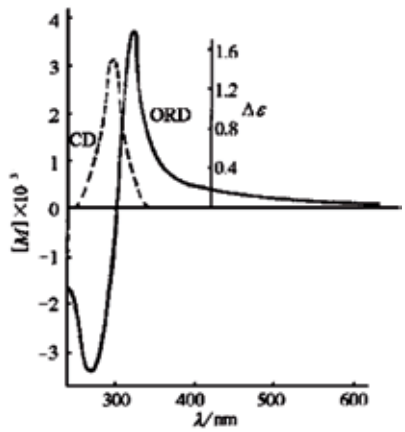

Figure 12.
The ORD and CD spectrum of (+)-camphor.

4.3.5.2 Circular dichroism (CD) spectrum

Optically active compounds have different molar absorption coefficients for left-circularly and right-circularly polarized light that make up plane polarized light, which is called circular dichroism (CD). The difference value between the two molar absorption coefficients ($\Delta\epsilon = \epsilon_L - \epsilon_R$) changes with the wavelength of the incident polarized light. With $\Delta\epsilon$ as the ordinate, the wavelength as the abscissa, the spectrum obtained is called circular dichroism spectrum. Because the absolute value of $\Delta\epsilon$ is very small, it is often replaced by molar ellipticity [θ]. The relationship between [θ] and $\Delta\epsilon$ is as follows.

$$[\theta] = 3300\Delta\epsilon. \quad (1)$$

Because $\Delta\epsilon$ could be positive or negative, the circular dichroism curve also could be classified as positive and negative. In the CD spectrum showing positive Cotton effect, only a peak appears near the λ_{max} of the chromophore in the molecule. Conversely, a trough appears in the CD spectrum showing negative Cotton effect. Therefore, CD spectra are simpler and easier to analyze than ORD spectra. For example, the ORD and CD spectra of (+)-camphor are shown in **Figure 12**. CD is more widely used than ORD in the study of chiral compounds.

4.3.6 Single crystal X-ray diffraction method

Single crystal X-ray diffraction could be applied independently to analyze the structures, components, contents, configurations, conformations, solvents, and crystal forms of samples. It is widely used in the stereostructural study of natural compounds, synthetic compounds, peptides, proteins, etc. Therefore, X-ray diffraction analysis is a necessary physical method in the field of structure and function research of modern natural drugs.

Single crystal X-ray diffraction is a kind of quantitative analysis technology, which can provide three-dimensional structural information of molecules, including atomic coordinates, bond length, bond angles, dihedral angles, hydrogen bonds, salt bonds, coordinate bonds, and so on. In addition, it is also a reliable method to determine the absolute configuration of chiral drug molecules and the epimers in the stereochemical structures. For example, see [29].

5. Conclusions and future directions

In recent years, study on phytochemicals from plants becomes more and more popular due to their demonstrated health benefits. A number of plants having high contents of phytochemicals (particularly phenolic acids and flavonoids) with associated antioxidant activities have been increasingly utilized. Complementary research is also needed to enhance the potential functionalities of the phytochemicals in future, where such plants have shown to contain numerous phytochemicals that may be beneficial to human health. The compiled results indicated that many of their bioactive compounds remain to be fully isolated, identified, and characterized (alkaloids, diterpenoids, and so on).

Therefore, phytochemicals can be considered as the source of natural medicines. The compounds of plants are bioaccessible and bioavailable in humans with some demonstrated health benefits, including antioxidant, anti-inflammatory, anti-cancer, anti-microbial, hypoglycemic action, etc. Additional well-designed human intervention studies and clinical trials are needed to validate the health benefits of phytochemicals.

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Chapter 4

The Phytochemical Composition of Medicinal Plants: Brazilian Semi-Arid Region (Caatinga)

Iago Almeida da Ponte, Murugan Muthuvel, Sudarsini Saravanabhavan and Stephen Rathinaraj Benjamin

Abstract

Carnauba wax, the most important vegetable wax under the economic and extracted from the leaves of the carnauba (*Copernicia prunifera* (Miller) H. E. Moore), is extensively applied in food due to its physiochemical characteristics with a majority of esters. *p*-Methoxycinnamic acid diesters obtained from the ceriferous powder of carnauba wax (PCO-C) have been associated with biological actions. However, being a versatile product, many types of research have been carried out seeking to expand the possibilities of applications of this raw material. Furthermore, different experimental studies on the pharmacological activities have also been undertaken in recent years and have tested various biological activities, such as hypolipidemic, hypocholesterolemic and hypoglycemic effects in mice. Therefore, in this book chapter, it is reviewing the development of a process of extraction of 4-hydroxycinnamic acid diesters of carnauba wax powder and investigates their biological actions and physical and chemical characteristics.

Keywords: Caatinga, *Copernicia prunifera*, *p*-methoxycinnamic acid, phytochemistry, biotechnological uses

1. Introduction

Caatinga is a Brazilian biome with a semi-arid climate, vegetation with small leaves and adapted to dry periods, as well as great biodiversity. This biome is found in areas of northeastern Brazil, in the states of Maranhão, Piaui, Ceará, Rio Grande do Norte, Paraiba, Pernambuco, Alagoas, Sergipe, Bahia and part of Minas Gerais. This whole area covers about 844,000 km^2, or 11% of the Brazilian territory [1]. This ecosystem is very important from the biological point of view because it has unique fauna and flora, formed by vast biodiversity, rich in genetic resources and vegetation consisting of species, woody, herbaceous, cactus, and bromeliads. It has 932 species of plants, 148 mammals and 510 birds, for example, and many of these species occur only in the Caatinga.

The main characteristics of the Caatinga are: Strong presence of shrubs with twisted branches and deep roots; Presence of cacti and bromeliads; Shrubs usually lose their leaves almost completely in times of drought (property used to prevent evaporative water loss). The leaves of this vegetation type are small in size; Caatinga soil has low fertility and is stony. Caatinga biodiversity supports various economic

activities aimed at agroforestry and industrial purposes. Despite its importance, Caatinga vegetation is a type of vegetation adapted to the aridity of the soil and the scarcity of water in the region. They are classified depending on the natural conditions of the areas and different characteristics like strata: arboreal: with species ranging between 8 and 12 m in height; shrub: with species ranging between 2 and 5 m in height; herbaceous: with species below 2 m in height [2]. *Copernicia prunifera* (Miller) H. E. Moore (Arecaceae) family, a typical desert flora animal categories and exclusively located of areas through the Caatinga biome [3]. It is also known as "Tree of life", *carnauba*, *carandauba*, *carnaba*, *carnaubeira*, *caranaiba*, *carnaúva*, among others.

The carnauba is a palm tree very common in the northeast region whose main feature is its height, which can reach 15 m. The stem is straight and cylindrical, with a diameter that can vary from 10 to 20 cm and has thorns at the bottom. The tree provides fruits from November to March. They are greenish when young and turn purple when they mature. Its fruits are well used to feed farm animals. According to Brazilian specialized guidelines characterize the "pó de olho" and "pó de palha" wax powder as category A and B, respectively [4–6]. Meanwhile, the apical leaves have found lower chlorophyll content, type A wax has a pigmentation that shifts from white to light yellow and has a higher incentive than category B, which has a greenish-gray pigmentation.

Carnauba wax is derived besides the leaves regarding the *Copernicia prunifera* tree (**Figure 1**) and is made principally out of long-chain wax esters (80%), 20% contained fatty acids, fatty alcohols, and hydrocarbons [7–9]. Carnauba wax has the most maximum melting point conditions of all vegetable waxes and has been utilized in an assortment of items, including cosmetic and food products, nourishment items, and the paper area [9]. Additionally, this material is widely used in folk medicine, including the treatment of rheumatism and syphilis. However, carnauba

Figure 1.
Copernicia prunífera *tree (from Fortaleza, Brazil).*

wax is utilized as a stabilizer to different waxes, for example, beeswax to improve the melting point, taking into account expanded utilization of these waxes [10].

Despite that, a great deal of research has been carried out into an attempt to extend the chances of potential outcomes of utilizations of this crude material. With this goal, analysis has been finished streamlining customary applications and examining advancements, for example, the utilization of wax for the microencapsulation of flavors and as a wellspring of molecules following up on the avoidance and treatment of, diabetes, dyslipidemia, and others. The wax is an item to show the level of local consumption with extraordinary potential for use all through the Brazilian food production chain. Along these lines, it is imperative to experts in the food area to more likely comprehend this crude material so as to misuse its maximum capacity. In this way, this review talks about the utilization of carnauba wax in food ranging from the nutritional, phytochemical evaluation, ethnobotanical and biotechnological applications.

2. Nutritional and chemical composition

Carnauba wax consists of complex mixture regarding long-chain fatty acids, free alcohols, esters, aromatic acids, aliphatic acids, triterpene diols, cinnamic acids, proteins, and hydroxy acids and ω-hydroxycarboxylic free acids [10–14]. Recently, one triterpene carnaubadiol was also isolated and identified present in the leaves were reported. The inorganic compounds existing such as aluminum, copper, magnesium, zinc, manganese, calcium, iron, and sodium [15]. Recent studies continued to assess more genetic resource of carnauba wax while revealing a more extensive variety in the nutritional composition as described in the following sections.

2.1 Pectin

Paim et al. [16] extracted the pectin from the aqueous pulp extracts (APE) of *Copernicia prunifera* analyzed by chromatographic and spectroscopic methods. From this study, the pectin substance acquired from the pulp of unripe fruits of *C. prunifera* demonstrated an estimation of 2.9%. Additionally, the pectin was observed by using the absorption spectra by demonstrating several carbonyl groups in the form of esterified and carboxylate compounds. Furthermore, the thin layer chromatography (TLC) technique identified galactose, galacturonic acid patterns, and arabinose compounds, respectively. By using, ^{13}C NMR spectroscopy analysis method, various forms of polymers were recognized in the pectic polysaccharides chain compounds including D-galacturonic acid (major signs), D-galactose (lower signs) and the peak molar mass (*M*pk) was determined by gel permeation chromatography of 0.6×105 g mol^{-1}. All these studies highlighted that pectin presence of higher molecular weight and a igher degree of esterification displaying improved performances.

2.2 Triterpenes

Almeida et al. [11], explored phytochemical investigation of hexane and ethanolic extracts carnauba wax (types 1 and 4) was analyzed and identified 16 dammarane-type triterpenes, with 13 newly categorized as (24R*)-methyldammara-20,25-dien-3α-ol and a mixture of alkyl (24R*)-methyldammar25-en-20-ol-3β-carboxylates, and 3 triterpenes such as carnaubadiol,(24R*)-methyldammara-20,25-dien-3β-ol and (24R*)-24-methyldammara-20,25-dien-3-one. Furthermore, fatty alcohols such as docosanol, eicosanol, and hexacosanol, tetracosanol as well as four sterols

(campesterol, cholesterol, sitosterol and stigmasterol) were detected and identified. These finding isolated compounds were characterized by using Infrared (IR) spectra and confirmed by classical chromatographic techniques such as gas chromatography-flame ionization detections (GC-FID), ^{1}H and ^{13}C nuclear magnetic resonance (NMR) methods.

^{1}H and ^{13}C NMR spectroscopy techniques have been applied for structural elucidation of dammarane triterpenoids [basic skeleton as carnaubadiol] in carnauba wax powder obtained from the leaves of *Copernicia cerifera* [13]. Totally four types of triterpenes were identified from hexane extract of carnauba wax. Four of these compounds were, structure1, (24RŁ)-24-methyldammara-21,25- diene-3ˇ-ol, structure of 2 and 3 was distinguished as (24RŁ)-24-methyldammara-25-ene-3-one. Furthermore, the structure of 4, illustrated as (E)-25-hydroperoxydammar-23-ene-3ˇ,20-diol. The chemical composition analyzed after successive column chromatography using silica gel hexane followed by ethanol at room temperature, respectively.

2.3 Proteins

Cruz et al. [12] isolated the wax protein from "Carnauba" wax and the samples accomplished by SDS-Tricine-gel electrophoresis technique. It showed relative molecular masses of 26,000 (β-1,3-glucanase) and 24,000 Da (class III chitinase), respectively. However, these proteins have been involved in the resistance systems of plants against insects and pathogens. In addition, the authors found that proteins segregated from the different portions of carnauba wax have antifungal enzymatic action. These chemicals, chitinase and β-1,3-glucanases, can hinder early development of organisms and modify hyphal (threadlike fibers like mycelium of parasites) morphology of growths developing within the proteins.

2.4 Ethnobotanical study of carnauba wax

2.4.1 Antioxidant activity

Phenolic bioactive and polyphenolic compounds occur normally and significant segments of the human diet due to their antioxidant capacity that decreases oxidative stress-inducing cellular damage associated with severe pathologies such as cardiovascular, neurodegenerative diseases and cancers [17]. The simplest bioactive phytochemicals containing a single substituted phenolic ring, like cinnamic acid and caffeic acid. Cinnamic acid is a naturally proceeding organic acid in plants, has low toxicity and a broad spectrum of biological activities. However, cinnamic acid derivatives comprise a series of trans-3-phenylpropenoic acids which differ in their substituents on the aromatic ring. The presence of a benzene ring and a low unsaturated hydrocarbon chain determines its low polarity and solubility in water. The most common cinnamic acid derivatives in plants are *p*-coumaric, caffeic, and chlorogenic acids and hydroxybenzoic and hydroxycinnamic acids, respectively [18].

Claisa et al. [19] studied the antioxidant activity by ABTS and FRAP methods and *in-vivo* cellular antioxidant activity assay. The antioxidant activity of ethyl acetate and hexane extracts of *p*-methoxy cinnamic diester (**Figure 2**) (PCO-C) showed the values 107.27 ± 3.92 μM Trolox/g and 73.3 ± 1.83 μM iron sulfate/g, respectively. From these results showed significant antioxidant activity values due to the presence of derivative of cinnamic acid compounds [20]. In addition, the *in-vivo* antioxidant activity showed lower ROS values in PCO-C alone (50 and 250 μg/mL). Accordingly, PCO-C did not produce any cellular oxidation significantly it produces low level of ROS, because of the oxidation of lymphocytes endured with

H_2O_2. However, PCO-C had a best antioxidant effect in high dose level (250 μg/mL) similar of Trolox (80 μM) and found an oxidation inhibition capacity in human peripheral blood lymphocytes (HPBLs). According to the authors, it is revealed that antioxidant activates arise from *p*-methoxy cinnamic diesters presence of phenolic compounds PCO-C. Therefore, the presence of these excellent antioxidant potentials of produce reflects its ability to deliver bioactive substances that neutralize reactive oxygen species (ROS) and scavenge free radicals produced by oxidative stress.

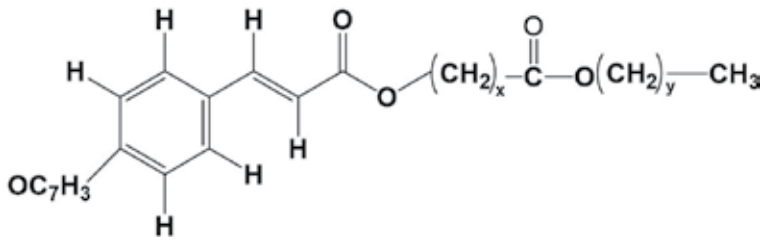

Figure 2.
Chemical structure of p-methoxy cinnamic acid diesters.

Rufino et al. [21] reported that significant antioxidant activities of polyphenol-rich extracts from tropical fruits and dry fruits especially carnauba, both DPPH, ABTS, FRAP, β-Carotene oxidation methods and total phenolic contents were performed. The antioxidant activity of the methanolic and ethanolic extracts of fresh fruits of carnauba found decreased values DPPH values 3549 ± 184 g fruit/g DPPH$^{\cdot}$ and increased ABTS values 10.7 ± 0.2 μmol Trolox/g, FRAP values 15.5 ± 0.4 μmol Fe_2SO_4/g and high β-Carotene bleaching values found 87.7 ± 2.7(% O.I) and extractable polyphenols values 830 ± 28.3 mg GAE/100 g, respectively. Additionally, the bioactive compounds values (mg/100 g fresh matter) such as, vitamin-C (78.1 ± 2.6), total anthocyanins (4.1 ± 0.1), yellow flavonoids (66.4 ± 2.3), and total carotenoids (0.6 ± 0.2), chlorophyll (4.2 ± 0.2), respectively. According to the authors, this study provides an adaptation of ABTS, DDPH, FRAP and β-carotene bleaching methods, along with an evaluation of the compounds related to antioxidant potential. The results showed promising perspectives for the exploitation of non-traditional tropical fruit species with considerable nutritional properties and antioxidant capacity.

2.4.2 Anti-microbial and anti-fungal activities

The prevention of the decomposition and assurance of the food safety can be attained by the use of compounds that act as preservatives of foodstuffs, by presenting antimicrobial properties, preventing the degradation by enzymatic and non-enzymatic reactions. The identification of new sources of compounds and increased demand for the prospection that has antimicrobial properties. However, its realization depends on some conditions, including solubility of the food and pH. Cinnamic acid derivatives (CAD) such as trans, hydroxy and methoxy cinnamic acid, 4-chlorocinnamate, cinnamic acid derived from oxazoline ions, antimicrobial and antifungal activities. These substances showed strong activity against Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and Gram-negative bacteria (*Escherichia coli* and *Proteus vulgaris*).

Gonçalves et al. [22] studied the effects on different concentration of carnauba wax (1, 2, 3 and 4.5%) on the brown rot, produced by *Monilinia fructicola* (G. Wint.) and Rhizopus rot, developed by *Rhizopus stolonifer* (Erhenb.:Fr.) *in vitro*

evaluation on infection of nectarine and plums. The authors, distinguished that no mycelial development of *M. fructicola* at any wax concentrations in post-contamination tests, however, *R. stolonifer* was totally restrained via by carnauba wax at all concentrations except at 1%. Additionally, *in vitro* evaluation for both *M. fructicola* and *R. stolonifera* no germination occurred of spores at any carnauba wax concentrations. There was 50% inhibition observed in spore germination for *M. fructicola* by utilizing 9% carnauba wax concentration and covered with nectarines 90% for *R. stolonifera*. The carnauba wax concentrations (4.5% and 9%) were applied to the protections with essentially reduced frequencies of both diseases in nectarines and plums. Nevertheless, the utilization of wax control was ineffective after infection by both diseases.

According to Jo et al. [23] studied quality and microbial safety of Fuji apples coated with CSW/LO (Carnauba-shellac wax nanoemulsion containing lemongrass oil). In this work, carnauba wax incorporated into shellac wax (Carnauba-shellac wax) with essential oils like lemongrass oil coating formulations and their effects on the coating and shelf life of the Fuji apples were evaluated. Total soluble solid content to titratable acid ratio, hardness, weight loss and color, sensory quality and microbial growth of fresh Fuji apples were studied during 5 months of storage at room temperature. According to the authors, results showed that carnauba extracts incorporated to shellac wax-based coatings together with lemongrass oil successfully maintained the firmness and color of coated freshly harvested apples in comparison with uncoated control samples, which presented severe texture softening. During storage conditions, the hardness of the uncoated apples exhibited the lowest conditions by 3.3 N and the weight loss was found by 7.7%. Interestingly, the weight loss was found to be 5.2% and the hardness of the coated apples did not change at any conditions, respectively. The total soluble solids and titratable acidity revealed that not significantly different between coated and uncoated apples.

Hence, the application of CSW-LO coated apples had better sensory scores with the sensory acceptability threshold for any attributes evaluated. In addition, the total aerobic bacteria population on the coated apples were deteriorated (1.4 log CFU/g) compared with uncoated apples after 5 months of storage. Additionally, the population of yeast and molds of the uncoated apples were found 2.2 log CFU/g after 5 months of storage, although yeast and molds were not detected on the coated apples, respectively. The results achieved demonstrate the feasibility of the addition of carnauba wax coating formulations for increasing the nutritional value of fresh apples without compromising their fresh-like quality attributes.

2.4.3 Antifungal activity

Different kinds of antimicrobial proteins have been purified from plants such as, β-1,3-glucanases, chitinases, ribosome-inactivating proteins, thionins, and defensins. In this case, β-1,3-glucanase and chitinase separated from type B wax of *Copernicia cerifera*, has revealed antifungal activity against phytopathogenic fungi medium [12]. Based on the results, the yeast *Saccharomyces cerevisiae* showed the patterns of growth for *Fusarium oxysporum* and *S. cerevisiae* in the presence of different fractions obtained from "Carnauba" wax and in control medium. Plant chitinases and β-1,3-glucanases are known as antifungal hydrolases since they inhibit fungal growth in model experiments by using on agar plates and in liquid media. The presence of isolated proteins by using SDS-Tricine-gel electrophoresis, and showed inhibit early growth of all fungi in their fractions in agar plates. Based on these results, defense proteins like chitinase and glucanases which appear to inhibit the early growth of all fungi and cause hyphal morphological alterations for fungi growing in the presence of these proteins (relative molecular masses of 26,000 and

24,000 Da) as compared with growth on control medium. According to the authors, *Copernicia cerifera* wax contains defense proteins ability to inhibit fungal growth. Moreover, the fungal cell walls together with β-1,3-glucans, recommend a protective role for these hydrolases.

2.4.4 Hypercholesterolemic activity

Paim et al. [24] first time studied *in vivo* study of the antihypercholesterolemic effect of the aqueous pulp extracts (APE) from the *C. prunifera* (APE 150 and 300 mg/Kg b.w./day) were directed to hyperlipidemic mice for 90 days. It showed that APE was promising results with lipidemic alterations were effective in both models causing significant changes in the values of total cholesterol, low-density lipoprotein cholesterol (LDL-C), HDL-C and triglycerides in serum. Nevertheless, it showed no renal toxicity and liver toxicity parameters (enzyme AST) and renal metabolites (urea and creatinine) to animals. Additionally, APE in high doses showed no renal and liver toxicity to animals. Despite the fact that the histological results bring about liver of mice treated with APE shows that doses (150 and 300 mg/Kg b.w./day) were not ready to alter the inflammatory procedure contrasted with the standard diet (SD) fed mice, all things considered, that better reaction opposing the hypercholesterolemic diet (HD). Besides, it was recognized the reduced intensity of inflammation in higher dose receiving present in the group. According to these results revealed that aqueous fruit pulp extracts of carnauba reduced hypercholesterolemia showing a potential preventive effect against cardiovascular diseases without side effects cause.

Furthermore, in this investigation, Filho et al. [25] revealed that the extract of PCO-C (100 mg/kg) found that productive in decreasing total cholesterol (TC) and triglyceride (TG) levels in both dyslipidemia induction models in hypercholesterolemic mice. This effect ascribed to the presence of high dietary and crude fiber content and antioxidant potential of PCO-C. Histological investigations demonstrated that PCO-C has no hepatotoxic impact and diminishes hepatic steatosis in animals that expended hyperlipidemic ration. In this manner, it was inferred that PCO-C separated from *Copernicia prunifera* may be helpful in the treatment of hyperlipidemia and atherosclerosis. Additionally, the authors highlighted that the results obtained in animals treated with PCO-C were pivotal compound had therapeutic potential in the prevention and treatment of diseases related with the metabolism of carbohydrates and lipids.

2.4.5 Hypoglycemic activity

Rodrigues et al. [26] studied that oral administration of *Copernicia cerifera* in glibenclamide diabetic mice at doses of 100 and 150 mg/kg bodyweight for 21 days.

According to the authors, the findings of this study indicated that 10% isopropanol in heptane leaf extract of Carnauba powder extract had antidiabetic activity when using therapeutic doses (100 and 150 mg/kg body weight (b.w.)). However, after treatment with 150 mg/kg b.w dose was found to be effective in significantly controlling blood glucose levels ($p < 0.05$), when compared to the reference drug glibenclamide. The observed hypoglycemic activity could be associated with the phytochemicals present in carnauba wax powder. These finding results suggested that PCO-C leads to diabetes by protecting beta-cells from oxidative damage. Indeed, the presence of the antioxidant effect of PCO-C may improve the pancreatic beta-cells to inhibit glucagon secretion and release more insulin levels. Finally, this study clearly shows that the leaf extract of carnauba wax powder possesses possible hypoglycemic activity in alloxan-induced diabetic mice.

2.4.6 Antiprotozoal activity

Almeida et al. [22] identified antiprotozoal metabolites from the *Copernicia prunifera* (Miller) showed *in vitro* action against promastigote and amastigote types of *Leishmania infantum*, trypomastigote forms of *Trypanosoma cruzi*. Among the separated dammarane-type triterpenoids, from the hexane and ethanolic extracts 'carnauba' wax (type 1 and 4) indicated antiprotozoal activity against promastigotes of Leishmania *infantum*, which showed the values of IC_{50} of 46.2 mM in tested extract 1. Besides, considering the positive controls miltefosine and benznidazole, the obtained results recommended that the impact of tested extract 1 against *L. infantum* is less noticeable than that observed against trypomastigotes of *Trypanosoma cruzi*. The intracellular amastigotes of *L. infantum* were sensitive to three types of triterpenoids, with IC_{50} estimations of 7.8, 37.6 and 51.9 μM, individually. Regardless of triterpenoid 2 and 3 exhibited absence of activity against the extracellular promastigotes, they killed the intracellular structures with selective index (SI) esteems more than 5.3 and 3.8, respectively, proposing a conceivable commitment of macrophages at the end of parasites. Notwithstanding, the tested extract 1 and 2 were less effective than standard drug miltefosine, which showed an IC_{50} of 16.4 μM. Finally, this study provided useful information about the antiprotozoal activity of 'carnauba' (*C. prunifera*) wax as well as the identification of compounds responsible to this potential.

2.5 Pharmaceutical processing

Carnauba wax has a wide scope of utilizations and, as a result, is industrially accessible in an assortment of blends. Carnauba wax utilized in fruit and vegetable coating is constantly connected as a microemulsion made with unsaturated fatty acids and an essential counterion [27]. These produce an anionic emulsifier where the carnauba wax is dispersed. In addition, various types of unsaturated fats utilized incorporate oleic, linoleic, palmitic, myristic or lauric acids. The fundamental counterion may be hydroxides of sodium, potassium salts, ammonium, morpholine [28] and triethanolamine [29]. Since carnauba wax is just utilized as a fruit coating in the mix with different substances, the adequacy and consistence of different substances should likewise be considered.

Nart et al. [30] studied carnauba wax demonstrates a pivotal reinforcement to support the sustained release of high soluble medications in relationship with Ethocel™ (EC) and Kollicoat® SR 30D utilizing reservoir and matrix systems, respectively. However, melt granulation of the medication with carnauba wax was connected as an intermediary of the key to sustained release mini-tablets, utilizing captopril (6.25 mg/mini-tablet) and metformin hydrochloride (15.0 mg/mini-tablet) as profoundly soluble model drugs. In addition, investigating the impacts of carnauba wax as a granulating excipient in the arrangement of mini-scale tablets, unmistakably the excipient diminished the contact of the drug particles with the disintegration medium, decreasing the release rate of the drugs and submitting the disintegration of the smaller than usual tablets. In this manner, it was seen that the melt granulation technique with carnauba wax improved the rheology of the considered drugs. The carnauba wax added to diminish the diffusion rate of the drug to the medium by expanding the hydrophobicity and lessening the disintegration rate of the structure of the measurements, impeding water dispersion a while later. The blend of carnauba wax with the EC at 50% indicated promising profiles for sustained release formulations.

Neto et al. [31] developed methionine microencapsulated with lipid matrix using carnauba wax by the melt emulsification technique. Different compositions of

carnauba wax: methionine (MEM 2:1 and MEM 4:1) were prepared and compared with pure methionine. In addition, scanning electron micrograph results showed no invade by ruminal microorganisms of both formulations after *in situ* testing. Taking into account that carnauba can apply an impact of protective on amino acids by covering their degradation in the rumen due to its hydrophobic distinguishing. In addition, it is a characteristic result of low degradability because of its concoction structure in unsaturated fats, and it is easy to obtain. Notwithstanding, carnauba wax sustained its thermal degradation temperatures and typical melting after the microencapsulation procedure, this diminishing in thermal stability of methionine is not because of its collaboration with the wax however most likely is because of the forces of intermolecular level (presences of hydrogen bridges) among the methionine particles. Finally, the formulation MEM 4:1 showed that promising results of the lower level of thermal degradation and higher yield and efficiency of microencapsulation.

2.5.1 Post-harvest storage

The valuable role of carnauba wax is outstanding for improving shelf life and supporting postharvest quality of a few fruits, for example, mango [32], avocado [33] and mamey sapote organic product [34]. Barmen et al. [35] studied pomegranate (*Punica granatum* L., cv. Mridula) fruits were treated with putrescine, carnauba wax and putrescine + carnauba wax combination prior at 2°C cold storage temperature. Further, carnauba wax is additionally stated to reduce the improvement of chilling injury (CI) manifestations. Respiration rate of stored fruits has been discovered expanded with the progression of the capacity period under every one of the medicines. Up to the fifteenth day of capacity, there was no critical contrast in breath rate in the organic products treated with polyamine like putrescine (PUT), carnauba wax and their mix. The low breath rate in carnauba wax treated organic product ascribed because of diminished gas exchange and thusly low oxygen accessibility to the natural product tissues for breath. The utilization of carnauba wax gave higher maintenance of fruit solidness, most likely because of the less drying out happened and furthermore to a slower degradation of cell divider segments. In this way, in control group and carnauba wax treated pomegranate fruits, the expansion in juice recuperation after 30th day of storage capacity may be ascribed to CI intervened activities of cell degrading enzymes such as pectin methylesterase and polygalacturonase. In addition, the utilization of carnauba wax covering in blend with PUT may have applied synergistic impact which aided in keeping up higher juice recuperation by diminishing loss of moisture from the fruits.

Germano et al. [36] studied, a galactomannan-carnauba wax-based coating improved the guava fruit in postharvest quality and storability over preservation of firmness in ambiental conditions (25°C). The authors prepared edible coating galactomannan (0.75%) and carnauba wax (0.9%) were treated with guava fruits (Paluma). At day 15, coated and refrigerated (FR) guava fruits were showed a climacteric rise in 59.3 mg CO_2 kg^{-1} h^{-1} and however, coated guava at ambient (FA) showed a diminished value 168.6 mg CO_2 kg^{-1} h^{-1} at 15 days of storage and firmness of 14.3 N attributed to lower lipid peroxidation and cell wall hydrolysis. In addition, no increase values of control refrigerated fruit (CR) and no further evaluation of control-uncoated 'Paluma' guava stored at ambient (CA) for 9 days. Additionally, coating improves increased antioxidant enzymes CAT and SOD activities refrigerated samples presented 35% lower H_2O_2 levels ($p < 0.05$) while compared to uncoated control samples. However, symptoms of chilling injury (CI) inhibition of softening and respiratory peaks are exhibited in refrigerated uncoated fruits. According to the authors, galactomannan carnauba wax coating was effective

in guava postharvest quality and maintaining firmness and color, also preventing chilling symptoms under refrigerated conditions, respectively.

3. Conclusion and future perspectives

Despite the current recognition of *Copernicia prunifera* as a quintessential Brazilian plant with growing interest of research and a boost in its commerce and industrial application for the formulation of therapeutic products, it can be safely postulated that its therapeutic potentials have not been fully explored. At present, researches and commercial interest on carnauba wax are skewed toward its cosmeceutical, food and pharmaceutical applications which have dwarfed research interest in its potential as a remedy for other diseases. Thus, further researches on its pharmacological activity recommended with the end-goal of unraveling the pharmacodynamics, pharmacokinetics and clinical relevance. In addition, toxicity risk assessment studies of both the bioactive extracts and isolated constituents need to be given more attention. Nevertheless, further studies and long-term human trials should be carried out in order to clarify the relationship between the consumption of 4-methoxy cinnamic acid diesters and its derivatives such as isolated pectin and their benefic impact on the human body, there seems to be certainly a promising future for new investigations.

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Conflict of interest

The authors declare no conflicts of interest to disclose in relation to this book chapter.

Acronyms and abbreviations

PIP	precipitation index permits
TLC	thin layer chromatography
Mpk	peak molar mass
CSW	carnauba-shellac wax
LO	lemongrass oil
APE	aqueous pulp extracts
LDL-C	low-density lipoprotein cholesterol
TC	total cholesterol
TG	triglyceride
HD	hypercholesterolemic diet
GC-FID	gas chromatography-flame ionization detections
IR	infrared spectroscopy
PCO-C	4-methoxy cinnamic acid diesters
b.w	body weight

DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	effective concentration
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt
FRAP	ferric reducing antioxidant power
NMR	nuclear magnetic resonance spectroscopy
SDS	sodium dodecyl sulfate
AST	aspartate transaminase
ROS	reactive oxygen species
PG	polygalacturonase
PUT	putrescine
CI	chilling injury
PPO	polyphenol oxidase
SI	selective index

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Section 2

Coumarins: Bioactivity, Synthesis and Labelling

Chapter 5

From Rat Poison to Medicine: Medical Applications of Coumarin Derivatives

Robbert Bipat

Abstract

Historical reports mention the application of medicinal plants containing coumarins against various ailments. Current research suggests that at least some of the actions described may be attributable to the action of these coumarins. Warfarin and its derivatives are coumarins used today in medical practice. Their mechanism of action lies in the competitive antagonism of vitamin K, through which they inhibit coagulation in the body by preventing the production of prothrombin. Due to this action, these coumarins are a major group of drugs with anticoagulant activity. Anticoagulants reduce the risks of undesirable blood clots leading to myocardial infarction, pulmonary embolism, and ischemic stroke among others. The anticoagulant activity can also lead to undesired bleeding. Extreme caution is warranted when given to menstruating women, patients suffering from disorders prone to bleeding like gastric ulcer and rheumatoid arthritis, and to persons with a high likelihood of blunt and sharp trauma. In addition, there is a significant augmentation of the anticoagulant activity when used in combination with non-steroidal anti-inflammatory agents and agents interfering with the metabolism of the coumarins. Recent findings propose additional uses like anti-tumor and antibiotic actions for coumarins. The clinical application of these actions has yet to be demonstrated.

Keywords: warfarin, coumarin, anticoagulant, prothrombin, coagulation cascade, adverse reaction, rodenticide, embryopathy, vitamin K

1. Introduction

Coumarins are members of the benzopyrone class of organic compounds that are found in many plants [1] and possess a variety of pharmacological properties such as antimicrobial, anti-inflammatory, antidiabetic, and antioxidant activity, as well as a significant influence on physiological processes like enzyme inhibitory activity [2]. Despite the wide availability of coumarins and their lead compounds and metabolites in natural products [3], their application up till now has been mostly limited to the anticoagulant activity of warfarin derived from dicoumarol and its analogues [4]. The mechanism of action of these anticoagulants lies in the competitive antagonism of vitamin K, through which they inhibit coagulation of blood in the body by preventing the production of prothrombin and several other coagulation factors [5]. Due to this action, these coumarins are a major group of oral drugs with anticoagulant activity. Anticoagulants reduce the risks of undesirable

blood clots leading to myocardial infarction, pulmonary embolism, and ischemic stroke among others. This chapter gives an overview of medical applications of coumarins, in particular the history and evolution of warfarin and related compounds as important anticoagulant agents.

2. History

The medical application of plants containing coumarins probably started long before the isolation of this chemical compound from the Tonka bean in 1820 by Nicholas Jean Baptiste Gaston Guibourt [6]. Ancient Romans produced a cough syrup from the marshmallow (*Althea officinalis*) [7], which contains the coumarin scopoletin [8]. This coumarin demonstrated inhibition of leucocyte migration in mice [9], a process that can be linked to the alleged antitussive effect. Cough is a result of the reaction of the airways to leukotrienes and other factors secreted by leucocytes [10–12]. Inhibition of the migration of these to the affected region consequently reduces the availability of these paracrine factors. In addition, at least one of the herbs mentioned by the famous Roman General Pliny in his pharmacopeia [13] contain coumarins with proven action. For example, the extracts of the common rue or herb-of-grace *Ruta graveolens* contain xanthotoxin [14], a coumarin that reduces the mobility of human spermatozoa possibly through inhibition of membranaeous potassium channels [15].

The application of coumarin and its derivatives in current western medicine dates to the fifties of the past century with the clinical recognition of coumarins as anticoagulant agents. This event was the result of observations of poisoning of animals with coumarin derivatives that led to massive internal organ bleeding [16]. Soon it became clear that the substance that was responsible for the deadly internal bleeding of cattle was dicoumarol [17]. Shortly after this, the proposal was made to develop a coumarin derivative with rodent killing ability and gradually warfarin found its application as a potent rodenticide [18]. When it became clear that this substance also led to bleeding disorders after poisoning in human beings [19], its application as a therapeutic anticoagulant found its way in medicine [20, 21]. Seven decades later, warfarin is still in use as an anticoagulant [22]. In the meantime, several other coumarins with anticoagulant properties like acenocoumarol, phenprocoumon, and fluindione have been developed, and they are used in a variety of clinical settings [23–25].

3. Physiology of hemostasis

Hemostasis in mammals and humans is the result of three sequential processes. The first of these is the acute vasoconstriction within seconds after damaged arteries and veins, by local activity of the potent vasoconstrictor thromboxane among others [26]. The second step is the formation of a blood clot through the entrapment of platelets by fibrin within hours and finally followed by the organization of the fibrin mesh into an adhesive structure on the vessel wall [27]. In one of the last steps of the coagulation, prothrombin converts to thrombin, an enzyme that converts the plasma protein fibrinogen to fibrin monomers and activates factor XIII of the coagulation cascade. Activated factor XIII synthesizes fibrin from these monomers. The acquired fibrin molecules then trap the platelets and eventually form the blood clot [27, 28]. The coagulation process has both an intrinsic and an extrinsic pathway. The difference is that the intrinsic pathway only requires ionized calcium to be activated while the extrinsic pathway requires both calcium and tissue factor that

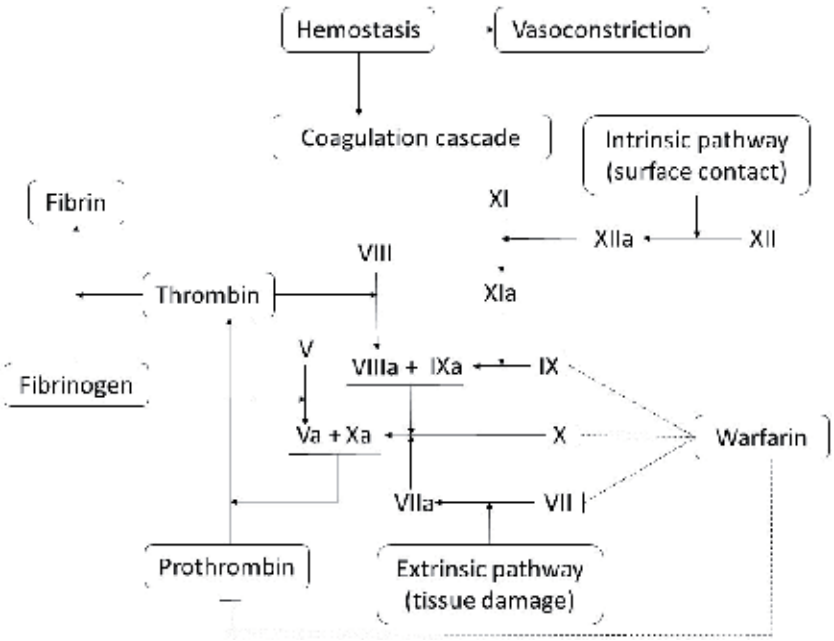

Figure 1.
Overview of the coagulation process along with the interference sites of warfarin.

is released with trauma [28]. **Figure 1** gives an overview of the coagulation process along with the interference sites of warfarin.

4. Pharmacology

4.1 Pharmacokinetics

Warfarin is highly lipid soluble [29–31]. Between 70 and 100% of the oral intake is rapidly absorbed by the intestines with a maximum plasma concentration within 2 h after oral intake [30]. The half-life of the drug is generally more than 20 h, with a large individual variety [32]. Due to the overt lipid solubility, the major part of the drug is protein bound with less than 3% being biologically available [29]. Consequently, the agent has a slow onset of action and a long duration of activity [33]. In fact, the optimal effect is delayed for a few days, until all remaining activated factors II, VII, IX, and X are depleted from the liver and the circulation [33]. Warfarin accumulates in the liver where it exerts its effect and is inactivated through oxidative metabolism by cytochromes P450 to several isomers of water-soluble hydroxywarfarin with negligible anticoagulant activity [34, 35]. These metabolites are almost completely cleared by the kidneys [36]. The hepatic accumulation and relative easy absorption in the intestines result in an enterohepatic circulation of the drug [37]. Enterohepatic circulation is a process in which substances are secreted by the liver with bile to the intestines and subsequently absorbed again by the latter [28]. This results in recycling of the product with very little elimination.

4.2 Mechanism of action

Warfarin inhibits the enzyme vitamin K epoxide reductase that recycles oxidized vitamin K [38]. Vitamin K activates the coagulating factors prothrombin

(factor II) and the structurally related serine proteases known as factors VII, IX, and X in the liver cells [27]. Decreasing the biological availability of vitamin K inhibits the synthesis of these essential factors and eventually leads to inhibition of the coagulation process. This means that this compound affects both the intrinsic as well as the extrinsic cascade of coagulation since prothrombin plays a central role in both of these pathways [28] and renders it a highly effective anticoagulant drug.

5. Indications and contraindications

Hemostasis is an essential process to prevent significant external as well as internal blood loss after injury. However, under certain circumstances, it is not desirable to activate or continue this homeostatic process like in disorders with spontaneous thrombosis such as deep venous thrombosis in the legs often resulting in pulmonary embolism [39]. In addition, there are conditions that are prone to a reasonable chance of forming a blood clot during stasis of the blood circulation like in atrial fibrillation and in the limbs of patients with prolonged immobility after surgery [39]. Moreover, conditions like myocardial infarction or ischemic stroke form a preventable group of disorders with inhibition of the thrombotic process [5]. Based on its anticoagulant properties, warfarin is thus an ideal compound for the treatment and prevention of these thromboembolic conditions [5].

Based on the pharmacokinetic properties and the challenges they present, dosing of warfarin is not simple, and a careful approach is necessary. On one hand, a low plasma concentration will not achieve the effect of sufficient anticoagulation and, on the other side of the spectrum, there is the constant chance of overdosing with potential lethal internal or external bleeding. Another problem is the great variety of absorption, body distribution, and metabolism of the agent with individual patients based on the pharmacokinetic properties of warfarin [30]. Frequent monitoring of therapeutic efficiency with adequate laboratory tools like prothrombin time (PT) or international normalized ratio (INR) is absolutely necessary [40, 41] and a fixed or constant dose is close to impossible. Nevertheless, warfarin is highly effective in anticoagulation regiments when carefully dosing and assessing the potential bleeding sites as well as other potential side effects. Warfarin is initially dosed at 5–10 mg daily [42]. Subsequent doses depend on the international normalized ratio, with a therapeutic value between 2 and 4. Concomitant administration of heparins like fraxiparine is necessary when fast anticoagulant activity is desirable [43].

Warfarin is not an ideal agent in conditions when immediate treatment of thromboembolism is imminent due to the long time of onset. In cases of pulmonary embolism and acute ischemic stroke, it is desirable to start with both the oral anticoagulant and fast-acting agent like heparins [44]. The long duration of action harbors another challenge. When acute termination of anticoagulation is necessary with unwanted bleeding like in menstruating women and after blunt and sharp trauma leading to hemorrhage, it could take days before the process of coagulation completely restores after quitting oral administration [27, 30] due to the depletion of coagulant factors in liver and blood. In these cases, intravenous administration of prothrombin complex, fresh frozen plasma with coagulation factors, and high doses of vitamin K may be helpful [45].

Warfarin readily passes the placenta and may result in spontaneous abortion due to retroplacental bleeding [46], as well as prematurity [47], fetal deformity [48], stillbirth [48], and fetal cranial bleeding [49]. Administration during the first trimester of pregnancy has a high risk of embryopathy [50]. This is accompanied by deformities of bone and cartilage [51], blindness, mental retardation, and other

neurologic abnormalities [52]. The occurrence of these complications and defects seem to be dose dependent [47, 53] and are most probably the result of the interference with vitamin K-dependent coagulation [38] and bone formation [54]. The effects of the central nervous system and the blindness are probably the result of microhemorrhages in the developing brain as a result of the anticoagulant activity [55]. Clotting factors are easily depleted in the fetus due to the immature liver and small circulating volume [46]. Warfarin does not enter breastmilk and is thus completely safe during lactation [56].

In conclusion, warfarin must be administered with great caution to women in their child-bearing age [57]. Therapy with this agent must be ceased immediately when it becomes clear that the patient is pregnant. Low-molecular weight heparins are a good alternative, since they do not cross the placenta and have been proven to be safe for mother, embryo, and fetus [58].

6. Drawbacks and side-effects

To say that anticoagulant coumarins have only a few side effects is an absolute understatement. Warfarin is one of the leading drugs with adverse effects requiring hospital admission [59]. Most of all, there is the constant chance of severe bleeding [60]. This can include internal hemorrhagic conditions in the head, gastrointestinal tract, female genitalia, the bladder and urethra or skeletal joints and muscles [40, 61]. They generally present as severe headache, stomach pain, and black or bloody stool, heavier than normal menstrual bleeding, discoloration of urine, and pain and swelling of the joints or muscles. Prolonged bleeding from external sharp or blunt wounds is always present [61]. All these conditions are the result of inability of the affected tissues to initiate and continue the process of hemostasis after damage to the epithelial barrier [62].

Patients suffering from hypertension, disorders of the liver, bleeding lesions, and the elderly and patients using drugs and substances that affect coagulation are at higher risk to suffer from bleeding when using warfarin [63]. Hypertension poses mechanical defects in the blood vessels, especially the arteries. Disorders of the liver reduce the ability of the body to eliminate the warfarin and thus make it more biologically available. In bleeding lesions, warfarin inhibits hemostasis. Among substances that can lead to bleeding when used with warfarin are steroidal and non-steroidal anti-inflammatory drugs, antibiotics, and alcohol. These potentiate the activity, interfere with the protein binding, and reduce the metabolism of warfarin, respectively [63]. Other side effects include injury to the kidneys with potential nephritis [64–66], inflammation of the skin [67] and blood vessels [66], and potentiation of rhabdomyolysis by simvastatin [64].

Due to resistance of rodents against warfarin, superwarfarins have been created [68]. These have a much longer time of activity and hence need only to be consumed once by the rodents, contrary to warfarin. The result however is that their effect persists much longer when deliberately or accidently consumed by humans [69] and treatment of this intoxication is a more challenging enterprise.

7. Interaction with drugs and foods

The efficacy of the anticoagulant treatment with warfarin highly depends on its bioavailability, since inhibition of the target (epoxy reductase) enzyme depends on direct binding of the drug to this protein [38]. In addition, vitamin K from external sources does not rely on recycling through this enzyme [38]. Hence, the absorption,

	Agent	Category	Possible mechanism	Effect	Reference
Allopathic medications	Amiodarone	Antiarrhythmic	Inhibition of hepatic metabolism		[78]
	Ciprofloxacin	Antibiotic	Reduction of vitamin K synthesis by intestinal bacteria	Increased bleeding	[79]
	Paroxetine	Antidepressant	Inhibition of hepatic metabolism	Potentiation	[80]
	Citalopram	Antidepressant	Inhibition of hepatic metabolism	Potentiation	[80]
	Clopidogrel	Antiplatelet medication	Inhibition of coagulation cascade	Potentiation	[81]
	Dipyridamole	Antiplatelet medication	Inhibition of coagulation cascade	Potentiation	[81]
	Diclofenac	NSAID	Inhibition of coagulation cascade	Potentiation	[82]
	Naproxen	NSAID	Inhibition of coagulation cascade	Potentiation	[82]
	Acetaminophen	Analgesic	Interference with hepatic metabolism	Increased bleeding	[83]
Food supplements	Fish oil	Lipid profile improvement	Inhibition of coagulation cascade?	Potentiation	[84]
	Pomegranate juice	Antioxidant	Interference with hepatic metabolism	Potentiation	[85]
	Glucosamine	Cartilage improvement	Unknown	Potentiation	[86]
Traditional medications	Chamomile	Medicinal herbal tea	Unknown	Increased bleeding	[87]
	Ginseng	Improving cognitive functions	Unknown	Inhibition	[88]
	St John's wort	Against depression	Induction of metabolism	Inhibition	[89]

Table 1.
Brief overview of possible interactions with warfarin.

transport, delivery, and elimination of warfarin as well as the external availability of vitamin K are potential sites of interaction with other drugs and with food and dietary supplements.

Drugs and food that influence the enterohepatic circulation can all affect the absorption of warfarin. Examples of these are the drug cholestyramine [37] and the avocado fruit [70], which prevent the reabsorption of warfarin in the intestines Concomitant administration of other protein-bound drugs may lead to greater amounts of circulating warfarin and increased risks of bleeding. Valproate sodium increases the bioavailability of warfarin through dislocation of its protein-binding sites [71]. Interference with the metabolism of warfarin is a potential of most drugs that are eliminated by hepatic metabolism. Among these are aspirin [72], non-steroidal anti-inflammatory drugs [72], serotonin reuptake inhibitors [49], anti-platelet agents and some antibiotics [72]. It can go both ways with the metabolism. Induction of the cytochromes will increase the elimination, while occupation of the binding sites by the drugs will increase the availability of warfarin.

Since warfarin acts through elimination of available bioactive vitamin K, variations of the net intake of this vitamin will certainly interfere with the drug action. A high intake of the vitamin will keep the coagulant factors at a higher level and thus inhibit the anticoagulant activity. Likewise, a lower intake will potentiate the effect of warfarin. The vitamin occurs in food in the form of phylloquinone and menaquinone. Phylloquinone is the form mostly found in plants and is also the most abundant form in food [73]. Menaquinones are mainly the product of bacterial production or conversion [74]. Consequently, simple multivitamin and other supplements, food with high vitamin K content [74] as well as antibiotics are sources of fluctuation in vitamin K intake since intestinal bacteria significantly contribute to the production of menaquinones [75].

Recently, another source of interference came into focus. In addition to the previous mentioned parameters, genetic variation in the expression of cytochrome P450 seems to play a role in the metabolism of warfarin [76], thus influencing the availability of the drug [77]. All these considerations make it clear that close monitoring of the individual coagulation ability is necessary for a successful therapy with this agent.

The abovementioned interactions are just a few of the many that are possible. **Table 1** gives examples of a variety of interactions with drugs, food, natural products, and supplements. This is only to underscore the cautious approach patients should practice when taking warfarin.

8. Future prospects and conclusion

Today, coumarins find their application predominantly as anticoagulants in medicine. The narrow therapeutic index of warfarin and related compounds sometimes limit their applicability and consequently there is a constant search for more safe agents in this drug class [90]. Unfortunately, the development of these will probably limit the use of these oral anticoagulants.

Aside from these developments, coumarins with several applications in medical practice are progressively being introduced. Investigators found that coumarin-3-carboxylic acid could be utilized as a dosimeter for radiotherapy. This substance converts to the highly fluorescent 7-hydroxy-coumarin-3-carboxylic acid, with a near perfect linear correlation upon irradiation [91].

The coumarin 2-hydroxycinnamic acid demonstrated inhibitive properties on the enzyme carbonic anhydrase [92]. Inhibition of this enzyme leads to diuresis [93] and decreases intraocular pressure in glaucoma patients [93] with clear therapeutic potential and clinical perspective.

Furano(pyrano)coumarins found in the roots of the Korean angelica (*Angelica gigas*) showed antibacterial activity in hay bacillus (*Bacillus subtilis*) cultures [94]. The coumarine derivative cloricromene reduced the inflammatory parameters in rats subjected to collagen-induced arthritis [95]. In addition, several studies found that coumarins may be useful as anti-tumor agents [4, 96].

Probably since ancient times, coumarins found their application in medicine. Currently, however, coumarins with predominantly anticoagulant properties are applied in daily medical practice. These have been developed from the initial discovery of a cattle killing weed more than six decades ago. Initially applied as a rodenticide, soon a therapeutic usable oral anticoagulant was developed, and slowly other agents entered the market. They have a small therapeutic index, rendering them toxic in a number of circumstances. The search for more safe agents with anticoagulant effects is ongoing and this may result in a decline of the use of coumarins in this field. Nevertheless, coumarins gradually find their way in other fields of medicine. Nevertheless, all these developments promise a bright future for coumarins in medical applications.

Conflict of interest

The author declares no conflict of interest.

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Chapter 6

One-Pot Synthesis of Coumarin Derivatives

Inul Ansary and Abu Taher

Abstract

Coumarin derivatives have a myriad of applications in medical science, biomedical research, and many industrial branches. For this reason, many efforts are being dedicated to the development of novel and more practical methods for synthesizing these compounds. This chapter describes several methods of one-pot synthesis of coumarin derivatives, including von Pechmann condensation, Knoevenagel condensation, Baylis-Hillman reaction, Michael addition, Kostanecki reaction, vinyl phosphonium salt-mediated electrophilic reaction, and Heck-lactonization reaction. The methods are compared with each other, and the advantages and disadvantages of each of them are addressed.

Keywords: coumarin derivatives, one-pot synthesis, methods and procedures, advantages and disadvantages

1. Introduction

Coumarin (2H-chromen-2-one) derivatives have spawn great interest over the years because of their significant biological importance [1]. They are associated with various biological activities viz. antiviral [2, 3], antibacterial [4, 5], antimicrobial [6], anticoagulant [7], anti-inflammatory [8, 9], anticancer [10, 11], anticonvulsant [12], antioxidant [13], antifungal [14, 15], and anti-HIV [16]. They also possess the properties like inhibition of platelet aggregation [17] and inhibition of steroid 5α-reductase [18]. Besides, they are attracting considerable attention of chemists due to their wide range of applications such as optical brighteners [19], photosensitizers [20], fluorescent and laser dyes [21], and additives [22] in food, perfumes, cosmetics, and pharmaceuticals. The novel compounds are also utilized in drug and pesticidal preparations [23]. Considering these multifarious activities of coumarins, synthetic chemists are actively engaged in developing new and superior methods for the isolation of coumarin derivatives. The most widely used method for their synthesis is Pechmann reaction [24–27], which involves the condensation between phenols and β-keto esters, in the presence of an acid catalyst. This method employs both homogeneous catalysts such as concentrated H_2SO_4 [24, 25], trifluoroacetic acid (TFA) [28], and Lewis acids (LA) such as $AlCl_3$ [29], $ZnCl_2$ [30], $ZrCl_4$ [31], $TiCl_4$ [32], etc. and heterogeneous catalysts such as cation-exchange resins [33], Nafion resin/silica composites [34], zeolite H-BEA (H-beta, SiO_2/Al_2O_3 = 14) [35], and other solid acids.

2. Methods to synthesize coumarin derivatives

2.1 Pechmann condensation reaction

The general reaction sequence of Pechmann reaction and its mechanism, shown in **Figure 1**, involves an esterification/transesterification between the phenol **1** and β-keto ester **2** in the presence of protonic acid or Lewis acid (LA) catalyst to produce species **4** followed by an attack to the activated carbonyl carbon by the aromatic ring at ortho-position to yield the new ring in species **5**. Finally, dehydration of species **5** affords coumarin derivative **2**.

A series of substituted coumarins **8** have been synthesized in 25–77% yields by the reactions of substituted phenols **6** with ethyl acetoacetate **7** in the presence of zinc-iodine mixture in refluxing toluene (**Figure 2**) [36]. It is observed that phenols containing electron-donating substituent like —CH_3 group result in higher yields compared to unsubstituted phenols and phenols having electron-withdrawing group such as NO_2 group.

When 3-(*N*,*N*-dimethylamino)phenol **9** is subjected to react with ethyl 2-acetamide-3-oxobutyrate **10** in the presence of anhydrous $ZnCl_2$ in absolute ethanol under reflux condition, the acetamido coumarin **11** is obtained only in 12.4% yield (**Figure 3**) [30].

Substituted coumarins **14** have been achieved in moderate to good yields from substituted phenols **12** and methyl acetoacetate **13** under conventional and microwave heating, respectively, catalyzed by concentrated H_2SO_4 (**Figure 4**) [37]. It is found that the reactions using the latter method are faster coupled with product in better yields compared to former one.

Synthesis of substituted coumarins **16** in 62–98% yields has also been described by Maheswara et al. [38] via reactions of substituted phenols **1** with β-keto esters **15** in the presence of a heterogeneous catalyst, $HClO_4.SiO_2$ under solvent-free conditions (**Figure 5**, Condition A). The aforementioned method involves recoverable cheap catalyst and shorter reaction time with high product yields. However, relatively lower yields (35–55%) of substituted coumarins **16** have been isolated from the similar starting precursors catalyzed by Amberlyst-15 acidic catalyst [39] in toluene under refluxing condition (**Figure 5**, Condition B).

Pechmann condensation reactions for the synthesis of substituted coumarins using various homogeneous and heterogeneous catalysts have been reported in literature and some important ones are summarized in **Table 1**.

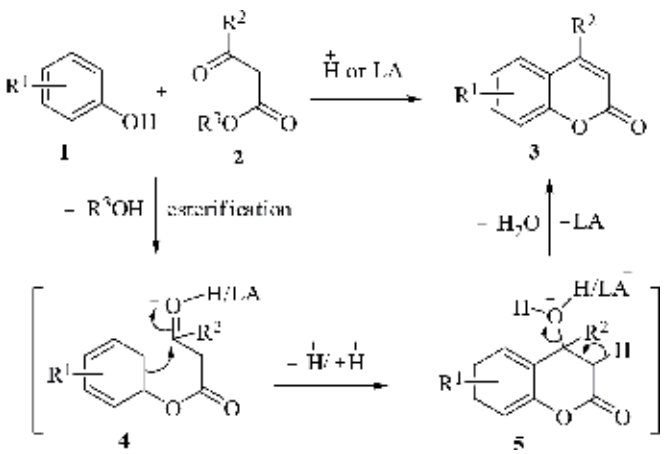


Figure 1.
Mechanism for the acid-catalyzed Pechmann condensation.

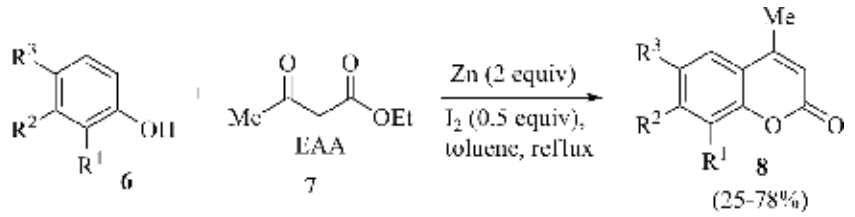


Figure 2.
Synthesis of substituted coumarins.

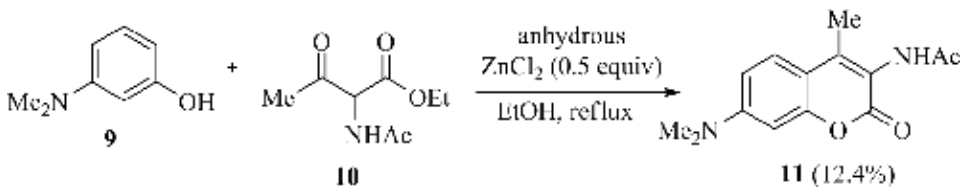


Figure 3.
Synthesis of acetamido coumarin.

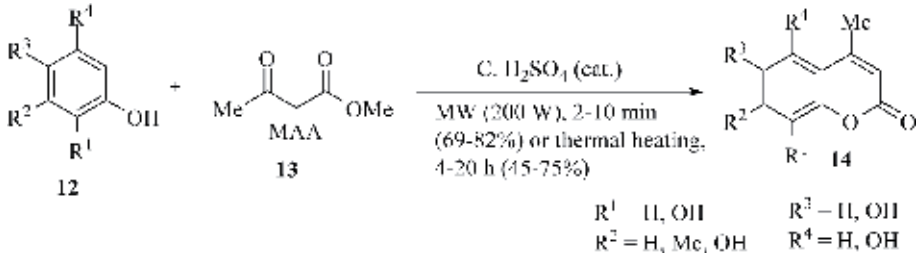


Figure 4.
Synthesis of substituted coumarins.

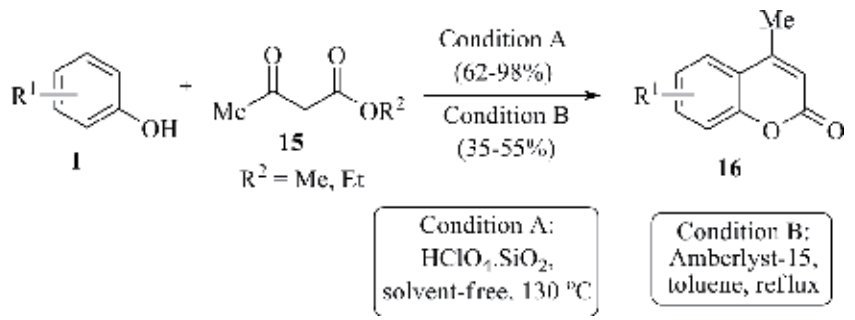


Figure 5.
Synthesis of substituted coumarins.

From **Table 1**, it is quite evident that the reactions under microwave as well as ultrasound irradiation occur at a faster rate than those of the conventional methods (entries 10, 14, 15, 16, 25, 31, 32, and 39). Unsubstituted phenol produces lower yields of corresponding coumarin derivatives and/or requires longer reaction time (entries 2–4, 7, 10, 12, 13, 24, 28, 30, and 38), higher temperature (entries 2, 3, 7, and 12), and excess amount of catalysts (entries 7 and 12) than di- and trihydric phenols. This may presumably be due to the less reactivity of unsubstituted phenol toward Pechmann condensation reaction compared to di- and trihydric phenols. In

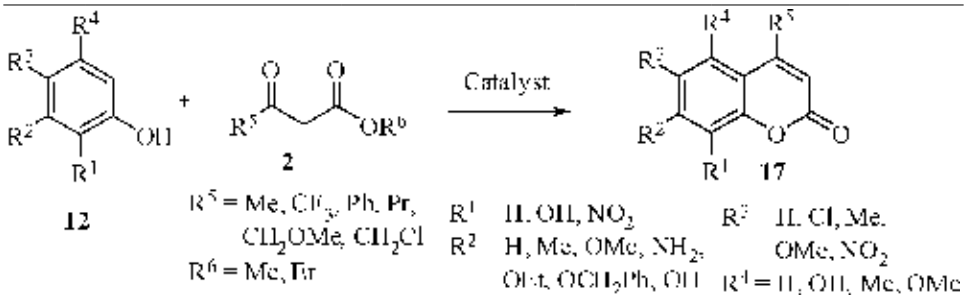
addition, the substitution of an electron-donating group such as *m/p*-Me or *p*-OMe in the phenols leads to decrease of catalytic activity and, hence, requires longer reaction time and/or gives rise to lower yields of products (entry 13). The reactivity of monohydric phenols having electron-withdrawing groups such as *m*-NH_2 and *m*-OMe is also lowered compared with simple di- and trihydric phenols (entries 19, 28, and 37). 1-Naphthol and 2-naphthol need longer reaction time (entries 13, 33, and 39) and/or furnish products with lower yields (entries 13, 37, and 40) compared to other phenols, due to the presence of another phenyl ring. However, better yield of benzocoumarin is obtained from the reaction between 1-naphthol and more reactive β-keto ester, ethyl 4-chloro-3-oxobutanoate (entry 37). It is interesting to note that β-keto ester having phenyl group at the β-position such as ethyl 3-oxo-3-phenylpropanoate is found to be less reactive in Pechmann condensation with resorcinol and 1,3-dihydroxy-5-methyl benzene due to the presence of conjugated keto center, which lengthens the reaction time than in the reactions of EAA and/or ethyl 4-chloro-3-oxobutanoate with resorcinol and 1,3-dihydroxy-5-methyl benzene (entries 21, 28, and 37). Besides, the reactivity of different types of phenols and β-keto esters, catalyst efficiency, and solvent effect of Pechmann condensation has also been studied. It is observed that $TiCl_4$ (entry 5) is the most effective catalyst as far as reaction time is considered, whereas montmorillonite K-10 (entry 1) and sulfated zirconia (SZr) (entry 9) are found to be less effective. Ionic liquids (ILs) such as 1-butyl-3-methylimidazolium hexafluorophosphate [bmim]PF_6 and 1,3-disulfonic acid imidazolium hydrogen sulfate (DSIMHS) have been used as effective and reusable catalysts and reaction media as well (entries 6 and 18).

Lewis acid–surfactant-combined catalyst (LASC) such as nano-TiO_2 on dodecyl-sulfated silica support (NTDSS) is used as a reusable and highly effective catalyst for Pechmann condensation of phenols containing different types of substituents in water led to excellent product yields (entry 20). Other recyclable solid acid catalysts have also been employed in Pechmann condensation reactions leading to coumarin derivatives in good to excellent yields under solvent-free (entries 22–24, 26–27, 29–30, and 42), microwave irradiation (entry 25) and/or ultrasound irradiation (entry 39) conditions.

More importantly, sulfonic acid-supported silica-coated magnetic nanoparticles (Fe_3O_4@SiO_2@$PrSO_3H$), $CuFe_2O_4$ nanoparticles, and zirconium(IV) complex grafted silica coated magnetic nanoparticles are found to be the most efficient catalysts toward Pechmann condensation, in which case the catalyst can be effortlessly separated by external magnet after completion of the reaction and reused for 22, 6, and 5 consecutive runs, without any significant loss in catalytic efficiency (entries 33–35).

Pechmann condensation of pyrogallol and resorcinol with ethyl acetoacetate over nanosponge MFI zeolite in comparison with conventional zeolites (MFI, BEA, and USY) and other layered MFI (lamellar, pillared, and self-pillared) have been investigated. It is important to note that the nanosponge catalysts exhibit the best catalytic performance with respect to the products' selectivity in the liquid-phase condensation reactions among all the investigated zeolites (entry 36).

On the other hand, the catalytic behavior of metal–organic frameworks such as Cu-benzene-1,3,5-tricarboxylate (CuBTC) and Fe-benzene-1,3,5-tricarboxylate (FeBTC) is investigated and compared with large-pore zeolites, beta (BEA), and ultrastable Y (USY) (entry 41). It is clear that zeolites BEA and USY are found to be more active catalysts in transformations of the most active substrates like resorcinol and pyrogallol but a low conversion of naphthol is observed. However, almost total transformation of naphthol (93–98% conversion) to the target product occurs within 23 h of the reaction time over metal–organic frameworks, CuBTC and FeBTC.



Entry	Catalyst	Reaction conditions	Time	Yields (%)	Reference
1	Montmorillonite K-10	K-10 (30 wt% of **12**), toluene, reflux	8–10 h	66–94	[40]
2	1-Butyl-3-methylimidazolium chloroaluminate [bmim]Cl. $2AlCl_3$	[bmim]Cl.$2AlCl_3$ (1.1 equiv. of **12**), 30–120°C	10–120 min	40–95	[41]
3	$InCl_3$	$InCl_3$ (10 mol%), 65–130°C	30–240 min	65–98	[42]
4	$ZrCl_4$	$ZrCl_4$ (2 mol%), 70°C	5–30 min	56–95	[31]
5	$TiCl_4$	$TiCl_4$ (0.5 equiv. of **12**), rt	50–70 s	56–95	[32]
6	1-Butyl-3-methylimidazolium hexafluorophosphate [bmim]PF_6	[bmim]PF_6 (4 ml), solvent-free, 100°C	45 min	90–95	[43]
7	$Bi(NO_3).5H_2O$	$Bi(NO_3).5H_2O$ (5–10 mol%), 80–130°C	15–300 min	47–94	[44]
8	SO_4^{2-}/CeO_2-ZrO_2	SO_4^{2-}/CeO_2-ZrO_2 (10 wt% of **12**), 120°C	4–143 min	80–94	[45]
9	SZr (sulfated zirconia)	SZr (1 wt% of **12**), 80°C	24 h	52–92	[46]
10	Ceric ammonium nitrate (CAN)	Condition A: CAN (10 mol%), solvent-free, 110°C Condition B: CAN (10 mol%), solvent-free, MW (300 W)	10–15 min 2–3 min	92–96 94–97	[47]
11	$ClSO_3H$	$ClSO_3H$ (0.2 ml), solvent-free, 10°C	10 min	91–98	[48]
12	LiBr	LiBr (10–20 mol%), 75–125°C	15–90 min	54–92	[1]
13	Nanocrystalline-cellulose-supported sulfonic acid ionic liquid	NCC-supported sulfonic acid IL (10 wt% of **12**), solvent-free, 80°C	18 min-24 h	20–98	[49]
14	$Cu(ClO_4)_2$	$Cu(ClO_4)_2$ (20 mol%), solvent-free, US (35 kHz), 45–50°C	30–50 min	70–96	[50]
15	Selectfluor	Condition A: Selectfluor (10 mol%), solvent-free, rt. Condition B: Selectfluor (10 mol%), solvent-free, US (30 kHz, 780 W)	85–90 min 15–40 min	70–79 82–94	[51]

Entry	Catalyst	Reaction conditions	Time	Yields (%)	Reference
16	I_2	Condition A: I_2 (25 mol%), toluene, 90°C Condition B: I_2 (1 mol%), MW	18 h 1.5–5 min	42–89 80–96	[52] [53]
17	AgOTf	AgOTf (10 mol%), solvent-free, 60°C	3–12 h	60–95	[54]
18	1,3-Disulfonic acid imidazolium hydrogen sulfate (DSIMHS)	DSIMHS (7 mol%), solvent-free, 70°C	2–27 min	80–96	[55]
19	*N,N′*-dimethylaminoethanol hydrosulfate ($[N_{112}OH][HSO_4]$)	$[N_{112}OH][HSO_4]$ (5 mol%), solvent-free, 90°C	3–24 h	20–99	[56]
20	Nano-TiO_2 on dodecyl-sulfated silica support (NTDSS)	NTDSS (5 mol% TiO_2), H_2O, reflux	3–8 h	89–98	[57]
21	$ZrOCl_2.8H_2O/SiO_2$	$ZrOCl_2.8H_2O/SiO_2$ (10 mol%), solvent-free, 90°C	5–80 min	75–99	[58]
22	Polydivinylbene-bound perfluoroalkylsulfonyl imide polymers (H-PDVB-*x*-SSFAI)	H-PDVB-*x*-SSFAI (10 mol%), solvent-free, 140°C	2 h	78–94	[59]
23	Polyaniline–fluoroboric acid–dodecyl hydrogen sulfate (PANI–HBF_4–DHS)	PANI–HBF_4–DHS (20 wt.% of **12**), solvent-free, 150°C	6 h	94–98	[60]
24	Silica sulfuric acid (SSA)	SSA (15 mol%), solvent-free, 80°C	0.5–2 h	70–97	[61]
25	ZrPW (Zirconium IV Phosphotungstate) 12-TPA/ZrO_2 (12-Tungstophosphoric acid supported onto ZrO_2)	Condition A: ZrPW (0.2 g), solvent-free, 130°C Condition B: ZrPW (0.2 g), solvent-free, MW (250 W), 130°C Condition C: 12-TPA/ZrO_2 (0.2 g), solvent-free, 130°C Condition D: 12-TPA/ZrO_2 (0.2 g), solvent-free, MW (250 W), 130°C	8 h 30 min 8 h 30 min	42–65 47–66 38–63 41–65	[62]
26	12-Tungstophosphoric acid supported on SnO_2 nanoparticles (12-TPA-SnO_2)	12-TPA-SnO_2 (30 wt% of TPA), solvent-free, 120°C	2 h	78	[63]
27	Poly(4-vinylpyridine)-supported copper iodide	P_4VPy-CuI (0.1 g), solvent-free, 80°C	10–90 min	84–92	[64]
28	Polystyrene-supported $GaCl_3$ (PS–$GaCl_3$)	PS–$GaCl_3$ (10 mol%), ethanol, reflux	45–300 min	45–96	[65]
29	Silica tungstic acid (STA)	STA (5 mol%), solvent-free, 80°C	20–90 min	75–97	[66]
30	CMK-5 supported sulfonic acid (CMK-5-SO_3H)	CMK-5-SO_3H (3 mol%), solvent-free, 130°C	15–120 min	60–97	[67]
31	FeF_3	FeF_3 (0.05 g), solvent-free, MW (450 W), 110°C	6–9 min	61–98	[68]

Entry	Catalyst	Reaction conditions	Time	Yields (%)	Reference
32	$FeCl_3$	$FeCl_3$ (10 mol%), solvent-free, US (20 kHz, 130 W)	1–20 min	55–99	[69]
33	Sulfonic acid supported silica coated magnetic nanoparticles (Fe_3O_4@SiO_2@$PrSO_3H$)	Fe_3O_4@SiO_2@$PrSO_3H$ (1.6 mol%), solvent-free, 130°C	3–50 min	87–98	[70]
34	$CuFe_2O_4$ nanoparticles	$CuFe_2O_4$ (5 mol%), H_2O, rt	15–34 min	82–98	[71]
35	Zr(IV)-HMNQ@ ASMPs [Zirconium(IV)-3-hydroxy-2-methyl-1,4-naphthoquinone (HMNQ)@3-aminopropylated silica coated magnetic nanoparticles (ASMPs)]	Zr(IV)-HMNQ@ ASMPs (20 mg), solvent-free, 110°C	10 min	95–100 (selectivity)	[72]
36	MFI nanosponge zeolite (MFI-NSZ)	MFI-NSZ (0.1 g), dodecane (0.5 g, internal standard), nitrobenzene, 120–150°C	70 h	80–90 (selectivity)	[73]
37	$In(OTf)_3$	$In(OTf)_3$ (1 mol%), solvent-free, 80°C	10–87 min	68–98	[74]
38	$Mg(NTf_2)_2$	$Mg(NTf_2)_2$ (1 mol%), solvent-free, 80°C	25–60 min	85–98	[75]
39	Poly(4-vinylpyridinium) hydrogen sulfate (PVPHS)	PVPHS (2 mol%), solvent-free, US (35 kHz, 200 W)	3–18 min	62–96	[76]
40	Polyvinylpolypyrrolidone-bound boron trifluoride (PVPP-BF_3)	PVPP-BF_3 (33 mol%), ethanol, reflux	2–3 h	76–96	[77]
41	Zeolites e.g., beta (BEA) and ultrastable Y (USY) Metal–organic frameworks (MOFs) such as Cu-benzene-1,3,5-tricarboxylate (CuBTC) and Fe-benzene-1,3,5-tricarboxylate (FeBTC)	Condition A: Zeolite (0.2 g), nitrobenzene, 130°C Condition B: MOF (0.2 g), nitrobenzene, 130°C	23 h 23 h	23–91 (conversion) 2–98 (conversion)	[78]
42	$Zn_{0.925}Ti_{0.075}O$ NPs	$Zn_{0.925}Ti_{0.075}O$ (10 mol%), solvent-free, 110°C	3–5 h	51–89	[79]

Table 1.
Synthesis of substituted coumarins via Pechmann condensation reactions.

Catalytic activity of many other catalysts under different reaction conditions is delineated in the recently published review [80].

2.2 Knoevenagel condensation reaction

An efficient green one-pot synthetic method for the synthesis of 3-substituted coumarin derivatives **21/22** has been observed by Knoevenagel condensation of various *o*-hydroxybenzaldehydes **18/19** with 1,3-dicarbonyl compounds **20** using

nano-ZnO catalyst under microwave or thermal conditions, which affords moderate to good yield of the products (**Figure 6**) [81]. Reactions under microwave-irradiation conditions are found to be more convenient than thermal conditions.

Various coumarin-3-carboxylic acid derivatives **25/26** have been synthesized in good yields using catalytic amounts of $SnCl_2.2H_2O$ under solvent-free condition (**Figure 7**) [82].

Ultrasound irradiation technique is also useful to synthesize 3-aryl coumarin derivatives. Treatment of *o*-hydroxybenzaldehydes **18** with aryl substituted acetyl chloride **27** in the presence of K_2CO_3 as a catalyst in tetrahydrofuran (THF) using ultrasound irradiation leads to the formation of 3-aryl coumarin derivatives **28** in moderate to high yields (**Figure 8**) [83]. This green method appears to be a convenient and simple pathway than that of conventional heating.

Coumarin-substituted benzimidazole or benzoxazole derivatives **32** that are known as coumarin dyes have been synthesized in good yields from 4-diethylamino-2-hydroxybenzaldehyde **29**, ethyl cyanoacetate **30**, and ortho-phenylenediamine/phenylenehydroxyamine derivatives **31** in the presence of reusable green solid acid like HZSM-5 zeolite, heteropoly acids, e.g., tungstophosphoric acid ($H_3PW_{12}O_{40}$), and/or tungstosilicic acid ($H_4O_{40}SiW_{12}$) in *n*-pentanol or water and even solvent-free conditions (**Figure 9**) [84].

Cellulose sulfonic acid (CSA) is an efficient catalyst for the synthesis of 3-substituted coumarin via Knoevenagel condensation reaction. Thus, 3-acetyl coumarin **34** is obtained in 88% yield in the reaction between salicylaldehyde **33** and ethyl acetoacetate **7** in the presence of CSA under solvent-free conditions (**Figure 10**) [85].

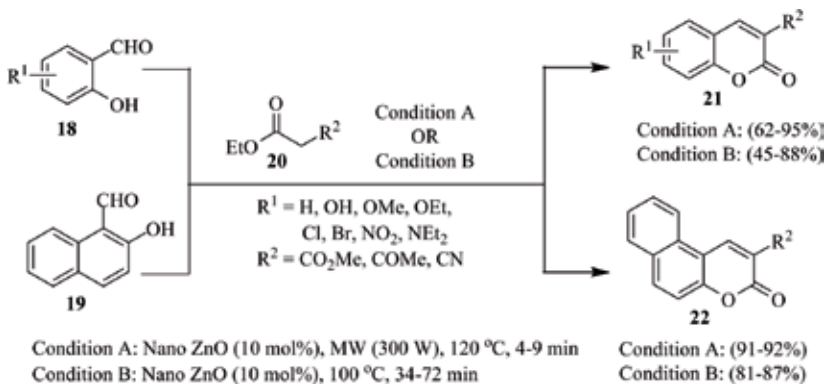


Figure 6.
Synthesis of 3-substituted coumarins.

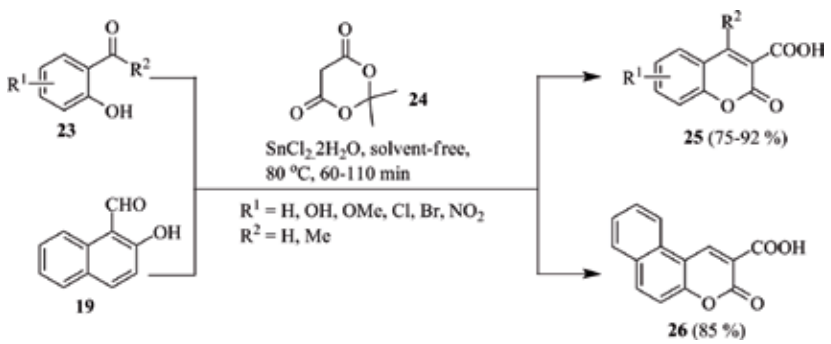


Figure 7.
Synthesis of coumarin 3-carboxylic acid derivatives.

Shaabani et al. [86] have described the synthesis of 3-substituted coumarins **21** in good yields via Knoevenagel condensation of 2-hydroxybenzaldehydes **18** with β-dicarbonyl compounds **35** in the presence of a recyclable ionic liquid 1,1,3,3-*N*,*N*,*N*′,*N*′-tetramethylguanidinium trifluoroacetate (TMGT) under thermal heating (**Figure 11**, Condition A) and/or microwave irradiation conditions (**Figure 11**, Condition B). 3-Substituted coumarins **21** are also synthesized from similar starting precursors using the 1,3-dimethylimidazolium methyl sulfate [MMIm][MSO_4] ionic liquid in the presence of L-proline as an additional promoter under heating condition (**Figure 11**, Condition C) [87].

Imidazolium based phosphinite ionic liquid (IL-$OPPh_2$) catalyzed synthesis of 3-substituted coumarin derivatives has been reported in literature; when *o*-hydroxy benzaldehydes **18** are treated with active methylene containing compounds **35** in the presence of IL-$OPPh_2$ catalyst at 60°C, 3-substituted coumarin derivatives are obtained in moderate to good yields (**Figure 12**) [88]. TSIL plays both the reaction media and catalyst as well.

Reactions of *o*-hydroxybenzaldehydes **18** with activated methylene compounds **35** catalyzed by Bronsted acid ionic liquid (BAIL) and 1-(4-sulfonic acid)butyl-3-methylimidazolium hydrogen sulfate [$(CH_2)_4SO_3HMIM$][HSO_4] in water lead to 3-substituted coumarin derivatives in good yields (**Figure 13**) [89].

Synthesis of substituted coumarins via Knoevenagel condensation using various organic catalysts such as piperidine, ammonia, L-lysine, L-proline, benzoic acid, etc. has been reported in literature and some are summarized in **Table 2**.

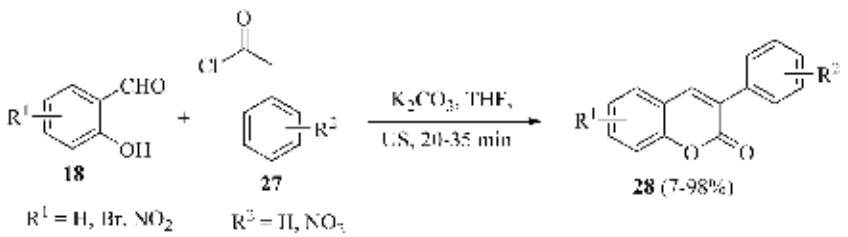


Figure 8.
Synthesis of 3-aryl coumarin derivatives.

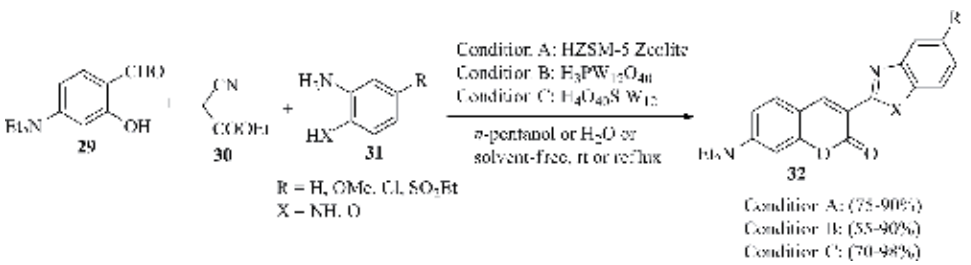


Figure 9.
Synthesis of coumarin-substituted benzimidazoles/benzoxazoles.

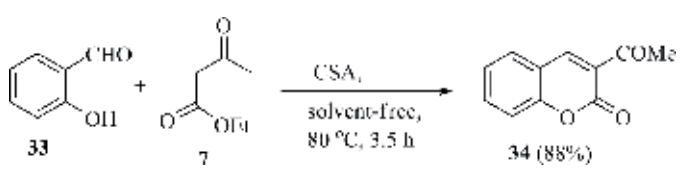


Figure 10.
Synthesis of 3-acetyl coumarin.

It is quite evident that in **Table 2** several methodologies for the synthesis of substituted coumarins using different organic catalysts are established. Among these, L-proline-catalyzed reactions offer high yields (entry 3), which explains synthesis of 3-substituted coumarins by the condensation of *o*-hydroxybenzaldehydes with a variety of active methylene compounds catalyzed by 1,3-dimethyl imidazolium methyl sulfate [MMIm][MSO_4] and L-proline. Another L-proline-catalyzed synthesis of coumarins is known, but in that case, the yield is very poor (entry 4). Similar result is also observed under L-lysine-catalyzed synthesis of coumarins (entry 5).

A series of 3-phenyl substituted coumarin analogues have been achieved via a two-step process involving esterification using 1,1-carbonyldiimidazole (CDI) followed by condensation reaction in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) under mild conditions (entry 1).

Microwave-assisted synthesis of coumarins is also known, which not only reduces the reaction time but also increases the yields of the products (entries 2, 6, and 7).

Benzocoumarin derivatives have been synthesized from 1-hydroxy-4-methyl-naphthalene-2-carbaldehyde and compounds containing active methylene group via piperidine-catalyzed Knoevenagel condensation reaction (entry 8). Moreover, benzothiazolyl coumarins with isothiocyanate functionality have been synthesized from commercially available 2-hydroxy-4-nitro benzoic acid in the presence of piperidine in ethanol (entry 9).

Application of sonochemistry for the synthesis of different coumarin derivatives is also useful due to better yield and shorter reaction time compared with the classical procedures (entry 10).

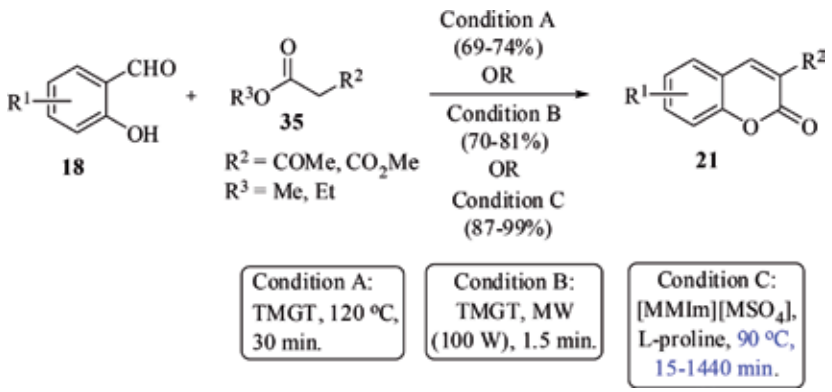


Figure 11.
Synthesis of 3-substituted coumarins.

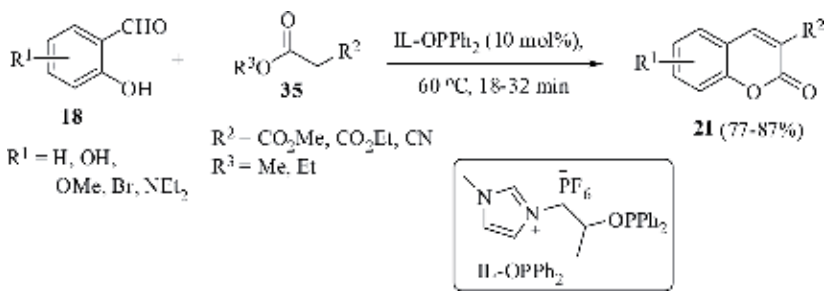


Figure 12.
Synthesis of 3-substituted coumarins.

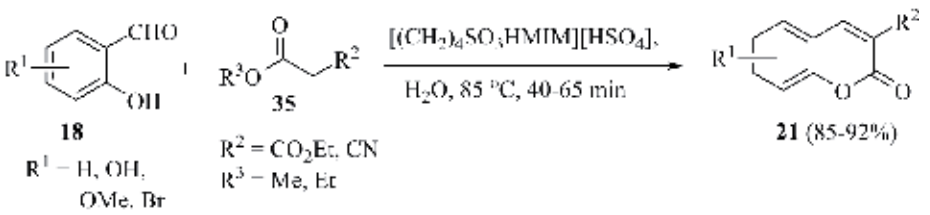
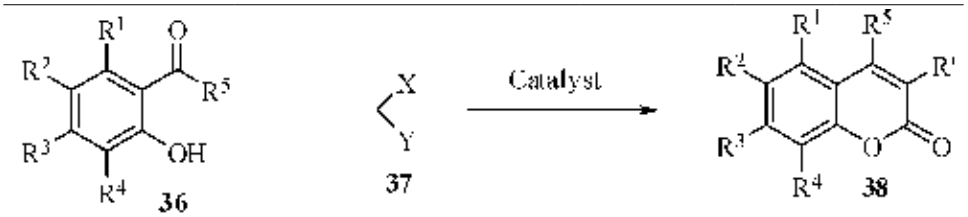


Figure 13.
Synthesis of 3-substituted coumarins.



R1 H, OH, OMe, Cl, Br, NO_2,
R2 H, Me, OMe, CO_2Et, CN, Br, I
R3 = H, Cl, Br, NEt_2, OH, OMe, NO_2
R4 = Me, OMe, OEt, I
R5 H, Me

X = H, Me, CN, CO_2H, CO_2Me, CO_2Et, COMe, COPh, SO_2Me, SO_2Ph, $SO_2C_4F_9$, $CONH_2$
Y CN, CO_2Me, CO_2Et, aryl
R' = CN, COMe, CO_2H, CO_2Et, aryl

Entry	Catalyst	Reaction conditions	Time	Yield (%)	Reference
1	CDI-DBU	(i) CDI (1.2 equiv.), DCM, rt. (ii) DBU (1.0 equiv.), DCM, rt	30 min 1–2 h	42–59	[90]
2	PhCOOH	Condition A: Polyphosphoric acid, MW (900 W), 100°C Condition B: H_2SO_4, Benzoic acid, MW (900 W), 90°C Condition C: benzoic acid, *n*-pentanol, MW (900°C), 110°C	4–6 min 3–4 min 3 min	60–75 58–75 85–95	[91]
3	L-proline	1,3-dimethyl imidazolium methyl sulfate, [MMIm][MSO4], L-proline (1 equiv.), 90°C	15–1440 min	87–99	[87]
4	L-proline	L-proline (20 mmol%), EtOH, rt	15–20 h	54–76	[92]
5	L-lysine	L-lysine (20 mol%), H_2O, rt. −80°C	6–24 h	50–90	[93]
6	Piperidine	Piperidine (catalytic), rt.	20 min	84	[94]
7	Piperidine	Piperidine (2.0 mol%), solvent-free, MW (400 W)	1 min	50–97	[95]
8	Piperidine	Piperidine (1.48 equiv.), EtOH, reflux	30 min	85–92	[96]
9	Piperidine	Piperidine (catalytic), EtOH, reflux	2 h	82	[97]
10	Piperidine	Piperidine (1.0 equiv.), AcOH (2.5 mol%), EtOH, US, rt	5–30 min	49–90	[98]
11	Piperidine	Piperidine, EtOH, rt-reflux	1–2 h	82–92	[99]
12	Piperidine	Piperidine (7.4 equiv.), EtOH, reflux	2 h	92	[100]

Table 2.
Synthesis of substituted coumarins via Knoevenagel condensation reactions.

6,8-Diiodocoumarin derivatives have also been synthesized in good yields by Knoevenagel condensation using piperidine as catalyst (entry 11). The reaction of 3-ethoxysalicylaldehyde with ethyl acetoacetate in the presence of piperidine leads to 3-acetyl-8-ethoxycoumarin (entry 12).

2.3 Baylis-Hillman reaction

Baylis-Hillman strategy has been employed to the synthesis of substituted coumarins as shown in **Figure 14**. When 2-hydroxybenzaldehydes **18** are subjected to react with methyl acrylate **39a** (R^2 = Me) in the presence of DABCO (1,4-Diazabicyclo[2.2.2] octane), a mixture of chromenes **40** and coumarins **41** are formed [101, 102]. However, similar reactions of 2-hydroxybenzaldehydes **18** with tert-butyl acrylate **39b** (R^2 = ^{t}Bu) under classical method [103] and/or microwave irradiation [104] afford corresponding Baylis-Hillman adducts **42**, which undergo cyclization under reflux in AcOH yielding a mixture of 3-substituted chromene **43** and coumarin **44**. Treatment of the Baylis-Hillman adducts **42** with concentrated HCl in refluxing AcOH produces 3-(chloromethyl) coumarins **45** in excellent yields. Moreover, the reaction of **42** with HI under reflux in a mixture of Ac_2O and AcOH furnishes 3-methyl coumarins **46**, which upon further reaction with SeO_2 affords the corresponding 3-formyl coumarins **47**.

The suggested mechanism for the formation of the coumarin derivatives **44/45/46** is shown in **Figure 15**.

Kaye et al. have also demonstrated the synthesis of substituted coumarins employing Baylis-Hillman strategy in different ways as shown in **Figure 16** [105, 106].

2.4 Kostanecki reaction

4-Arylcoumarins **59** have been synthesized in good yields employing Kostanecki reaction between 2-hydroxybenzophenones **57** and acetic anhydride **58** in the presence of DBU under mild condition (**Figure 17**) [107].

The mechanism of the Kostanecki reaction is outlined in **Figure 18**.

Similarly, 3,4-disubstituted coumarins **65** are isolated from readily available 2-acyloxybenzophenones **64** under Kostanecki reaction conditions (**Figure 19**) [107].

2.5 Michael addition reaction

Michael addition could be applied [108] to the synthesis of 3-aroylcoumarins **68** in good yields from easily available 2-hydroxybenzaldehydes **66** and α-aroylketene dithioacetals (AKDTAs) **67** in the presence of a catalytic amount of piperidine in refluxing THF (**Figure 20**).

The reaction proceeds via initial Michael addition followed by intramolecular aldol condensation reaction as depicted in **Figure 21**.

2.6 Wittig reaction

Kumar and coworkers [109] have reported the synthesis of substituted coumarins **3** from phenolic compounds **23** containing ortho-carbonyl group and triphenyl (α-carboxymethylene)phosphorane imidazole ylide **73** via intramolecular Wittig cyclization in good yields (**Figure 22**). All the reactions proceed via formation of the phosphorane intermediates **74** as established by spectroscopic results.

2.7 Vinyl phosphonium salt-mediated electrophilic substitution reaction

A series of 4-carboxy(ethyl/methyl) coumarins **76** have been synthesized in good yields from substituted phenols **1** and di(ethyl/methyl)

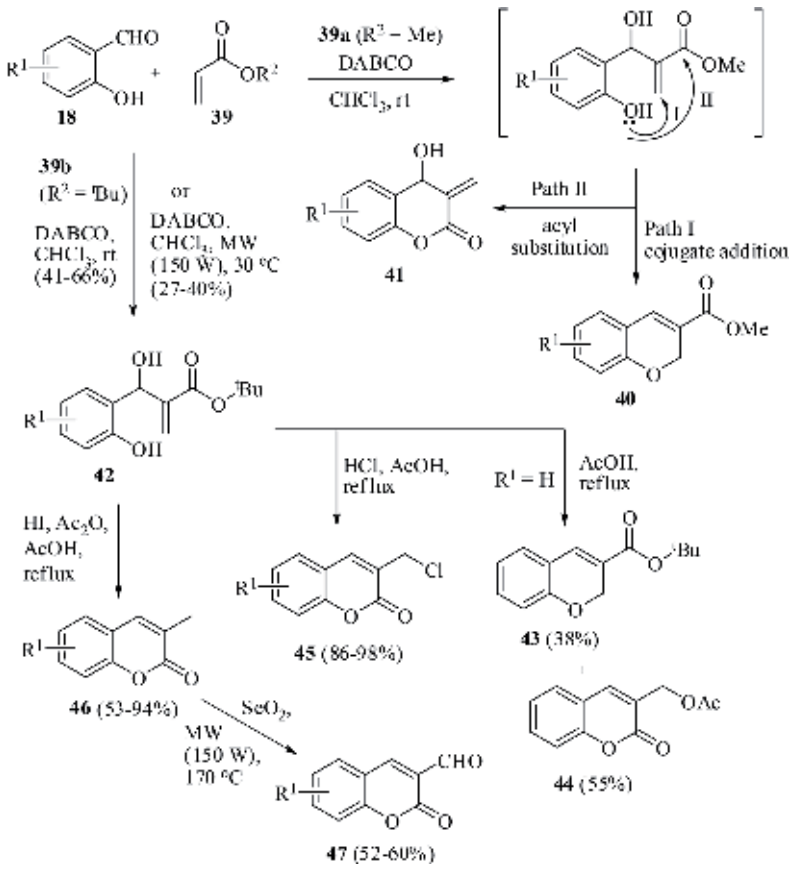


Figure 14.
Synthesis of 3-substituted coumarins.

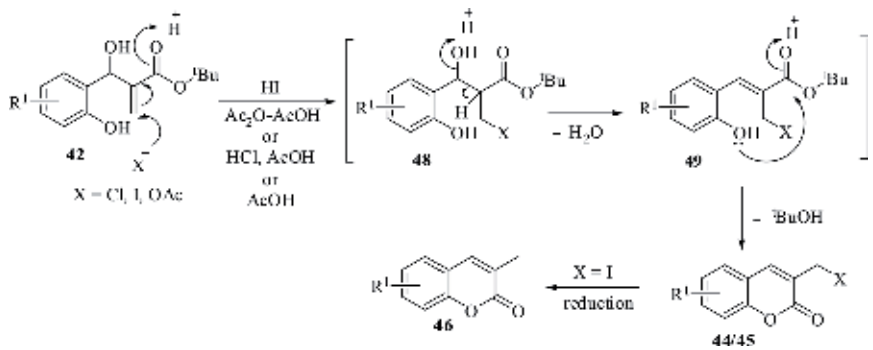


Figure 15.
Possible mechanism for the formation of 3-substituted coumarins.

acetylene-dicarboxylate **75** in the presence of phosphinite ionic liquid (IL-$OPPh_2$) under solvent-free microwave irradiation conditions (**Figure 23**) [110]. It is noticed that the diphenylphosphine group in ionic liquid accelerates the reaction.

The proposed mechanism for the formation of coumarins **76** via vinyl phosphonium salt-mediated electrophilic substitution is shown in **Figure 24**.

4-Carboxymethyl coumarins **82** have been synthesized by Yavari et al. [111] in moderate to excellent yields from the reactions of substituted phenols **1** and dimethyl acetylenedicarboxylate (DMAD) **81** in the presence of triphenylphosphine (**Figure 25**) via vinyl triphenylphosphonium salt-mediated aromatic electrophilic

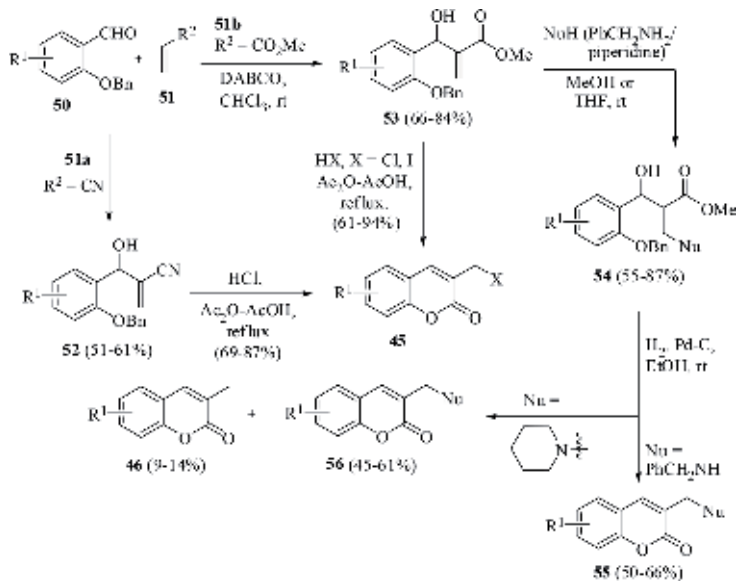


Figure 16.
Synthesis of 3-substituted coumarins.

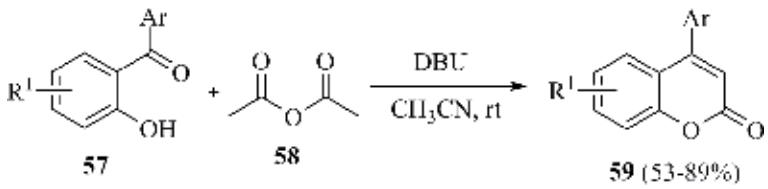


Figure 17.
Synthesis of 4-arylcoumarins.

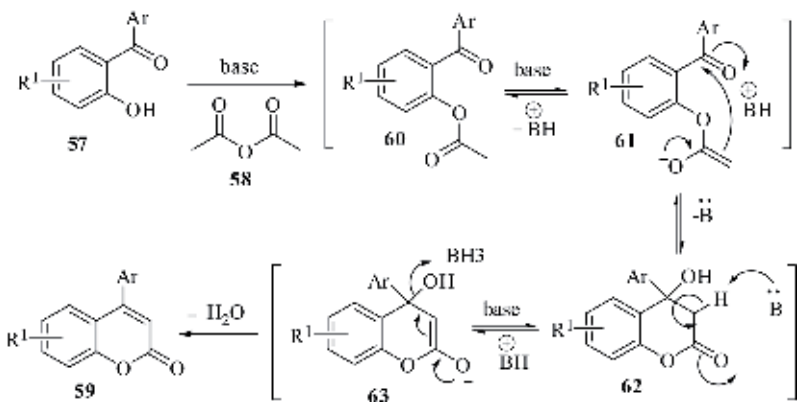


Figure 18.
Mechanism for Kostanecki reaction.

substitution reaction as mentioned in **Figure 24**. Similar results are found from the given starting materials under microwave irradiation in shorter reaction time [112].

However, reactions of di- and trihydric phenols with dimethyl acetylenedicarboxylate (DMAD) in the presence of triphenylphosphine in toluene under reflux afford polyfunctionalized coumarin analogues along with unwanted by-products in appreciable amount (**Figure 26**) [113].

Figure 19.
Synthesis of 3,4-disubstituted coumarins.

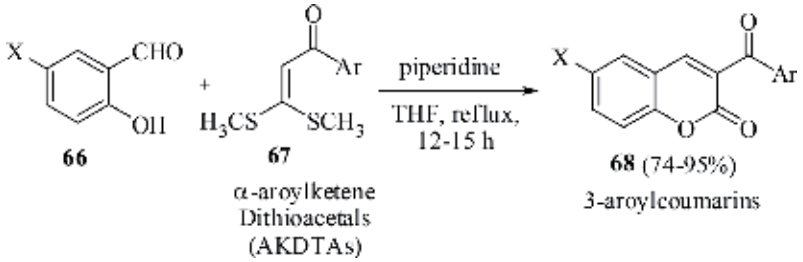


Figure 20.
Synthesis of 3-aroylcoumarins.

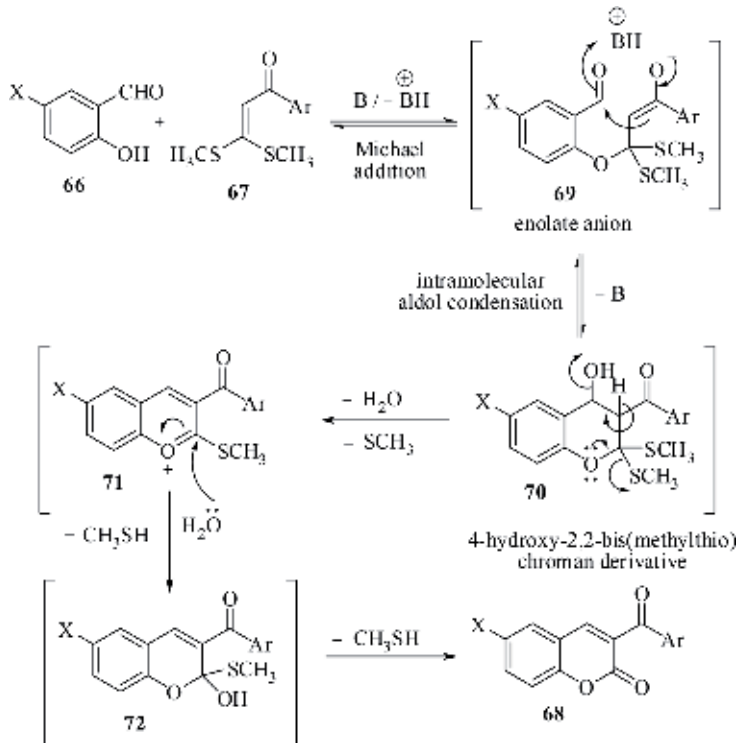


Figure 21.
Probable mechanism for the formation of 3-aroylcoumarins.

Similar reactions of 2-hydroxybenzaldehydes **18** with di(ethyl/methyl)acetylenedicarboxylates **75** leads to the corresponding 4-carboxy(ethyl/methyl)-8-formyl coumarins **93** in moderate to good yields (**Figure 27**) [114].

The methodology has also been employed to the synthesis of angular pyridocoumarins **97**/**98** and benzo-fused 6-azacoumarin **100** as shown in **Figure 28** [115].

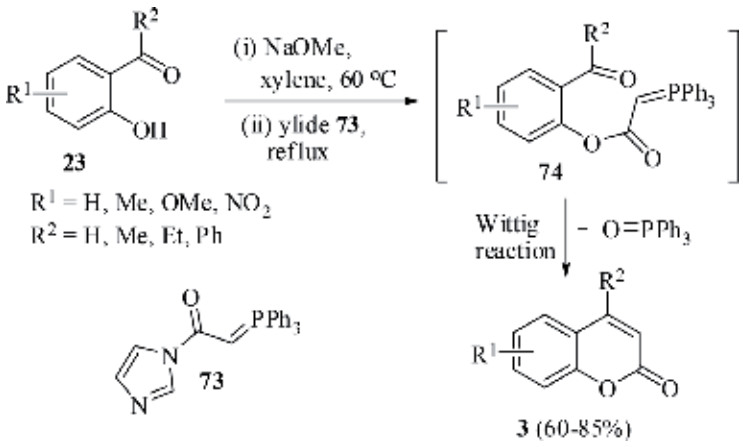


Figure 22.
Synthesis of substituted coumarins.

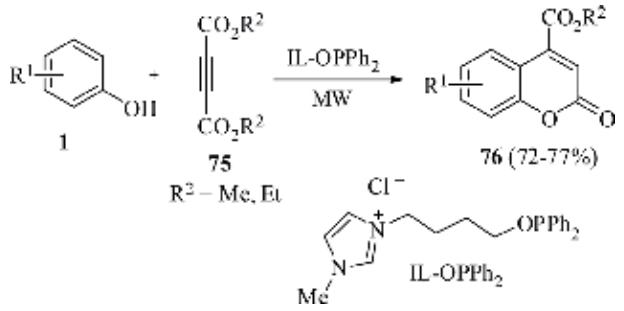


Figure 23.
Synthesis of 4-carboxy(ethyl/methyl) coumarins.

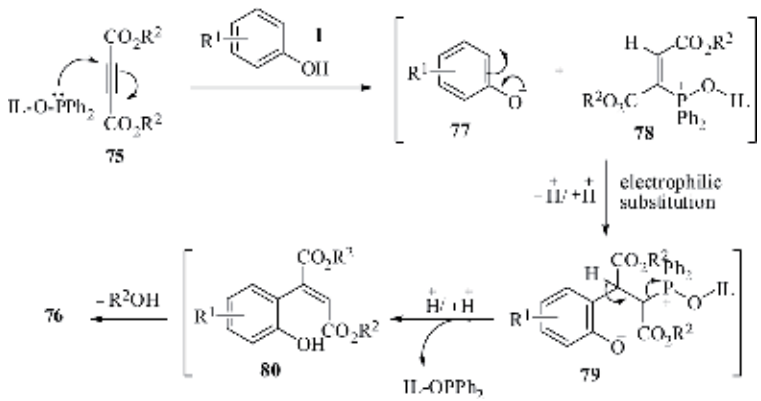


Figure 24.
Proposed mechanism for the synthesis of substituted coumarins via vinyl phosphonium salt-mediated electrophilic substitution.

2.8 Palladium-catalyzed reactions

Palladium-catalyzed reactions between substituted phenols **101** and ethyl propiolates **102** lead to substituted coumarins **103/104** (**Figure 29**) [116, 117].

Unsymmetrical monohydric phenols having *m*-OMe or *m*-Me substituent as respectively in 3-methoxyphenol and *m*-cresol show regioselectivity toward the

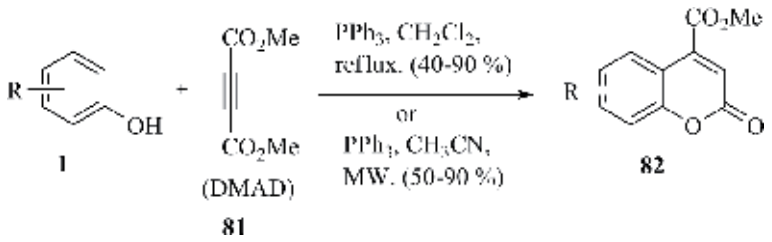


Figure 25.
Synthesis of 4-carboxymethyl coumarins.

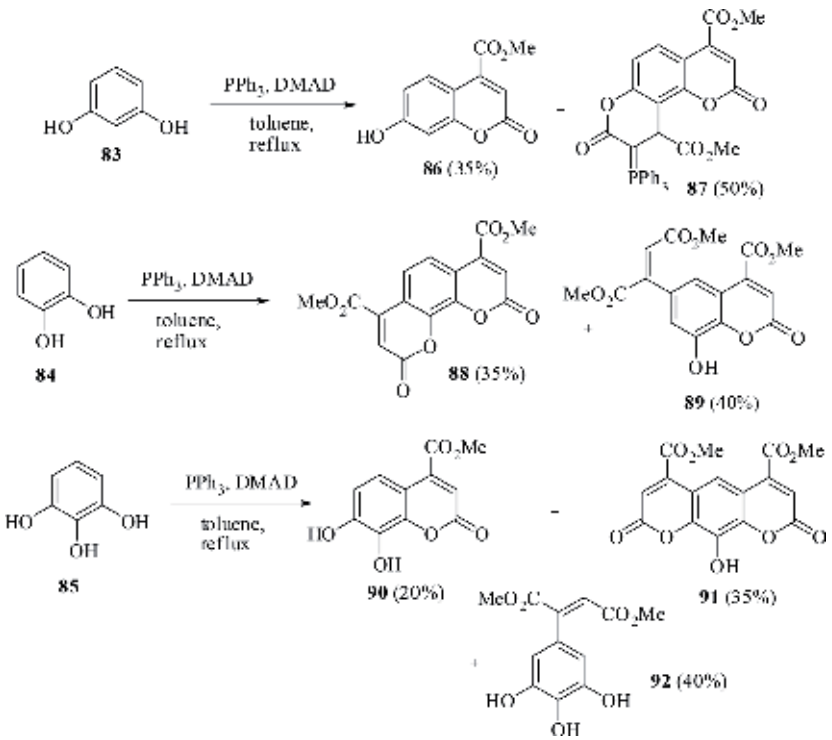


Figure 26.
Synthesis of polyfunctionalized coumarin analogues.

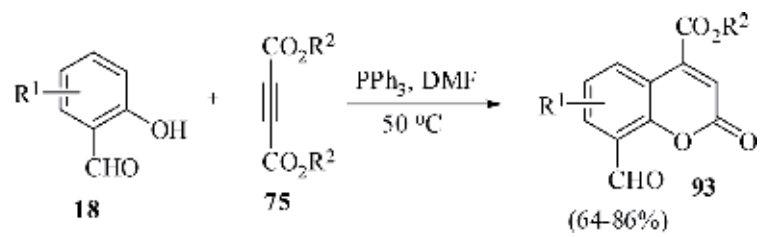


Figure 27.
Synthesis of 4-carboxy(ethyl/methyl)-8-formyl coumarins.

formation of a new bond in coumarins, which occurs at the *para* position to the methoxy group, and therefore, the regioisomers **103** are found to be formed predominantly over **104**. However, symmetrical dihydric phenol with OMe substituent like that in 5-methoxybenzene-1,3-diol affords the regioisomer **104** predominantly over **103** under the reaction condition applied. This may be due to the steric effects

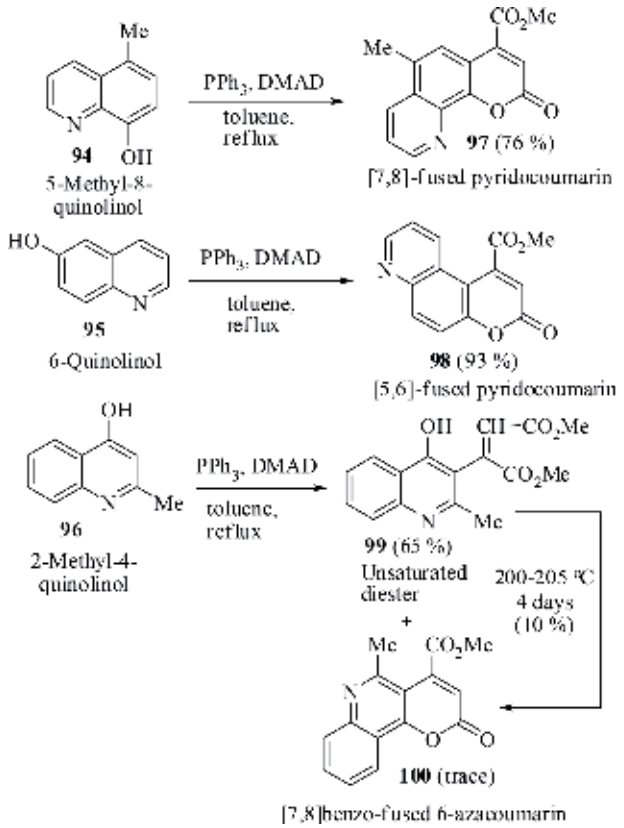


Figure 28.
Synthesis of pyridocoumarins and benzo-fused azacoumarin.

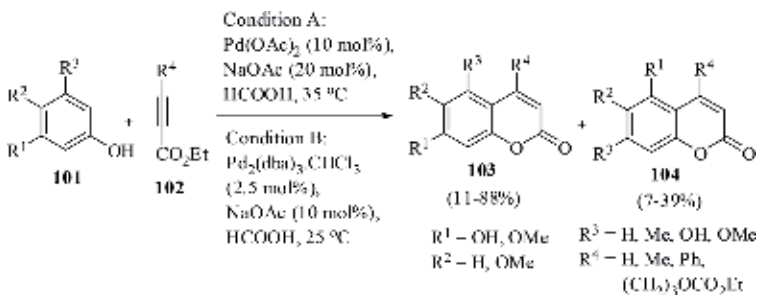


Figure 29.
Synthesis of substituted coumarins.

of the R^4 group of ethyl propiolate **102**, which dominates over the electronic effect of the methoxy group of the phenol.

A proposed mechanism for the formation of coumarins **103/104** is shown in **Figure 30**.

Substituted coumarins **3** have been synthesized in moderate yields (42–69%) via $Pd(OAc)_2$-catalyzed reaction of substituted phenols **1** with substituted propiolic acid **110** ($R^3 = CO_2H$) in TFA under mild conditions (**Figure 31**, Condition A) [118]. However, a mixture of catalysts $FeCl_3$ and AgOTf showed better catalytic efficiency toward yields (60–93%) of coumarin derivatives **3** (**Figure 31**, Condition B). Propiolic acid ester **110** ($R^3 = CO_2Et$) also furnishes the desired products **3** upon

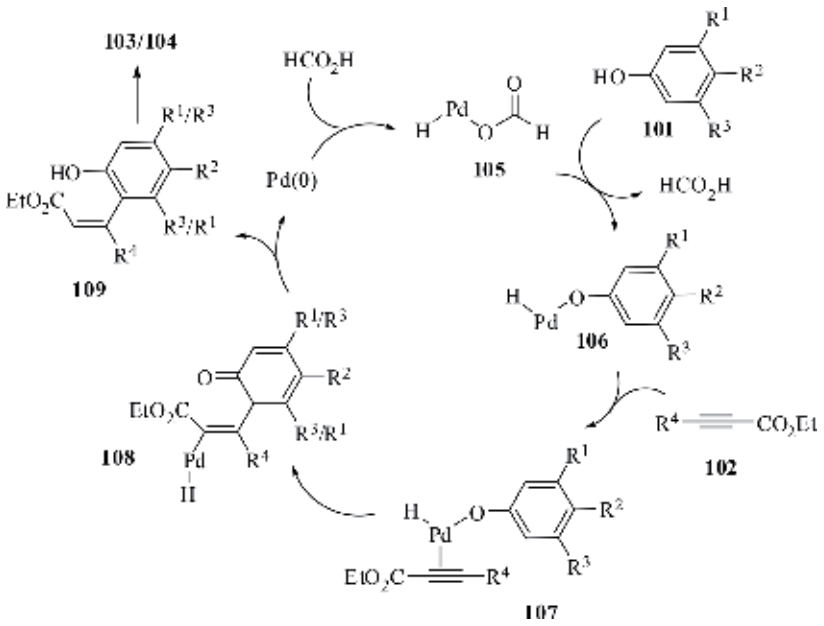


Figure 30.
Possible mechanism for Pd-catalyzed synthesis of coumarins.

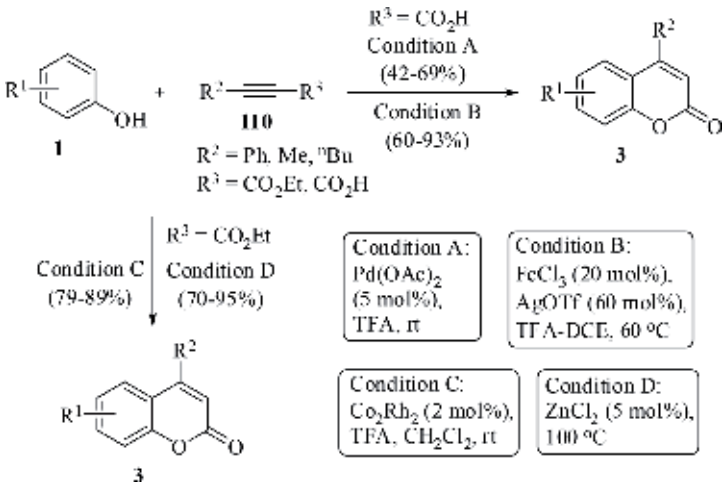


Figure 31.
Synthesis of substituted coumarins.

reactions with substituted phenols **1** under specified conditions as provided in **Figure 31** (Conditions C and D) [119–121].

4,6-Disubstituted coumarins **113** have been achieved employing palladium-catalyzed tandem Heck-lactonization of the *Z*- or *E*-enoates **112** with *o*-iodophenols **111** (**Figure 32**, Conditions A, B, and C) [122, 123].

For Heck-lactonization, the enoate *Z*-**112a** is found to be more reactive than its *E*-isomer, leading to the corresponding coumarin **113** in good yields (68–84%) under all reaction conditions studied. The enoate *Z*-**112b** leads to coumarin derivative **113** in relatively lower yields (42–56%), which may be due to the presence of the bulky ^{t}Bu ester group that hampers the lactonization step. Moreover, the reactivity of *E*-enoates depends on the β-substituent. *E*-enoates **112c** (R^2 = CH_2CHMe_2,

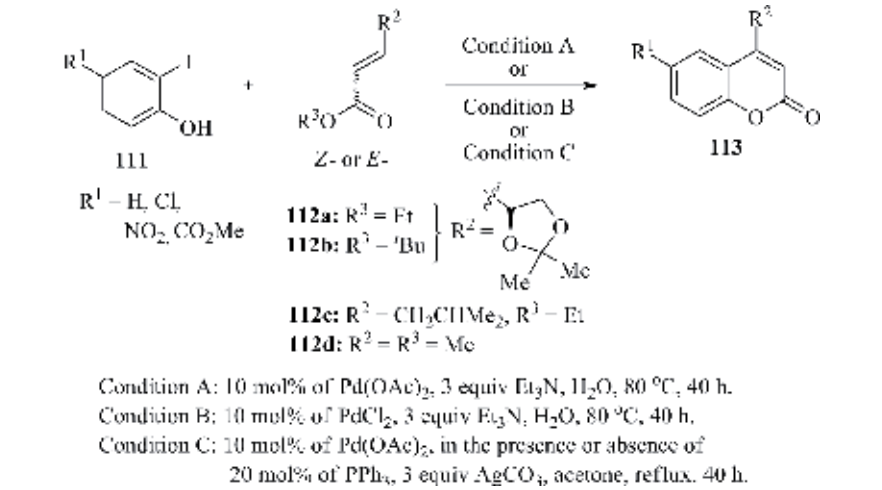


Figure 32.
Synthesis of 4,6-disubstituted coumarins.

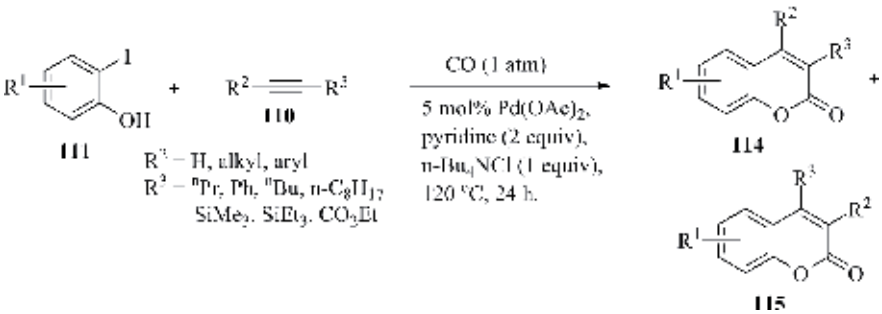


Figure 33.
Synthesis of 3, and 4-substituted and 3,4-disubstituted coumarins.

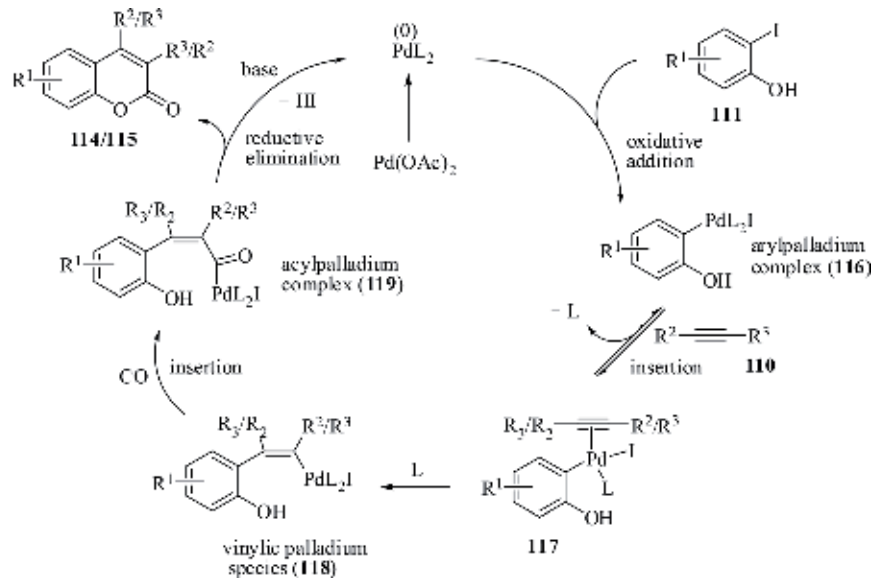


Figure 34.
Possible mechanism for the synthesis of coumarins via carbonylative annulation.

$R^3 = CH_3$) and **112d** ($R^2 = R^3 = CH_3$) having CH_2CHMe_2 and CH_3 group, respectively, at the β-carbon, and their double bonds are therefore less sterically hindered than that in *E*-enoate **112a.** This reduced hindering is a major factor for the higher reactivity of *E*-enoates **112c** and **112d** than *E*-enoate **112a**.

Palladium-catalyzed carbonylative annulation of terminal alkynes **110** (R^2 = H; $R^3 = {}^nPr$, Ph, $SiMe_3$, $SiEt_3$, CO_2Et, etc.) with *o*-iodophenols **111** affords 3-substituted coumarins **114** (R^2 = H) in poor yields (18–36%) (**Figure 33**) [124]. On the other hand, both 3- and 4-substituted coumarins **114** (R^2 = H) and **115** (R^2 = H) have been synthesized from *o*-iodophenols **111** and terminal alkynes **110** (R^2 = H; $R^3 = {}^nC_4H_9$, ${}^nC_8H_{17}$) bearing long alkyl chain. In addition, a wide variety of 3,4-disubstituted coumarins **114/115** (R^2, $R^3 \neq$ H) have also been achieved in moderate to good yields (43–78%) via carbonylative annulation between *o*-iodophenols **111** and internal alkynes **110** (R^2, $R^3 \neq$ H) [125].

The suggested mechanism of the carbonylative annulation is presented in **Figure 34**. The carbonylative annulation process is believed to proceed via (a) oxidative addition of *o*-iodophenol **111** to Pd(0), (b) insertion of alkyne **110** into the aryl-palladium complex **116**, (c) CO insertion into the resulting vinylic palladium species **118**, and (d) nucleophilic attack of the phenolic oxygen on the carbonyl carbon of the acylpalladium complex **119** with simultaneous regeneration of the Pd(0) catalyst.

3,4-Disubstituted coumarins **121** are also isolated in good to excellent yields from readily available 2-(1-hydroxyprop-2-ynyl)phenols **120** via palladium-catalyzed

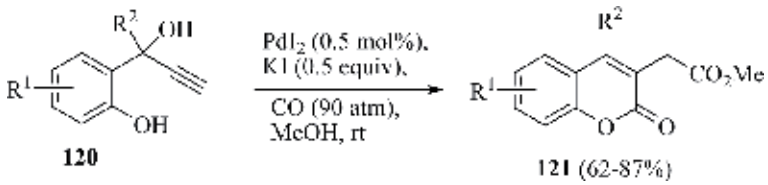


Figure 35.
Synthesis of 3,4-disubstituted coumarins.

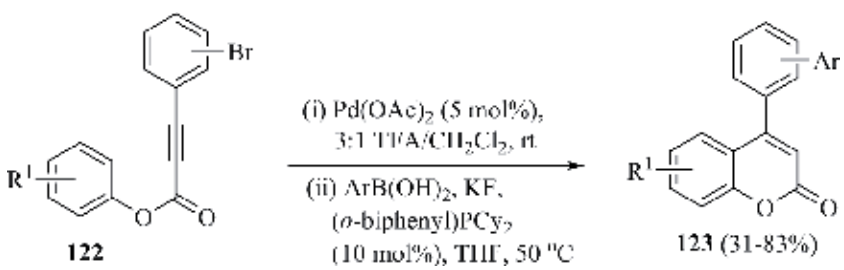


Figure 36.
Synthesis of 4-arylcoumarins.

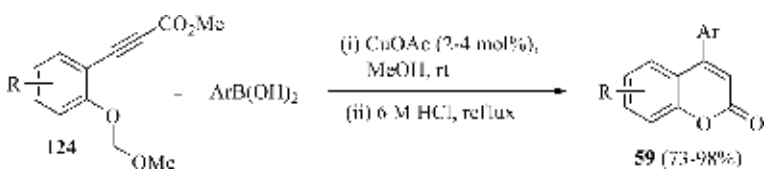


Figure 37.
Synthesis of 4-arylcoumarins.

dicarbonylation process in the presence of KI in MeOH at room temperature (**Figure 35**) [126].

Furthermore, electrophilic palladium-catalyzed cycloisomerization of brominated arylpropiolates **122** followed by Suzuki coupling with arylboronic acids furnishes 4-arylcoumarins **123** in moderate to good yields (**Figure 36**) [127]. This strongly suggests that a single loading of catalyst $Pd(OAc)_2$ could be used to conduct sequential reactions for the synthesis of substituted coumarins.

2.9 Other methods

CuOAc-catalyzed hydroarylation of methyl phenylpropiolates **124** having a methoxy methyl (MOM)-protected hydroxyl group at the ortho-position with various arylboronic acids followed by acidic workup leads to 4-arylcoumarins **59** in good to excellent yields (**Figure 37**) [128].

Substituted coumarins **126** are obtained in moderate to excellent yields by $Yb(OTf)_3$-catalyzed reactions of substituted phenols **1** with alkylidene Meldrum's acid **125** in CH_3NO_2 at 100°C (**Figure 38**) [129].

A series of 3-alkylcoumarins **128** are obtained in moderate yields from 2-hydroxybenzaldehydes **18** and α,β-unsaturated aldehydes **127** via generation of *N*-heterocyclic carbenes (NHC) in ionic liquid under conventional heating (**Figure 39**, Condition A) and/or microwave irradiation conditions (**Figure 39**, Condition B) [130].

3-Benzoylcoumarins **130/131** and coumarin-3-carbaldehydes **47** have also been isolated in moderate to good yields from the reactions of 2-hydroxybenzaldehydes **18/19** with phenylpropionyl chloride **129a** and/or propionyl chloride **129b** under esterification conditions (**Figure 40**) [131].

An electrochemical method has been developed for the synthesis of 6*H*-benzo[*c*] chromen-6-ones **133** in good to excellent yields from biphenyl-2-carboxylic acids **132** via radical arene carbon–oxygen bond formation reaction (**Figure 41**) [132].

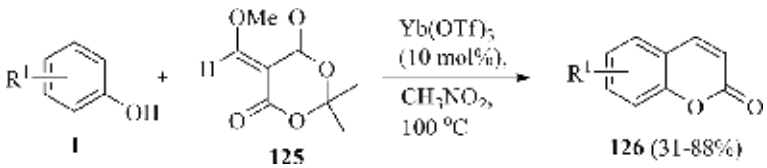


Figure 38.
Synthesis of substituted coumarins.

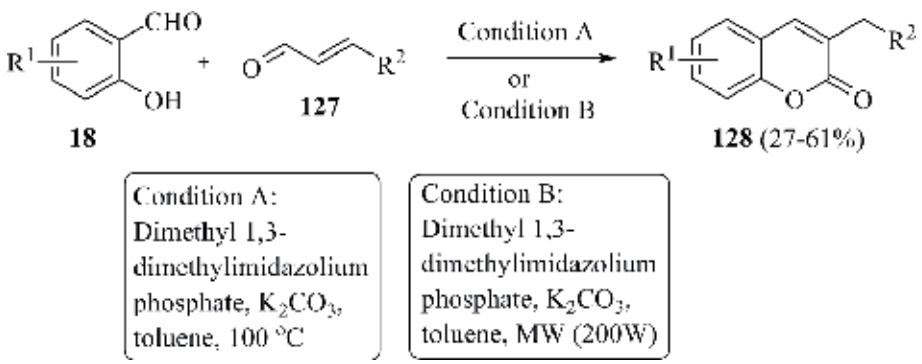


Figure 39.
Synthesis of 3-alkylcoumarins.

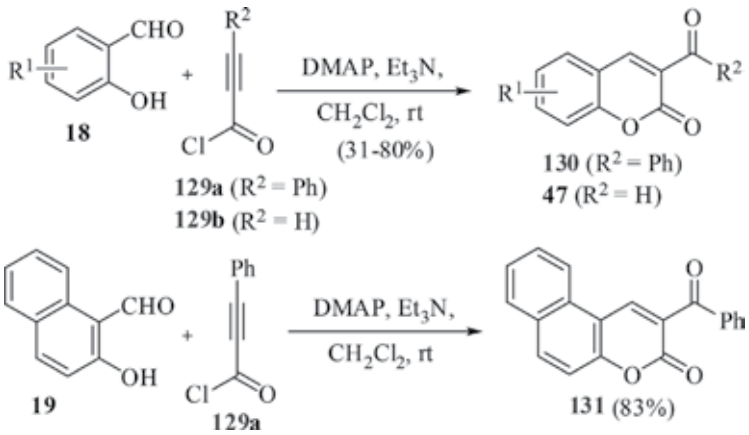


Figure 40.
Synthesis of 3-benzoyl coumarins and coumarin-3-carbaldehyde.

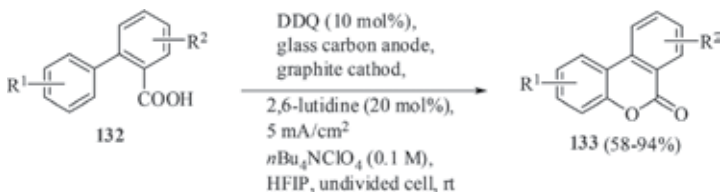


Figure 41.
Synthesis of 6H-benzo[c]chromen-6-ones.

The method involves DDQ as a redox mediator, inexpensive glassy carbon electrodes to facilitate an intramolecular lactonization of biphenyl-2-carboxylic acid derivatives, and 2,6-lutidine as an additive, in 0.1 M $^{n}Bu_4NClO_4$ electrolyte mixture of 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP).

3. Concluding remarks

In this chapter, we have discussed a plethora of methods for the one-pot synthesis of coumarin derivatives and their advantages and/or demerits compared to other methods. Both the Pechmann as well as Knoevenagel condensation reactions under microwave and/or ultrasound irradiation conditions, and catalyzed by ionic liquids and/or solid acids have several advantages including high products yields, diminutive reaction times, ease of isolation of products, recycle of catalysts, and green aspects by avoiding toxic catalysts and solvents. Chemo- and regioselective syntheses of 3-substituted coumarins have been reported via Baylis-Hillman reactions under mild conditions. On the other hand, vinyl phosphonium salt-mediated electrophilic substitution reactions of phenols afford 4-carboxyalkyl coumarin derivatives in good yields under neutral conditions. This method offers significant advantages for the synthesis of coumarins having acid sensitive functional groups. In contrast, the most widely used method von Pechmann condensation requires acidic conditions. Moreover, palladium-catalyzed Heck lactonization protocol has been employed for the regioselective synthesis of coumarin derivatives from *o*-iodophenols and enoates. It is revealed that this reaction is sensitive to steric hindrance around the double bound in the enoates. Regioselective synthesis of 3,4-disubstituted coumarins

achieved from substituted 2-iodophenols and alkynes containing different substituents via palladium-catalyzed carbonylative annulative process is sensitive to the steric bulk of the alkynes, and alkynes bearing tertiary alkyl substituents generally fail to undergo annulation. Unsymmetrical alkynes produce mixtures of regioisomers with generally only modest selectivity. Kostanecki reaction protocol furnishes a notable improvement in reaction conditions for coumarin synthesis and gives rise to the advantage of its synthetic capability, especially for highly functionalized 4-arylcoumarins with structural diversity.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 7

Coumarin Derivatives with Antimicrobial and Antioxidant Activities

Gabriela Tataringa and Ana Maria Zbancioc

Abstract

Coumarin derivatives are structurally interesting compounds for synthesizing antimicrobial and antioxidant agents. Starting from 4-methyl-7-hydroxycoumarin, several derivatives with these properties have been obtained through different reaction steps. Their molecular structures were established by Fourier-transform infrared spectroscopy and nuclear magnetic resonance spectroscopy. The synthesized coumarin derivatives exerted meaningful activities against Gram-positive and Gram-negative bacteria as well as strains of *Candida* spp. All compounds also exhibited high and moderate antioxidant activity in assays for DPPH inhibition, total reducing power, and nitric oxide (NO) inhibition when compared to ascorbic acid.

Keywords: 4-methyl-7-hydroxycoumarin, synthesis, coumarin derivatives, antibacterial activity, antifungal activity, antioxidant activity

1. Introduction

Natural and synthetic coumarins have drawn much attention due to its broad pharmacological activities. Literature review reveals that coumarin (2-oxo-2H-chromene) and its derivatives represent one of the most active classes of heterocyclic compounds which possess a wide spectrum of biological activities [1–9]: antitumor [1, 2], antibacterial [3, 4], antifungal [5–7], anticoagulant [8], antioxidant [9], and anti-inflammatory [10].

1.1 Coumarins as antimicrobial agents

Over the past few decades, the search for newer antimicrobials remains an area of intensive investigation in the field of medicinal chemistry due to resistance developed by microorganism to conventional antibiotics. Antimicrobials are one of most significant weapons in fighting bacterial infections. Throughout history, there has been a continual battle between humans and the multitude of microorganisms that cause infection and disease [11, 12]. Coumarin derivatives have a wide range of structural modifications [13], and they can serve as molecular templates for new drugs. Coumarin derivatives are also considered as potential antimicrobial agents [14].

Medimagh-Saidana et al. reported synthesis and antimicrobial activity of some coumarin esters (**1**) (**Figure 1**). These compounds showed good activity against

Bacillus sp. and moderate activity against *Aspergillus niger*. For the data of the antibacterial activity, these compounds were found to be active against *Pseudomonas* sp. [15].

Al-Amiery et al. have synthesized some coumarin derivatives, and their antifungal activity was determined based on the growth inhibition rates of the mycelia of strains of *Aspergillus niger* and *Candida albicans* in Potato Dextrose Broth (PDB) medium against concentrations ranging from 10 to 100 μg/ml. The compound (**2**) (**Figure 1**) showed good activity as antifungals against fluconazole as standard drug [16].

Behrami et al. synthesized 8-amino-4,7-dihydroxy-chromen-2-one coumarin derivatives. The antibacterial activities of all the compounds and standard streptomycin and cefalexine at concentrations of 2, 3, and 5 mg/ml were studied against *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli*. One compound (**3**) (**Figure 1**) was more active than cefalexine and lesser active than streptomycin, and it was most active among synthesized compounds [17].

Some coumarin derivatives containing thiazolidin-4-one ring were synthesized by Rama Ganesh et al. and were screened for their antibacterial activity against Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* and Gram-negative bacteria *Klebsiella pneumonia* and *Escherichia coli* at the concentration of 0.001 mol/ml compared with the standard drug ciprofloxacin. Zone of inhibition of highly active compound (**4**) (**Figure 1**) was 20 mm against *Staphylococcus aureus* and *Bacillus subtilis* [18].

1.2 Coumarins as antioxidant agents

Free radicals are molecular species capable of independent existence that contain an unpaired electron in an atomic orbital; they are usually unstable and very reactive. These species are normally produced in the human body from essential metabolic processes, but they may also occur from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals [19].

There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body and to prevent the deterioration of fats and other constituents of food stuffs. In both cases, there is a preference for antioxidants from natural rather than from synthetic

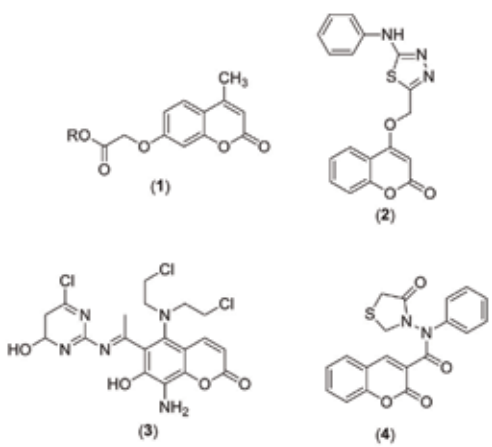


Figure 1.
Coumarin derivatives with antimicrobial activity.

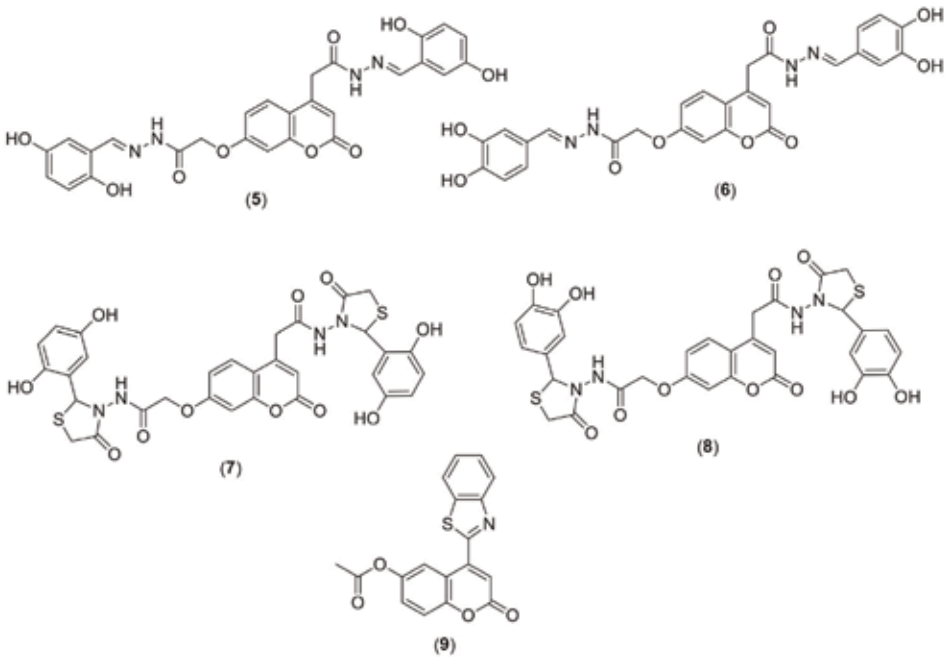


Figure 2.
Coumarin derivatives with antioxidant activity.

sources [20]. As improved antioxidant status helps to minimize the oxidative damage and thus delays or prevents pathological changes, potential antioxidant therapy should be included either as natural free-radical-scavenging antioxidant enzymes or as an agent which is capable of augmenting the activity of antioxidant enzymes [21].

The human organism possesses natural systems to annihilate these species, but when the body's ability to regulate them is overwhelmed, a condition known as oxidative stress appears, free radicals attacking important macromolecules leading to cell damage and homeostatic disruption [22].

Many coumarin derivatives have a special ability to scavenge reactive oxygen species and to influence processes involving free radical injury [23, 24].

Maja et al. synthesized a series of carbohydrazide with coumarin ring (**5**, **6**) and some coumarin derivatives with a heterocyclic ring (**7**, **8**) (**Figure 2**). All these compounds prove a good antioxidant activity [25].

Shivani et al. synthesized new coumarin-substituted derivatives of benzothiazole, and they were evaluated for antioxidant activity by DPPH radical scavenging activity. The test compound (**9**) (**Figure 2**) showed good in vitro antioxidant activity [26].

2. Synthesis of coumarin derivatives

Looking to the medicinal importance of the coumarin ring, we employed coumarin as a naturally occurring skeleton for the construction of new derivatives which might exhibit promising antimicrobial and antioxidant activities [27].

The starting materials, 4-methyl/propyl-7-hydroxycoumarin, were prepared by Pechmann synthesis which involved the condensation of resorcinol and ethylacetoacetate/ethylbutyrylacetate in the presence of H_2SO_4 concentrate [28].

2.1 Mechanism of the Pechmann condensation

The reaction is conducted with a strong Brønsted acid such as methanesulfonic acid or a Lewis acid such as $AlCl_3$. The acid catalyzes transesterification as well as keto-enol tautomerization [29]. A Michael addition leads to the formation of the coumarin skeleton. This addition is followed by rearomatization and then by elimination of water which gives the product (**Figure 3**).

The coumarin compounds have wide interest due to their diverse pharmacological properties. In particular, these biological activities make coumarin compounds more attractive and testing as novel therapeutic compounds.

As part of our aim in research of biologically active coumarin derivatives, the free hydroxyl group on the coumarin ring has allowed us to introduce some radicals that can improve the biological activity.

The fourth scheme describes the reactions of 4-methyl/propyl-7-hydroxycoumarin with ethyl bromoacetate. 2-Ethyl-((4-methy/propyl-2-oxo-2H-chromen-7-yl)oxy)acetate (**IIa–IIb**) was prepared by heating a mixture of 4-methyl/propyl-7-hydroxycoumarin and ethyl bromoacetate in the presence of K_2CO_3 anhydrous in dry acetone. After filtration, the solution was evaporated, and the solid products (**IIa** or **IIb**) were recrystallized from ethanol [30] (**Figure 4**).

The fifth scheme describes the reactions of compounds **IIa–IIb** with hydrazine hydrate. The chemistry of hydrazide and its derivatives has obtained great interest in both organic chemistry and biological science with remarkable impact. Hydrazides and hydrazones are possessing -NH-NH_2 and -NH-N=CH- groups, respectively. The availability of proton in hydrazides constitutes them as an important class of compound for new drug discovery. Therefore, researchers have shown great interest in developing these compounds as target structures for evaluating new biological activities [31].

Hydrazinolysis of compounds **IIa–IIb** gave the corresponding acetohydrazides (**IIIa–IIIb**) in good yields [30, 32]. One mole of the compound **IIa** or **IIb** in ethanol

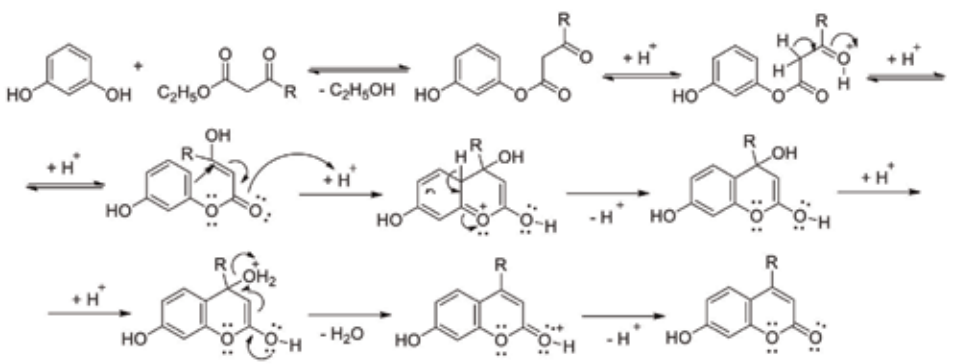


Figure 3.
Mechanism of the Pechmann condensation.

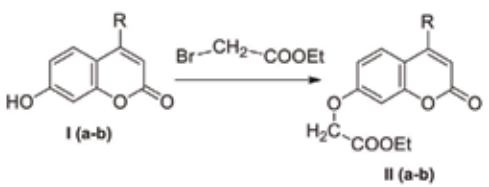


Figure 4.
*Synthesis of coumarin esters **II(a-b)**.*

was heated for 4–6 h with hydrazine hydrate (two moles). After this period of time, the mixture was cooled at room temperature, and the precipitate was filtrated and then purified by recrystallization (**Figure 5**).

The reaction of the acid hydrazides (4-methyl/propyl-2-oxo-2H-benzopyran-7-oxyacetic acid hydrazide) with CS_2 in ethanol containing KOH at room temperature has been presented in **Figure 6**. The corresponding potassium dithiocarbazate derivatives, **IVa–IVb,** are obtained [32].

The obtaining of coumarin derivatives with a thiadiazole ring has been described in **Figure 7**. Literature data show that pyrrole, pyrazole, thiadiazoles, and triazoles and their derivatives are very attractive targets due to their biological properties. In view of the above observations, we have synthesized compounds with thiadiazole ring in order to evaluate the potential antimicrobial and antioxidant activities.

These compounds were obtained following the reaction between potassium 4-methyl/propyl-2-oxo-2H-benzopyranyl-7-oxymethyldithiocarbazate and acetic acid. The reaction occurred under refluxing. The solid product was separated by filtration and then purified by recrystallization from acetic acid [33, 34].

In the synthesis of coumarin derivatives, elemental analysis and two basic spectroscopic techniques, infrared spectroscopy (IR) and nuclear magnetic resonance

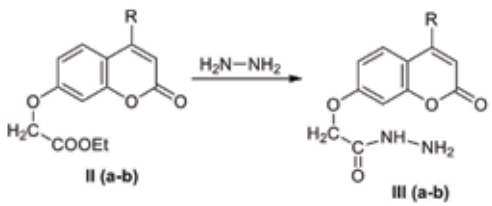


Figure 5.
*Synthesis of coumarin acetohydrazides **III(a-b)**.*

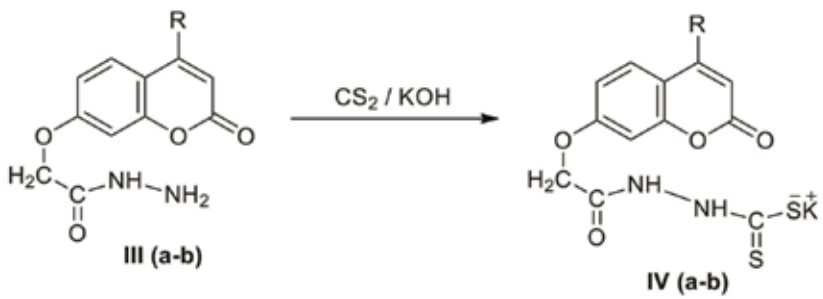


Figure 6.
*Synthesis of coumarin potassium salts **IV(a-b)**.*

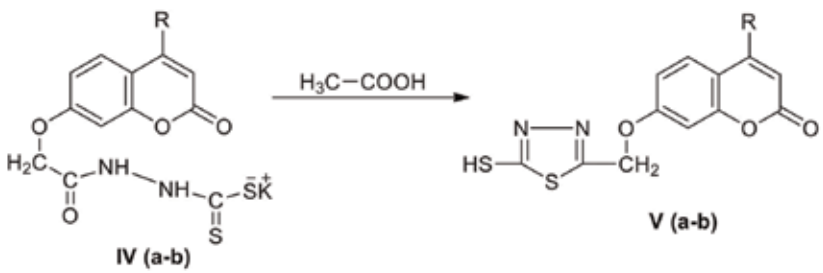
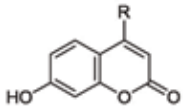


Figure 7.
*Synthesis of coumarin thiadiazoles derivatives **V(a-b)**.*

spectroscopy (NMR), were used to characterize the structures of the target compounds [35].

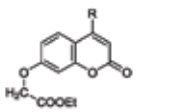
The physical constants and analytical data of compounds **Ia–Ib**, **IIa–IIb**, **IIIa–IIIb**, **IVa–IVb**, and **Va–Vb** have been given in **Tables 1–5**.



4-R-7-hydroxy-coumarin

Compd.	R	MP °C	Molecular formula	Analysis found (calculated) (%)	
				C	H
Ia	H_3C–	185	$C_{10}H_8O_3$	68.18 68.02	4.58 4.55
Ib	H_3C–CH_2–CH_2–	130	$C_{12}H_{12}O_3$	70.57 70.21	5.92 5.69

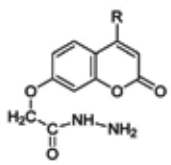
Table 1.
*Physical constants and analytical data of compounds **Ia–Ib**.*



2-ethyl-((4-R-2-oxo-2H-chromen-7-yl)oxy)acetate

Compd.	R	MP °C	Molecular formula	Analysis found (calculated) (%)	
				C	H
IIa	H_3C–	100–102	$C_{14}H_{14}O_5$	64.12 64.10	5.38 5.22
IIb	H_3C–CH_2–CH_2–	98	$C_{16}H_{18}O_5$	66.19 65.89	6.25 6.05

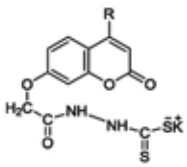
Table 2.
*Physical constants and analytical data of compounds **IIa–IIb**.*



4-R-2-oxo-2H-benzopyran-7-oxyacetic acid hydrazide

Compd.	R	MP °C	Molecular formula	Analysis found (calculated) (%)	
				C	H
IIIa	H_3C–	204–205	$C_{12}H_{12}N_2O_4$	58.06 57.98	4.87 4.80
IIIb	H_3C–CH_2–CH_2–	147–148	$C_{14}H_{16}N_2O_4$	60.86 60.56	5.84 5.76

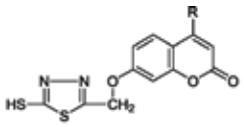
Table 3.
*Physical constants and analytical data of compounds **IIIa–IIIb**.*



potassium 4-R-2-oxo-2H-benzopyranyl-7-oxymethyldithiocarbazate

Compd.	R	MP °C	Molecular formula	Analysis found (calculated) (%)	
				C	H
IVa	H_3C–	184	$C_{13}H_{11}N_2O_4KS_2$	43.08 42.87	3.06 3.01
IVb	H_3C–CH_2–CH_2–	176–178	$C_{15}H_{15}N_2O_4KS_2$	46.13 46.02	3.87 3.76

Table 4.
*Physical constants and analytical data of compounds **IVa–IVb.***



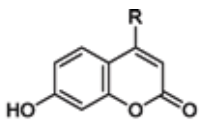
7-((5-mercapto-1,3,4-thiadiazol-2-yl)methoxy)-4-R-2H-chromen-2-one

Compd.	R	MP °C	Molecular formula	Analysis found (calculated) (%)	
				C	H
Va	H_3C–	267–268	$C_{13}H_{10}N_2O_3S_2$	50.97 50.63	3.29 3.06
Vb	H_3C–CH_2–CH_2–	172	$C_{15}H_{14}N_2O_3S_2$	53.87 53.33	4.22 4.12

Table 5.
*Physical constants and analytical data of compounds **Va–Vb.***

The IR spectra of all synthesized compounds showed some characteristic peaks indicating the presence of particular groups (**Tables 6–10**).

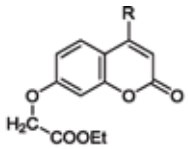
^{1}H-NMR spectra of the synthesized compounds are in accordance with the assigned structures (**Table 11**). The aliphatic protons resonated in the range of 1.20–4.77 ppm. It can be seen that all the compounds exhibited the respected proton chemical shifts in the same range.



4-R-7-hydroxy-coumarin

Compd.	R	υ_{O-H} cm^{-1}	$\upsilon_{C-H\ aliph}$ cm^{-1}	$\upsilon_{C=O\ lactone}$ cm^{-1}	υ_{C-O} cm^{-1}
Ia	H_3C–	3280	2950	1680	1150
Ib	H_3C–CH_2–CH_2–	3195	2970	1695	1140

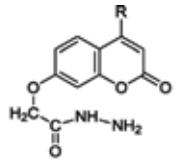
Table 6.
*IR spectral data of compounds **Ia–Ib.***



2-ethyl-((4-R-2-oxo-2H-chromen-7-yl)oxy)acetate

Compd.	R	$\upsilon_{C-H\ arom}$ cm^{-1}	$\upsilon_{C=O\ side\ chain}$ cm^{-1}	$\upsilon_{C=O\ lactone}$ cm^{-1}
IIa	H_3C–	3070	1750	1680
IIb	H_3C–CH_2–CH_2–	3080	1740	1690

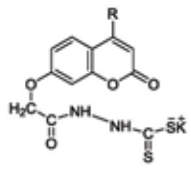
Table 7.
*IR spectral data of compounds **IIa–IIb.***



4-R-2-oxo-2H-benzopyran-7-oxyacetic acid hydrazide

Compd.	R	υ_{NH2} cm^{-1}	υ_{CO-NH} cm^{-1}	υ_{C-N} cm^{-1}
IIIa	H_3C–	3423, 3331	1612	1271
IIIb	H_3C–CH_2–CH_2–	3411, 3340	1610	1260

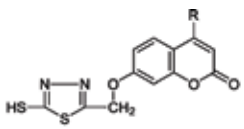
Table 8.
*IR spectral data of compounds **IIIa–IIIb.***



potassium 4-R-2-oxo-2H-benzopyranyl-7-oxymethyldithiocarbazate

Compd.	R	υ_{N-H} cm^{-1}	$\upsilon_{C=S}$ cm^{-1}
IVa	H_3C–	3210	1240
IVb	H_3C–CH_2–CH_2–	3150	1210

Table 9.
*IR spectral data of compounds **IVa–IVb.***



7-((5-mercapto-1,3,4-thiadiazol-2-yl)methoxy)-4-R-2H-chromen-2-one

Compd.	R	υ_{S-H} cm^{-1}	$\upsilon_{C=N}$ cm^{-1}	υ_{S-C} cm^{-1}
Va	H_3C–	2380	1610	621
Vb	H_3C–CH_2–CH_2–	2350	1620	638

Table 10.
*IR spectral data of compounds **Va–Vb.***

Compd.	R	Chemical shift (δ ppm) 500 MHz
Ia	H_3C–	2.73 ppm s, 3H: CH_3; 6.04 ppm s, H_3; 6.72 ppm s, H_8; 6.83–6.85 ppm d, H_6, $J_{H6,H5}$ = 7.5 Hz; 7.62–7.64 ppm d, H_5, $J_{H5,H6}$ = 7.5 Hz; 10.85 ppm s, 1H: OH
Ib	H_3C–CH_2–CH_2–	1.20 ppm t, 3H: CH_3 (12); 1.66 ppm m, 2H: CH_2 (11); 2.81–2.84 ppm m, 2H: CH_2 (10); 6.11 ppm s, H_3; 6.72 ppm s, H_8; 6.84–6.85 ppm d, H_6, $J_{H6,H5}$ = 7.5 Hz; 7.63–7.65 ppm d, H_5, $J_{H5,H6}$ = 7.5 Hz; 10.86 ppm s, 1H: OH
IIa	H_3C–	1.26–1.28 ppm t, 3H: CH_3 (14); 2.73 ppm s, 3H: CH_3; 4.25–4.28 ppm m, 2H: CH_2 (13); 4.61 ppm s, 2H: CH_2 (10); 6.04 ppm s, H_3; 6.84 ppm s, H_8; 7.05–7.07 ppm d H_6; 7.62–7.64 ppm d, H_5
IIb	H_3C–CH_2–CH_2–	0.95–0.98 ppm t, 3H: CH_3 (17); 1.20–1.23 ppm t, 3H: CH_3 (14); 1.60–1.65 ppm m, 2H: CH_2 (16); 2.72–2.75 ppm t, 2H: CH_2 (15); 4.15–4.20 ppm m, 2H: CH_2 (13); 4.92 ppm s, 2H: CH_2 (10); 6.17 ppm s, H_3; 6.98 ppm s, H_8; 6.96–6.97 ppm d H_6; 7.73–7.75 ppm d, H_5
IIIa	H_3C–	2.74 ppm s, 3H: CH_3; 3.86 ppm s, 2H: NH_2 (13); 4.42 ppm s, 2H: CH_2 (10); 6.03 ppm s, H_3; 6.83 ppm s, H_8; 7.05–7.07 ppm d; 7.62–7.64 ppm d, H_5; 8.02 ppm s, NH (12)
IIIb	H_3C–CH_2–CH_2–	0.95–0.98 ppm t, 3H: CH_3 (16); 1.59–1.66 ppm m, 2H: CH_2 (15); 2.72–2.75 ppm t, 2H: CH_2 (14); 4.35 ppm s, 2H: NH_2 (13); 4.61 ppm s, 2H: CH_2 (10); 6.17 ppm s, H_3; 6.98 ppm s, H_8; 6.99–7.00 ppm d H_6; 7.74–7.76 ppm d, H_5; 9.42 ppm s, NH (12)
IVa	H_3C–	2.71–2.74 ppm d, 3H: CH_3; 4.41–4.43 ppm s, 2H: CH_2 (10); 6.02–6.05 ppm q, H_3; 6.82–6.84 ppm d, H_8; 7.05–7.08 ppm q H_6; 7.62–7.64 ppm d, H_5; 9.94 ppm s, NH (12); 11.23 ppm s, NH (13)
IVb	H_3C–CH_2–CH_2–	1.20–1.22 ppm t, 3H: CH_3 (18); 1.62–1.66 ppm m, 2H: CH_2 (17); 2.79–2.82 ppm t, 2H: CH_2 (16); 4.40 ppm s, 2H: CH_2 (10); 6.10 ppm s, H_3; 6.84 ppm s, H_8; 7.04–7.08 ppm q H_6; 7.62–7.66 ppm d, H_5; 9.95 ppm s, NH (12); 11.25 ppm s, NH (13)
Va	H_3C–	2.39 ppm s, 3H: CH_3; 4.77 ppm s, 2H: CH_2 (10); 6.23 ppm s, H_3; 6.99 ppm s, H_8; 7.02–7.04 ppm d H_6; 7.70–7.72 ppm d, H_5; 10.29 ppm s, SH (16)
Vb	H_3C–CH_2–CH_2–	0.95–0.98 ppm t, 3H: CH_3 (19); 1.60–1.65 ppm m, 2H: CH_2 (18); 2.72–2.75 ppm t, 2H: CH_2 (17); 4.77 ppm s, 2H: CH_2 (10); 6.17 ppm s, H_3; 6.98 ppm s, H_8; 6.99–7.00 ppm d H_6; 7.75–7.76 ppm d, H_5; 11.27 ppm s, SH (16)

Table 11.
*^{1}H-NMR spectral data of compounds **I–V**.*

3. Pharmacological activities of coumarin derivatives

3.1 Antimicrobial activity

The compounds were screened for their antibacterial and antifungal activity according to standard protocols [36].

The antimicrobial activity was studied using Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Sarcina lutea* ATCC 9341, *Bacillus cereus* ATCC 14579), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853), and pathogenic yeasts (*Candida albicans* ATCC 10231, *Candida glabrata* ATCC MYA 2950, *Candida parapsilosis* ATCC 22019). All these strains were obtained from the Culture Collection of the Department of Microbiology, Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, Romania.

Antimicrobial activity was evaluated by agar disc diffusion method (CLSI, 2014). A small amount of each microbial culture was diluted in sterile 0.9% NaCl until the turbidity was equivalent to McFarland standard no. 0.5 (106 CFU/ml). The suspensions were further diluted 1:10 in Mueller-Hinton agar for bacteria and Sabouraud agar for yeasts and then spread on sterile Petri plates (25 ml/Petri plate). Sterile stainless steel cylinders (5 mm internal diameter; 10 mm height) were applied on the agar surface in Petri plates. Then, 0.1 ml of each compound (10 mg/ml in DMSO) was added into the cylinders. The DMSO solvent was also tested in order to assess its intrinsic antimicrobial activity. Commercial available discs containing ampicillin (25 μg/disc), chloramphenicol (30 μg/disc), and nystatin (100 μg/disc) were also placed on the agar surface. The plates were incubated at 37°C for 24 h (bacteria) and at 24°C for 48 h (yeasts). After incubation the diameters of inhibition zones were read in triplicate. Statistical analysis of the results included the calculation of standard deviation (**Tables 12** and **13**).

The qualitative screening of the antimicrobial activity was performed in order to identify the antimicrobial spectrum of the tested compounds. The inhibitory effects of the synthetic compounds against Gram-positive and Gram-negative bacteria and fungi are given in **Tables 12** and **13** [35].

According to the results of the antibacterial studies, the efficacy of the tested compounds against Gram-positive bacteria was higher than that exhibited for Gram-negative bacteria. All the synthesized compounds were very active against *S. aureus* ATCC 25923, the most active compounds being **Ib**, **IIb**, **IIIb**, and **IVb**. The replacement of the methyl radical in the fourth position with the propyl group was correlated with an increased activity against *S. aureus* ATCC 25923.

The tested compounds exhibited excellent antibacterial activity against *S. lutea*, the most active derivatives being **IIb**, **IVb**, **Ib**, and **IIIb**.

Compd./reference	Diameters of the growth inhibition zone (mm)				
	S. aureus ATCC 25923	*S. lutea* ATCC 9341	*B. cereus* ATCC 14579	*E. coli* ATCC 25922	*Pseudomonas aeruginosa* ATCC 27853
Ia	14 ± 0.52	25 ± 0.79	25 ± 1.52	12 ± 0.79	8 ± 0.93
Ib	27 ± 1.29	29 ± 0.83	NA	NA	NA
IIa	14 ± 0.91	22 ± 0.79	26 ± 0.79	11 ± 0.52	8 ± 1.43
IIb	27 ± 0.52	30±	NA	NA	NA
IIIa	15 ± 0.54	20 ± 0.79	22 ± 0.79	10 ± 0.79	9 ± 0.79
IIIb	25 ± 0.52	28±	NA	NA	NA
IVa	17 ± 1.08	25 ± 0.91	24 ± 1.52	12 ± 0.93	9 ± 1.43
IVb	25 ± 1.08	30 ± 0.83	NA	NA	NA
Va	14 ± 0.52	25 ± 0.52	20 ± 1.43	10 ± 1.52	8 ± 0.52
Vb	21 ± 1.43	25 ± 0.79	NA	13 ± 0.83	NA
Ampicillin (25 μg/disc)	26 ± 0.04	36 ± 0.00	NA	21 ± 0.79	NA
Chloramphenicol (30 μg/disc)	22 ± 0.00	38 ± 0.00	24 ± 0.00	21 ± 0.52	NA

Data are mean ± SD (n = 3); NA, no activity.

Table 12.
*Antibacterial activity of compounds **I–V**.*

Compd./reference	Diameters of the growth inhibition zone (mm)		
	C. albicans ATCC 10231	*C. glabrata* ATCC MYA 2950	*C. parapsilosis* ATCC 22019
Ia	24 ± 1.83	21 ± 0.52	34 ± 1.83
Ib	10 ± 0.91	10 ± 0.79	10 ± 0.54
IIa	25 ± 0.52	27 ± 0.54	35 ± 1.83
IIb	9 ± 1.83	NA	NA
IIIa	19 ± 1.79	24 ± 0.52	24 ± 1.79
IIIb	12 ± 1.83	11 ± 0.54	11 ± 0.54
IVa	23 ± 0.91	16 ± 0.52	25 ± 1.08
IVb	10 ± 0.54	12 ± 1.08	NA
Va	16 ± 1.79	21 ± 1.83	21 ± 0.54
Vb	9 ± 0.54	9 ± 0.52	NA
Nystatin (100 μg/disc)	25 ± 0.52	25 ± 0.52	24 ± 0.00

Data are mean ± SD (n = 3); NA, no activity.

Table 13.
*Antifungal activity of compounds **I–V**.*

We found a moderate action against *B. cereus* ATCC 14579, the most active being the umbelliferone derivatives with a methyl group attached to C4: **IIa**, **Ia**, and **IVa**.

Against *Escherichia coli* ATCC 25922, the investigated compounds had a weaker action than the controls ampicillin and chloramphenicol. The most active was the compound that contains a thiadiazole ring, **Vb**.

The presence of the methyl group attached to the coumarin ring in the fourth position had a positive influence on the anti-*Pseudomonas* ATCC 27853 potential of the compounds, all the tested 4-propyl-coumarin derivatives being inactive.

We have noticed a very important action against the investigated *Candida* strains; all tested compounds were found to be very active against fungi. The compounds **IIa** and **Ia** had a greater inhibitory potential against *C. parapsilosis* ATCC 22019 than nystatin. The introduction of the sulfur atom appeared to be correlated with a good anti-*Candida* activity.

3.2 Antioxidant activity

In order to evaluate the antioxidant activity of synthesized compounds, we use three antioxidant assays: DPPH radical inhibition, total reducing power, and nitric oxide (NO) inhibition.

The DPPH assay is based on assessing the substances' ability to reduce the stable radical (diphenylpicrylhydrazyl) to diphenylpicrylhydrazine. The DPPH free radical, bearing an odd electron, gives a strong absorption maximum at λ = 517 nm (purple color). When the odd electron of the DPPH radical pairs with a hydrogen atom from an antioxidant, the reduced form DPPH-H is created, and the color turns from purple to yellow [36, 37].

A possible mechanism that can explain the antioxidant effect of the coumarin hydrazide derivatives is related to the keto-enol forms of the substances, the enol group being capable to easily donate the hydrogen (**Figure 8**) [38].

The experimental procedure for the DPPH assay was adapted from literature [27, 28, 37], only slight modifications being made. Briefly, 2.5 ml solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical 0.1 mM in methanol was added over 0.5 ml

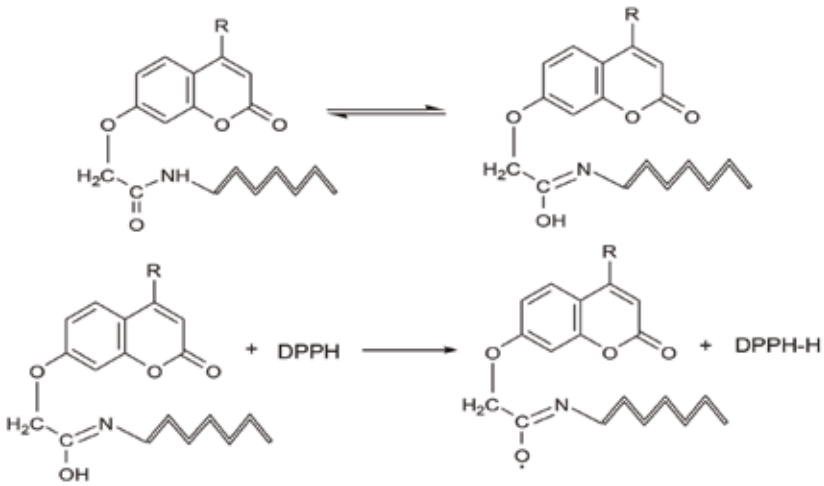


Figure 8.
Proposed mechanism for antioxidant activity of coumarin hydrazides.

of methanolic solution of the tested compound (1 mg/ml). The absorbance of the DPPH solution at 517 nm was determined spectrophotometrically before ($A_{control}$) and 15 minutes after adding the solutions of the compounds (A_{test}), and the percentage of activity was calculated. Ascorbic acid was used as a reference compound:

$$\%\ radical\ scavenging\ activity = (A_{control} - A_{test}) \times 100/A_{control}$$

where $A_{control}$ is the absorbance of the control sample (DPPH solution without test sample) and A_{test} is the absorbance of the test sample (DPPH solution + test compound).

Out of the tested compounds, the most active DPPH free radical scavengers were the coumarin hydrazide derivatives (**IIIa–IIIb**, **IVa–IVb**). The activities of **IVa** were similar to that of the standard, the inhibition percentage being over 90%, the introduction of sulfur atoms in the molecule having a positive influence on the scavenging potential. Compounds **IIIa** and **IVa**, containing a methyl group, were slightly more active than their analogues with propyl radical (**Table 14**) [19].

Fe(III) reduction is often used as an indicator of electron-donating activity. In the reducing power assay, antioxidants with electron-donating abilities reduce ferricyanide to ferrocyanide by donating an electron. The amount of ferrocyanide is monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reducing ability [39]. Within this assay, EC_{50} values are the effective concentrations at which the absorbance is 0.5.

The solution of the test compound (0.5 ml) at different concentrations in methanol was mixed with phosphate buffer (1.25 ml, 0.2 mol/l, pH 6.6) and potassium ferricyanide 1% (1.25 ml), and the mixture was incubated at 50°C for 20 min. At the end of the incubation period, trichloroacetic acid 10% (1.25 ml) was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer solution was collected, and 2.5 ml were mixed with distilled water (2.5 ml) and ferric chloride 0.1% (0.5 ml). The absorbance was measured after 15 min at 700 nm against a blank. The EC50 values were calculated by linear interpolation between values above and below 50% activity. Ascorbic acid was used as reference [28, 30, 36, 37].

The reducing power of the tested compounds was modest, and the results are presented in **Table 15**. The only substances that were moderately active were the hydrazide derivatives **IIIa** and **IVa**, but their activity was inferior to that exhibited by the reference substance (ascorbic acid) [19].

Compd.	Inhibition percentage (%)
Ia	21.4
Ib	21.2
IIa	19
IIb	19.83
IIIa	73
IIIb	53.56
IVa	95
IVb	87.67
Va	31.51
Vb	25.13
Ascorbic acid 1 mg/ml	96.8

Table 14.
*DPPH inhibition percentages of compounds **I–V** (1 mg/ml).*

Compd.		Extinction
Ia		0.0599
Ib		0.0105
IIa		0.0234
IIb		0.0010
IIIa	(1 mg/ml)	1.3432
	(0.8 mg/ml)	1.1237
	(0.6 mg/ml)	1.0821
	(0.4 mg/ml)	0.9674
	(0.2 mg/ml)	0.5499
IVa	(1 mg/ml)	0.6592
	(0.8 mg/ml)	0.5900
	(0.6 mg/ml)	0.4854
	(0.4 mg/ml)	0.4258
	(0.2 mg/ml)	0.2785
IVb		0.4887
Va		0.0472
Vb		0.3063
Ascorbic acid 1 mg/ml		2.8261

Table 15.
*The reducing power of compounds **I–V**.*

The calculated values for EC_{50} are shown in **Table 16**. This method could not be applied to compound **IIIb** due to the formation of an abundant precipitate in the process.

Nitric oxide is involved in a variety of biological functions (neurotransmission, vascular homeostasis, antimicrobial and antitumor activities). NO was primarily

Compd.	EC_{50} (mg/ml)
Ia	>>1
Ib	>>1
IIa	>>1
IIb	>>1
IIIa	0.176
IVa	0.627
IVb	1.02
Va	>>1
Vb	>1
Ascorbic acid 1 mg/ml	0.049

Table 16.
The calculated values of EC_{50}.

described as a regulator of vascular tones in the cardiovascular system. Beyond this function it can prevent platelet activation, limit leukocyte adhesion to the endothelium, and regulate myocardial contractility, and it is involved in immune system reactions.

Despite the possible beneficial effects of NO, it also contributes to oxidative damage. In general, the overwhelming production of NO contributes to the pathogenesis of both acute and chronic inflammatory processes, and NO has been recognized as one of the main signaling molecules involved in these processes [23, 40]. Therefore, compounds that act like nitric oxide inhibitors have beneficial effects.

The NO inhibition assay is based on the diazotization of sulfanilic acid at acid pH by nitric oxide. The reaction product is subsequently coupled stoichiometrically with N-(1-naphthyl)ethylenediamine, forming a colored azo compound which is measured spectrophotometrically at a peak absorbance of 548 nm [36].

0.5 ml of the tested coumarin derivative solution, as well as ascorbic acid (standard compound), was taken in separate tubes, and 2.0 ml of sodium nitroprusside

Compd.	NO inhibition (%)
Ia	15.2
Ib	19.8
IIa	12.11
IIb	14.7
IIIa	22.8
IIIb	55.5
IVa	28.45
IVb	29.44
Va	22.6
Vb	26
Ascorbic acid 50 mg/ml	77.19

Table 17.
*NO inhibition activity of compounds **I–V**.*

(10 mM) and 0.5 ml phosphate buffer saline (pH = 7.4) were added to each tube. The solutions were incubated at 25°C for 150 minutes. After the incubation, over 0.5 ml of the incubated solution 1 ml of sulfanilic acid 0.33% was added, and the mixture was left for 5 min at room temperature; after this period of time, 1 ml naphthylethylene diamine (NED) HCl reagent 0.1% was added, and the solutions were incubated for another 30 min. The absorbance was measured at 546 nm [38].

Most of the investigated compounds were moderate NO inhibitors (**Table 17**) [19].

4. Concluding remarks

We have synthesized some coumarin derivatives starting from 4-methyl-7-hydroxycoumarin with antimicrobial and antioxidant activities to different reaction steps. The IR and NMR spectra of the synthesized compounds were in accordance with the assigned structures. All the synthesized compounds were very active against *S. aureus* ATCC 25923, and they exhibited excellent antibacterial activity against *S. lutea*. The presence of the methyl group attached to the coumarin ring in the fourth position had a positive influence on the anti-*Pseudomonas* ATCC 27853 potential of the compounds, all the tested 4-propyl-coumarin derivatives being inactive. Against the investigated *Candida* strains, all tested compounds were found to be very active. The introduction of the sulfur atom appeared to be correlated with a good anti-*Candida* activity. The most active DPPH free radical scavengers were the coumarin hydrazide derivatives, the activities of these being similar to that of the standard. The reducing power of the tested compounds was modest, and only the hydrazide derivatives were moderately active. Most of the investigated compounds were moderate NO inhibitors.

The interest in the synthesis of coumarin derivatives has been gaining importance over the last decades, reflecting the importance of such compounds in both medical and chemical research. Future goals for this field of research include the discovery, synthesis, and development of compounds which display increased potency, as well as fueling structure–activity relationship studies aimed at understanding the modes of action of the most biologically active members of these classes of products.

Although coumarin is a simple molecule and many of its derivatives have been known for more than a century, it continues to maintain the interest of researchers being a plentiful source of potential drug candidate because of their significant therapeutic potential.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 8

Coumarins as Fluorescent Labels of Biomolecules

António Pereira, Sérgio Martins and Ana Teresa Caldeira

Abstract

Important areas such as environmental sciences, medicine, pharmacy, and cellular biology are dependent on very sensitive analytical techniques. One of the most common methodologies used for their bioanalytical purposes is the fluorescent labelling. The synthesis of new fluorophores and the great development of fluorescent-labelling techniques combined with the enormous technological advances in the field of fluorescence microscopy allowed to deepen the structural knowledge of biomolecules. This new organic fluorophores form covalent bonds with the sample to be analyzed, producing stable bioconjugates that show fluorescence in a wide range of wavelengths, depending on the label used. Coumarin derivatives represent one of the most important chemical classes of organic fluorescent materials being one of the most extensively investigated and commercially significant groups of organic fluorescent materials. In this chapter, it is reviewed the use of fluorescent coumarin derivatives and their application to labelling biomolecules. These fluorescent labels allow researchers to study, and understand, biomolecular assemblies that exhibit complex sensitivity and selectivity. Reactive fluorescent coumarin derivatives are actually widely used in labelling biomolecules as peptides, proteins, oligonucleotides, nucleic acids, and carbohydrates, among other biological molecules.

Keywords: coumarins, fluorophores, labelling, biomolecules, bioconjugation

1. Introduction

Important areas such as environmental sciences, medicine, medicinal chemistry, and cellular biology are dependent on very sensitive analytical techniques to detect and track biomolecules (amino acids, peptides, proteins, antibodies, oligonucleotides, nucleic acids, carbohydrates, and other biological molecules). Many of these techniques often require labelling with reporters or sensors, such as isotope labels [1], radioactive tracers [2], colorimetric biosensors [3], photoswitchable biomaterials [4], photochromic compounds [5, 6], electrochemical sensors [7], or fluorescent labels [8, 9]. The fluorescent labelling presents numerous advantages, when compared to the other techniques, due to the high sensitivity of the fluorescence technique and also due to its non-destructive nature that allows the use of small sample quantities and their fluorescent labels. The fluorescence process occurs in certain molecules called fluorophores or fluorescent dyes, and a fluorescent probe is nothing more than a fluorophore enabled to detect particular components of complex biomolecular assemblies, including live cells, with complex sensitivity and selectivity [10]. The organic fluorophores may form covalent

or non-covalent linkages with the sample to be analyzed, producing the respective bioconjugates (or complexes) that can show fluorescence, from short to very long wavelengths, depending on the label used. The bioconjugation technique depends on two interrelated chemistries: the reactive functionality present on the fluorescent label and the functional groups present on the target biomolecules to be labeled. The knowledge of the basic mechanisms by which the reactive groups couple to target functionalities provides the means to intelligently design the bioconjugation strategy. Choosing the correct fluorescent label that can react with the chemical groups available on target biomolecules forms the basis for successful labelling [11].

In general, the fluorescent label should be small in size and chemically stable, with minimal interference on the structure and biological functions of the unlabeled biomolecules, producing high fluorescence quantum yield bioconjugates.

On the other hand, the labelling reaction should be extremely efficient with high yields, preferably establishing a stable covalent linkage between the fluorescent label and a specific residue in the target biomolecule. The efficiency and selectivity of several fluorescent-labeled biomolecules have been used to study and understand their dynamics, kinetics, and photophysical properties [12–18].

The amine reactive fluorescent labels are the most frequently used to prepare stable bioconjugates to a great number of biological applications since amino groups are either abundant or easily introduced into biomolecules. In contrast, to study some particular protein structures and functions, thiol-reactive reagents are chosen due to the smaller presence of thiol groups, when compared with lysine, in biomolecules [19]. In this context, cysteine is generally the amino acid chosen to label when it is desired to label selectively a protein in vitro, due to its relatively low abundance and high nucleophilicity compared to other amino acid side chains. Specific and noninterfering dual fluorescent labelling in a peptide or protein molecule allows conformational investigations in terms of intramolecular distances [20].

The expeditious development of the fluorescent-labelling techniques allowed to explore and discover several cellular functions. To study, and understand, the activity of signal transduction by visualizing protein binding or folding, the fluorescence correlation spectroscopy (FCS) and the fluorescence resonance energy transfer (FRET) are widely used [21]. Molecular tags that specifically bind to particular membrane-permeable dyes [22] allow to study protein dynamics and trafficking by fluorescence recovery after photobleaching (FRAP) as well the protein turnover [23, 24].

The great development of fluorescent-labelling techniques combined with the enormous technological advances in the field of fluorescence microscopy allowed to study, in vivo and in vitro systems, the protein distribution as well as their translocation and their interactions [25]. With specific and efficient fluorescent labelling, the proteins can be visualized in real time for the elucidation of their functions in a complex biological network, which also allows the detection of the protein-protein interactions, fundamental to understand intra- and intercellular communications [26].

Coumarins (benzopyranones or 2H-chromen-2-ones), whether natural products or synthetic ones, have also aroused a growing interest of the scientific community in the last decades due to their very significant pharmacological activity [27–37]. The nature and substitution pattern in the coumarins grant them diversified and exceptional optical properties with high fluorescence quantum yields [38]. Coumarins constitute the major class of fluorescent dyes [39–63], used as fluorescent labels and probes for physiological measurement [43–47], fluorescent whiteners [48], optical brighteners [49, 50], nonlinear optical chromophores [51–53], emission layers in organic light-emitting diodes (OLED) [54–57], and more recently, in caging [58–61], and labelling [62, 63]. Due to strong blue fluorescence of coumarin, it is easy to distinguish its light from green, yellow, and red,

an enormous advantage in multicolored fluorescence investigation. Developments from the last decade show that the introduction of appropriated substituents into the coumarin ring contributes to structures with improved photophysical and spectroscopic properties [64–66]. The synthesis of new fluorophores, with absorption and emission at long wavelengths, is of extreme importance for biological purposes, and the coumarins may play a leading role in this field.

2. Chemical labelling

Of all different fluorescent-labelling techniques, the chemical labelling is actually one of the most used as it allows novel types of experiments in biomolecules using a wider range of reactive fluorescent chromophores available. The covalent attachment of the chemical probes with specific amino acid has the advantage of being an irreversible process when compared to the non-covalent binding [67]. The chemical labelling methodology produces very stable bioconjugates, easy to manipulate with high efficiency, in a great number of available fluorophores that can be coupled covalently to the target biomolecule. Chemical labelling methods produce better results in in vitro studies rather than in vivo [18]. The most used methods in chemical labelling, in the biomolecules' native functional groups, under mild aqueous conditions, and using fluorescent coumarins, are discussed below.

2.1 Amine reactive fluorescent coumarins

Presently, amine reactive fluorescent coumarins are widely used to label biomolecules, as peptides, proteins, oligonucleotides, and nucleic acids, among others. The fluorescent bioconjugates obtained are very useful in fluorescence in situ hybridization (FISH), receptor labelling immunochemistry, cell tracing, and fluorescent analog cytochemistry studies. Almost all of the techniques used in these tests implicate a robust fluorescent conjugate able to support rigorous incubation, hybridization, and washing steps, which is provided by the stability of the covalent bond between the amine reactive dye and biomolecule. Chemically, the amine labelling reaction proceeds usually through acylation pathway producing stable amide (or thiourea) bonds. The "ideal" reactions are those which require the same conditions as proteins, like functional group tolerance, compatibility, selectivity, water as solvent (or pH ~ 7), room temperature, high reaction rates, low reactant concentration, and nontoxic reagents.

A number of fluorescent amino-reactive coumarins have been developed to label various biomolecules, and the resultant conjugates are widely used in biological applications. Four major classes of amine-reactive fluorescent reagents are currently used to label biomolecules: succinimidyl esters (SE), 4-sulfotetrafluorophenyl (STP) esters, sulfonyl chlorides, and isothiocyanates [68]. **Figure 1** represents, in a general schematic diagram, the referred labelling reactions, between an amine group of a biomolecule and a fluorescent amino-reactive coumarin.

2.1.1 Fluorescent coumarin succinimidyl esters

Succinimidyl esters (SE) are proven to be very good reagents for amine modifications. These kinds of reagents are generally stable and show good reactivity and selectivity with aliphatic amines, such as the amine group of lysine side chain. Some of these kinds of reactive dyes are hydrophobic molecules and should be previously dissolved in anhydrous dimethylformamide (DMF) or dimethylsulfoxide (DMSO), but the sulfo-succinimidyl esters are water soluble. The amine labelling

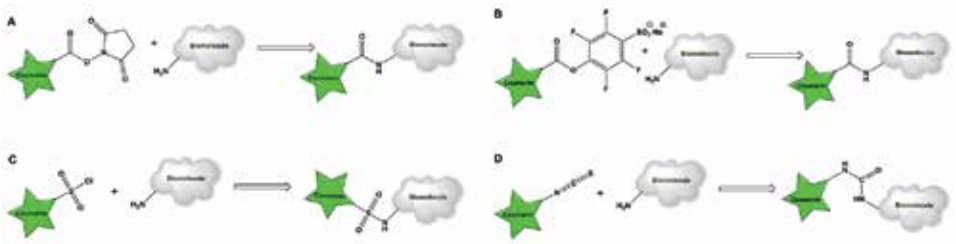


Figure 1.
Schematic diagram of amine labelling techniques using succinimidyl esters (A), 4-sulfotetrafluorophenyl esters (B), sulfonyl chlorides (C), and isothiocyanates (D).

reaction with succinimidyl esters has a handicap, due to its great pH dependence. Succinimidyl esters react with non-protonated aliphatic amine groups, and the amine acylation reaction must be carried out at pH > 7.5. In the specific case of protein labelling by succinimidyl esters, the reactions require a pH between 7.5 and 8.5. Buffers used in labelling reactions shall not contain nucleophilic compounds because they may react with the labelling reagent to form unstable intermediates that could destroy the reactive dye. Most conjugations are done at room temperature, but either high or low temperature may be required for a particular labelling reaction. Some of the fluorescent coumarin succinimidyl esters contain a seven-atom aminohexanoyl spacer between the fluorophore and the reactive group, providing better solubility and spatial separation between the fluorophore and the target molecule being labeled. This separation potentially reduces the quenching that typically occurs upon conjugation and makes the dye more available for recognition by secondary detection reagents [68]. The most important fluorescent coumarin succinimidyl esters used for labelling biomolecules are shown in **Table 1**, as the corresponding values of maximal excitation (Ex) and emission (Em) wavelengths and their physicochemical features and biological applications [19, 68].

2.1.2 Fluorescent coumarin 4-sulfotetrafluorophenyl (STP) esters

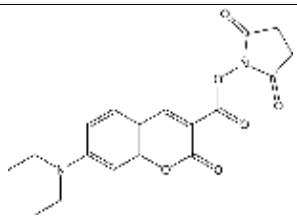
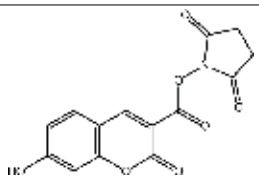
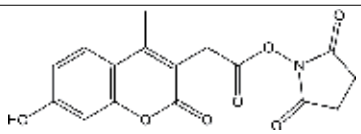
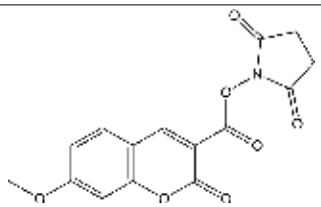
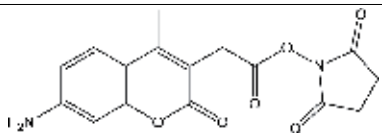
Some succinimidyl esters may not be compatible with a specific application due to their insolubility in aqueous solution. To overcome these limitations, the 4-sulfotetrafluorophenyl (STP) ester can be used. These sulfonated esters have higher water solubility than simple succinimidyl esters and sometimes eliminate the need for organic solvents in the conjugation reaction, which is a great advantage to maintain the native characteristics of biomolecules. They are, however, more polar than succinimidyl esters, which makes them less likely to react with buried amines in proteins or to penetrate cell membranes [68, 94]. **Table 2** presents the single fluorescent coumarin 4-sulfotetrafluorophenyl (STP) ester used for labelling biomolecules, as the corresponding values of maximal excitation (Ex) and emission (Em) wavelengths and their physicochemical features and biological applications [95, 96].

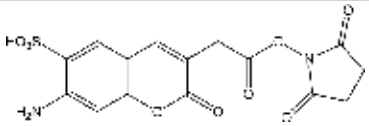
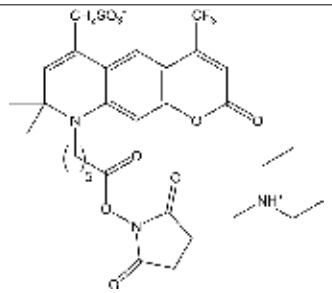
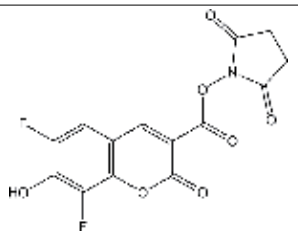
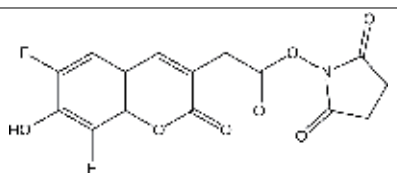
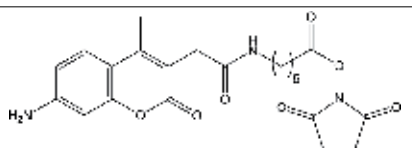
2.1.3 Fluorescent coumarin sulfonyl chlorides

Sulfonyl chlorides (SC) are highly reactive and are unstable in water, especially at high pH required for reaction with aliphatic amines. The labelling reactions with sulfonyl chlorides must be performed, carefully, at very low temperature in a place with local exhaust ventilation. Sulfonyl chlorides present a major reactive handicap as they can also easily react with other reactive groups present in biomolecules as phenols, thiols, aliphatic alcohols, imidazoles, and many others. Fortunately, this

kind of reactions rarely occurs in proteins or in aqueous solution, allowing the use of this type of chromophores to label proteins. Sulfonyl chloride dyes are generally hydrophobic molecules and should be dissolved in anhydrous dimethylformamide (DMF), but never in dimethylsulfoxide (DMSO) due to their highly instability in this solvent.

The labelling reactions of amines with SC reagents are strongly pH dependent, and the sulfonylation-based conjugations may require a pH 9.0–10.0 for optimal conjugations, which potentiates the sulfonyl chlorides' degradation by hydrolysis reactions. In general, sulfonylation-based conjugations have much lower yields than the succinimidyl ester-based conjugations. As in the case of succinimidyl esters, the buffers used in sulfonyl chloride reactions shall not contain nucleophilic compounds, because they may react with the labelling reagent to form unstable

Coumarin	Ex/Em (nm)	Physicochemical features and biological applications	Ref.
 2,5-dioxopyrrolidin-1-yl-7-diethylaminocoumarin-3-carboxylate (DEAC SE)	432/472	Strong blue-fluorescent bioconjugates. Quite hydrophobic fluorescent dye, used for labelling live cells	[19, 69–72]
 2,5-dioxopyrrolidin-1-yl 7-hydroxycoumarin-3-carboxylate	363/447	One of the most popular blue-fluorescent dyes for labelling proteins and nucleic acids and increasingly used to label peptides, nucleotides, and carbohydrates	[19, 73]
 2,5-dioxopyrrolidin-1-yl 2-(7-hydroxy-4-methylcoumarin) acetate	364/458	Widely used for preparing bioconjugates of blue fluorescence but pH-dependent and environment-sensitive fluorescence	[19, 74, 75]
 2,5-dioxopyrrolidin-1-yl 7-methoxycoumarin-3-carboxylate	358/410	Used to label peptides and nucleotides with strong blue fluorescence and also used to label cell membranes although its fluorescence is quite short	[19, 72, 76, 77]
 2,5-dioxopyrrolidin-1-yl 2-(7-amino-4-methylcoumarin-3-yl)acetate	350/450	Used for fluorohistochemical examination of human kidney glomeruli. Reacts under mild conditions	[78, 79]

Coumarin	Ex/Em (nm)	Physicochemical features and biological applications	Ref.
 7-amino-3-(2-((2,5-dioxo-pyrrolidin-1-yl) oxy)-2-oxoethyl)-4-methylcoumarin-6-sulfonic acid (Alexa Fluor™ 350 SE)	346/442	Blue-fluorescent dye, water soluble and pH insensitive from pH 4 to pH 10, used for stable signal generation in imaging and flow cytometry	[68, 80–83]
 Triethylammonium (9-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-8,8-dimethyl-2-oxo-4-(trifluoromethyl)-8,9-dihydro-2H-pyrano[3,2-g] quinolin-6-yl)methanesulfonate (Alexa Fluor™ 430 SE)	430/545	Bright green-fluorescent dye, water soluble and pH insensitive from pH 4 to pH 10. Used for stable signal generation in imaging and flow cytometry	[68, 80, 81, 84, 85]
 2,5-dioxopyrrolidin-1-yl 6,8-difluoro-7-hydroxycoumarin-3-carboxylate (Pacific Blue™ SE)	410/455	Conjugates of this dye are strongly fluorescent even at neutral pH. Ideally suited for 405 nm violet diode laser excitation on the Applied Biosystems® Attune™ Acoustic Focusing cytometer and similarly equipped fluorescence microscopes	[68, 86–88]
 2,5-dioxopyrrolidin-1-yl 2-(6,8-difluoro-7-hydroxy-4-methylcoumarin-3-yl)acetate (Marina Blue™ SE)	365/460	Conjugates that are strongly fluorescent, even at neutral pH. Optimally detected using optical filters configured for 4′,6-diamidino-2-phenylindole (DAPI)	[68]
 2,5-dioxopyrrolidin-1-yl 6-(2-(7-amino-4-methylcoumarin-3-yl)acetamido)hexanoate (AMCA-X SE)	353/442	Conjugates yield blue fluorescence that can be used as a contrasting color in multicolor applications. Because its fluorescence may not be as bright as that of other dyes or may be obscured by autofluorescence, it is only recommended for use with highly abundant targets	[68, 89–91]

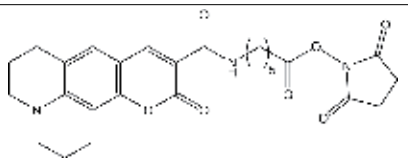
Coumarin	Ex/Em (nm)	Physicochemical features and biological applications	Ref.
 2,5-dioxopyrrolidin-1-yl 6-(11-oxo-2,3,5,6,7,11-hexahydro-1H-pyrano[2,3-f]pyrido[3,2,1-ij] quinoline-10-carboxamido)hexanoate (Coumarin 343 X SE)	437/477	Blue-emitting coumarin can be used to design fluorescence resonance energy transfer (FRET)-based assays with fluorescein amidite (FAM) as acceptor and to construct systems which harvest blue light energy	[92, 93]

Table 1.
Fluorescent coumarin succinimidyl esters used for biomolecule labelling.

intermediates that could destroy the reactive dye [19, 97–99]. **Table 3** shows fluorescent coumarin sulfonyl chlorides used for labelling biomolecules, as the corresponding values of maximal excitation (Ex) and emission (Em) wavelengths and their physicochemical features and biological applications. In addition to the coumarins presented in **Table 3**, new sulfonyl chloride coumarins have been developed, with high potential as fluorescent probes [100, 101].

2.1.4 Fluorescent coumarin isothiocyanates

Isothiocyanates form thioureas upon reaction with amines, but some thiourea products are much less stable than the conjugates that are prepared from the corresponding succinimidyl esters. Most part of isothiocyanate-reactive dyes are hydrophobic molecules and should be dissolved either in anhydrous dimethylformamide (DMF) or in dimethylsulfoxide (DMSO), and their reactions may require a pH 9.0–10.0 for optimal conjugations. As in the previous cases, the buffers used shall not contain nucleophilic compounds. The isothiocyanate conjugations are done at room temperature, but either high or low temperature may be required for a particular labelling reaction [19, 102]. The unique fluorescent coumarin isothiocyanate used for labelling biomolecules is shown in **Table 4**, but new isothiocyanate coumarins have been synthesized, with high potential as fluorescent probes [103, 104].

2.2 Thiol-reactive fluorescent coumarins

Cysteine is, in comparison with lysine, a rare amino acid present in biomolecules, and, for this reason, thiol-reactive reagents are used to label selectively a biomolecule at a defined site, probing their function, interaction, and biological structure. A great number of thiol-reactive dyes have been developed to analyze the proteins' topography in biological membranes, to measure the distances within (or between) proteins, and to observe and understand the changes in protein conformation using environmental sensitive probes.

Maleimides and iodoacetamides are the principal types of thiol-reactive coumarin dyes reported in the literature. Despite many similarities in their reactivity and selectivity toward thiol-reactive moieties, maleimides have a great advantage in relation to iodoacetamides, due to their high stability, solubility in simple solvent mixtures, and their high reactivity in the neutral pH range. Air oxidation of thiol compounds (to

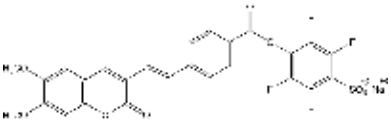
Coumarin	Ex/Em (nm)	Physicochemical features and biological applications	Ref.
 Sodium (E)-4-((4-(2-(6,7-dimethoxycoumarin-3-yl)vinyl)benzoyl) oxy)-2,3,5,6-tetrafluorobenzenesulfonate	392/490	Used to label proteins and nucleotides with strong blue fluorescence. Blue-fluorescent dye, water soluble and pH insensitive with excellent photostability	[95, 96]

Table 2.
Fluorescent coumarin 4-sulfotetrafluorophenyl (STP) ester used for biomolecule labelling.

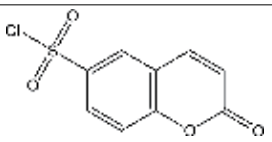
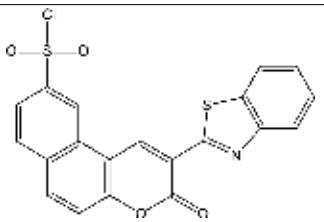
Coumarin	Ex/Em (nm)	Physicochemical features and biological applications	Ref.
 Coumarin-6-sulfonyl chloride	360/460	Used to label amines, amino acids, and phenols in mild conditions. Fluorescence produced in alkaline solution or in the presence of β-cyclodextrin	[99, 100]
 2-(benzo[d]thiazol-2-yl)-3-oxo-3H-benzo[f]chromene-9-sulfonyl chloride	405/435	Biosensor sensitive toward polarity changes in bio environments	[101]

Table 3.
Fluorescent coumarin sulfonyl chlorides used for biomolecule labelling.

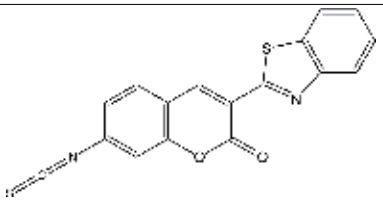
Coumarin	Ex/Em (nm)	Physicochemical features and biological applications	Ref.
 3-(benzo[d] thiazol-2-yl)-7-isothiocyanatocoumarin	485/535 (conjug.)	Selective determination of flu antigen	[102]

Table 4.
Fluorescent coumarin isothiocyanate used for biomolecule labelling.

disulfides) is a major competing reaction for the iodoacetamide modifications of thiol compounds [18, 19, 105]. Due to the disinterest on the development of new coumarin iodoacetamides, for the above reasons, only the fluorescent coumarin maleimides will be focused in this section. **Figure 2** represents, in a general schematic diagram, the thiol-labelling reaction with fluorescent coumarin maleimides.

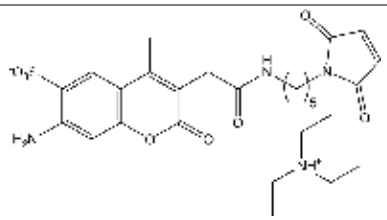
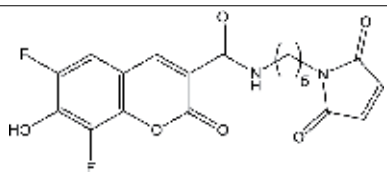
2.2.1 Fluorescent coumarin maleimides

Maleimides readily react with thiol moieties of biomolecules to form thioether conjugates even under neutral conditions. The thioether bond formed is quite stable and is known to be responsible for the light produced, especially in the solution. Maleimides require conjugation conditions less rigorous than those of iodoacetamides and do not react with histidine and methionine under physiological conditions. Most labelling reactions can be done at room temperature at neutral pH. However, either elevated or reduced pH or temperature may be required for a particular labelling reaction [18, 19, 68]. In **Table 5**, the most important fluorescent coumarin maleimides used for labelling biomolecules are presented, as the corresponding values of maximal excitation (Ex) and emission (Em) wavelengths and their physicochemical features and biological applications.

2.3 Tyrosine-reactive fluorescent coumarins

The hydroxyl groups of the amino acids can be labeled with the same reagents used for the lysine residues, but the labelling reaction is carried out in organic solvent, like anhydrous dimethylformamide (DMF) or dimethylsulfoxide (DMSO), which absorbs the formed water molecule avoiding possible hydrolysis reactions. The amino acid

Figure 2.
Schematic diagram of thiol-labelling technique using maleimides.

Coumarin	Ex/Em (nm)	Physicochemical features and biological applications	Reference
 Triethylammonium 7-amino-3-(2-((5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl) amino)-2-oxoethyl)-4-methylcoumarin-6-sulfonate (Alexa Fluor™ 350 C5 Maleimide)	345/444	Blue-fluorescent dye, with moderate photostability, water soluble and pH insensitive from pH 4 to pH 10, used for stable signal generation in imaging and flow cytometry	[68, 80, 81, 106–108]
 N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl) pentyl)-6,8-difluoro-7-hydroxycoumarin-3-carboxamide (Pacific Blue™ C5-Maleimide)	410/455	Excellent reagent for thiol-selective modification, quantitation, and analysis and usually requires a higher pH than reaction of maleimides with thiols. Does not react with methionine, histidine, or tyrosine	[68, 80, 109]

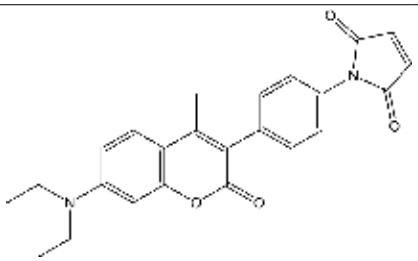
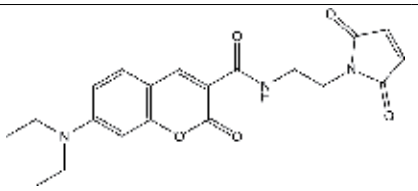
Coumarin	Ex/Em (nm)	Physicochemical features and biological applications	Reference
 1-(4-(7-(diethylamino)-4-methylcoumarin-3-yl)phenyl)-1H-pyrrole-2,5-dione	391/472	Labelling of protein thiol groups in tissue sections. Fluorescence probe for glutathione intact cells Used to monitor release of thiols, to quantitate thiol in microplate reactions, and to distinguish proliferating cancer cells by nuclear protein staining	[68, 105]
 7-(diethylamino)-N-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)coumarin-3-carboxamide	419/467	Used as a fluorescent biological sensing device and for real-time measurements for the release of inorganic phosphates during enzymatic reaction. Also, used for intramolecular fluorescence energy transfer (FRET) experiments	[68, 110–112]

Table 5.
Fluorescent coumarin maleimides used for biomolecule labelling.

hydroxyl groups do not allow highly specific labelling reactions due to the existence of several hydroxyl groups in biomolecules (serine, threonine, and tyrosine) [113].

One of the well-known labelling methods is the reaction with diazonium salts resulting in the formation of azo compounds, as 4-trifluoromethylcoumarin-7-diazonium chloride [114]. Although these aryl diazonium ions are promising for the desired application, their storage and delivery are challenging, and they often require in situ generation. The pH range should be between 8 and 10 for the formation of a phenolate anion [115].

3. Concluding remarks

Reactive fluorescent coumarins have been increasingly attracting special interest as fluorescent labels, with a wide range of applications in bioimaging and biolabelling, due to their extremely attractive and stable scaffold. Coumarins will allow the development of new low-cost fluorescent dyes due to its easy synthesis with high yields, large Stokes shift, pH independence of absorbance and emission, and excellent photostability, which represents a great value for the biological fluorescence imaging techniques.

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Conflict of interest

There are no conflicts of interest to declare.

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Section 3

Medical and Human Health Applications

Chapter 9

Ocimum Phytochemicals and Their Potential Impact on Human Health

Debjoy Bhattacharjya, Sinchan Adhikari, Arijit Biswas, Anil Bhuimali, Parthadeb Ghosh and Soumen Saha

Abstract

The genus *Ocimum* (Lamiaceae) is distributed all over the world and can be found in many environments. *Ocimum* species is a rich source of various phytochemicals including tannins, phenolic acids, anthocyanins, phytosterols, and policosanols. These phytochemicals have the potential to significantly impact human health. The economic importance of *Ocimum* is also evident; *Ocimum* oil and its constituents and derivatives are used as flavoring agents throughout the world in food, pharmaceutical, herbal, perfumery, and flavoring industry. The important advantages of *Ocimum* plants in various treatments are their safety besides being less expensive, efficacy and availability throughout the world. This paper will focus on the biological effects of *Ocimum* essential oils, with particular attention on the molecular mechanism underlying their action.

Keywords: *Ocimum* sp., essential oil, composition, biological activity

1. Introduction

Living plants produce a vast quantity of chemicals required for their performance and improvement. Some of these chemicals are primary metabolites, which consist of proteins (amino acids), carbohydrates, fats, nucleic acids, etc. but, besides these primary chemicals, the plants further provide just so-called secondary metabolites, which are specific to some taxonomic groups (families, genera). About 80% of the world's population still depends on the traditional system of medicine for curing several health hazards [1]. Despite the vast scientific development in contemporary medicine, Ayurvedic system of medicine is widely practiced and accepted by people not only in India but also in many developed countries. According to the World Health Organization (WHO), about 80% of patients in India still, rely on the practitioners of the traditional system of medicines. The therapeutic use of herbal crude medicines in different rural and urban communities is most of the time regulated by their traditional beliefs, and thus a majority of the herbal drugs are used as "folk" medicines and well practiced since long past. Furthermore, increasing dependency on medicinal plants in the industrialized communities have been found to the extraction and improvement of several remedies and chemotherapeutics from these plants and from traditionally established rural herbal remedies [2]. In these communities, herbal remedies become deeply engaged in the practice of minor conditions and again on the explanation of the increasing costs of specific health maintenance. Although synthetic drugs enhanced the

demand against green remedies because of their rapid-acting implements, people have been turned up to understand the benefits correlated with essential remedies. Chemically prepared drugs may act at earlier, but they side effects which influence the human body separately in the long run, because medicinal plants work in an integrated or probiotic with limited or no negative effects on the body [3].

The genus *Ocimum* includes approximately 150 species, possessing a great variation in plant morphology and biology, essential oil content, and chemical composition [4]. The economic importance and global dissemination of *Ocimum*, with its many uses in cooking and folk medicine, make it important to investigate its pharmacological and toxicological effects in order to ensure its efficacy and safety. In India, among the medicinal herbs known for their healing properties, the genus *Ocimum* (commonly known as 'Basil' or 'Tulsi') is very important for its curative potential. Basils contain a wide range of essential oils rich in phenolic compounds and a wide array of other natural products including flavonoids and anthocyanins [5] having great pharmacological importance.

Nowadays, scientists are mainly focused on exploring the potential of plant antioxidants for curing several diseases. Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions [6]. Antioxidants counteract the harmful reactive oxygen species (ROS) and free radicals generated in the living organism during regular metabolism, especially under stress conditions. Several in vitro and in vivo studies have already proved the antioxidative property of beta-carotene, alfa-tocopherol, ascorbic acids, phenolics, flavonoids present in different plants [7].

Presently due to our regular stressful lifestyle, we are suffering from several types of diseases like aging, diabetes, degenerative disorders, etc., which mainly develop due to the development of ROS in our body. A dynamic balance is already operating in our body to reduce the harmful effects of generated ROS that is not adequate enough. Therefore, it is obvious to enrich our diet with antioxidants for developing protection. The purpose of this paper will focus on the recent research of the major nutrients and phytochemicals of *Ocimum* and their potential health benefits related to the dietary prevention of chronic diseases.

2. Health benefits of phytochemicals

"Phyto" in the word of phytochemicals is derived from the Greek word "phyto," which means plant. Phytochemicals are a naturally occurring group of chemicals in plants and plant-derived foods, which may function in reducing the risk of chronic diseases [8]. Although it is estimated at least more than 5000 dietary phytochemicals have been discovered, it is believed that a high percentage of phytochemicals in foods still remain unknown [8]. Critical reviews of studies available in the literature support the concept that phytochemicals (polyphenols, tocopherols, tocotrienols, carotenoids, and ascorbic acid) has been associated with the maintenance of good health as well as prevention/treatment of many health conditions including cancer, cardiovascular diseases, diabetes, hypertension, stroke, metabolic syndrome, and other degenerative diseases. It is largely accepted that the additive effects of the combinations of various phytochemicals in whole plant-based foods are shown to have stronger protective actions than single, isolated phytochemical compounds [9].

3. Role of secondary metabolites

New drug model seeks to meet on bringing compounds active toward target proteins. Even though newly pharmaceutical companies and support organizations

take influence in molecular design, combinatorial chemistry and synthetic chemistry, natural productions, and especially those of plant source, remain as a prerequisite cause of new remedies, current medicine leads and other new synthetic entities (NCEs) [10, 11].

Plants produce a diverse array of compounds that can broadly be categorized into primary and secondary metabolites. Primary metabolites are the ones which are required for the normal growth and biological processes and are produced in the pathways that are crucial for plant survival. The other class of metabolites, though generally termed secondary, is also very crucial for plants from the ecological perspective.

These secondary metabolites are classified on the basis of their biosynthetic pathway and the following types are frequently observed-terpenes, phenylpropanoids, alkaloids, saponins, and glucosinolates. The availability of carbon, nitrogen, and sulfur along with energy from the primary metabolism governs the biosynthesis of these compounds [12].

4. Classification of secondary metabolites

Metabolites are the mediators and amounts of metabolism. The term metabolite is commonly confined to narrow fragments. Metabolites have specific functions,

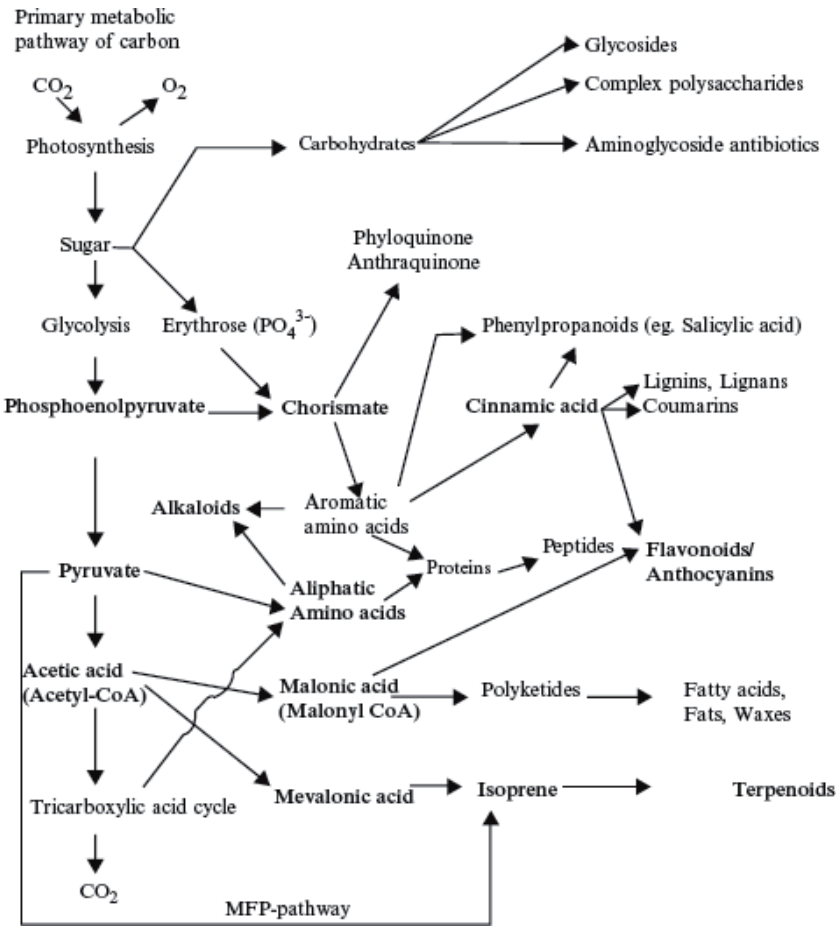

Figure 1.
Major pathways of biosynthesis of secondary metabolites [15].

consisting of fuel, structure, signaling, stimulatory and inhibitory effects on enzymes, and the catalytic activity of their holding (mostly as a cofactor to a stimulant), defense and interaction with distinct pathogens. Plant metabolites are categorized based on their biosynthetic pathways. The pathways of biosynthesis are responsible for the occurrence of both primary and secondary metabolites (**Figure 1**) [13, 14]. Plant secondary metabolites can be classified on the basis of chemical structure (for example, having rings, containing a sugar), composition (consisting of nitrogen or not), their solubility in numerous solvents, or the pathway by which they are synthesized (e.g., phenylpropanoid, which provides tannins).

5. Essential oil of *Ocimum* species

Since ancient times, essential oils are known for their medicinal use, and they are very much interesting and impressive natural plant commodities. They continue to be of paramount importance until the present day. Essential oils have been tested as perfumes, flavors for foods and beverages, or to provide both bodies and care for thousands of years [16].

Species	Class of compounds	Reference
O. basilicum L.	**Monoterpene hydrocarbons** α-Phellandrene, α-pinene, α-terpinene, α-terpinolene, α-myrcene, β-phellandrene, β-pinene, camphene, *cis*-β-ocimene, *cis*-ocimene, δ-3-carene, β-ocimene, (E)-β-ocimene, limonene, myrcene, *ρ*-cymene, sabinene, terpinolene, thujene, γ-terpinene	[18–20, 23]
	Oxygenated monoterpene α-Citral, α-fenchyl acetate, α-terpineol, borneol, bornyl acetate, camphor, carvacrol, carvone, 1,8-cineole, *cis*-linalool oxide, *cis*-rose oxide, citronellol, endo-fenchol, estragol, eugenol, exo-2-hydroxycineole-acetate, fenchone, geranial, geraniol, geranyl acetate, hotrienol, *iso*-neomenthol, *iso*-pinocamphone, *trans*-pinocamphone, L-camphor, L-carvone, lavandulol, linalool, linalool *cis*-furanoid, linalool *trans*-furenoid, linalyl acetate, menthol, menthone, methyl chavicol, myrtenal, myrtenol, neral, nerol, ocimene oxide, pinocarvone, P-menth-1,8-dien-4-ol, piperitone, pulegone, terpinen-4-ol, terpinyl formate, *trans*-linalool oxide, *trans*-myroxide, *trans*-sabinene hydrate, *trans-p*-menth-2-en-1-ol, thymol, verbenone, (Z)-sabinene hydrate	[18, 19, 21, 25]
	Sesquiterpene hydrocarbons β-acoradiene, β-bourbonene, β-caryophyllene, β-cedrene, β-copaene, β-cubebene, β-elemene, β-guaiene, β-ocimene, β-selinene, cyclohexane, 2,4 diisopropenyl-1-methyl-1-vinyl, 1,4,7-cycloundecatriene,1,5,9,9-tetramethyl, α-acoradiene, α-amorphene, α-bulnesene, α-cadinene, α-cedrene, α-copaene, α-cubebene, α-guaiene, α-gurjunene, γ-gurjunene, α-7-epi-selinene, α-humulene, aromadendrene, α-(Z)-bergamotene, α-zingiberene, epsilon-muurolene, dehydroaromadendrene, germacrene-A, bicycloelemene, bicyclogermacrene, cadinene, cadina-3,5-diene, *cis*-calamene, *cis*-muurola-4(14),5-diene, (E)-β-farnesene, (E)-caryophyllene, 1-epibicyclosesqui-phellandrene, germacrene-B, germacrene-D, guaia-1(10),11-diene, iso-caryophyllene, isoledene, longifolene, δ-selinene, St α-ylangene, *trans*-α-bisabolene, *trans*-α-bergamotene, *trans*-β-farnesene, *trans*-β-ocimene, *trans*-caryophyllene, valencene, γ-cadinene, γ-terpin, δ-cadinene, γ-muurolene, (Z)-calamenene	[19, 26, 21–24]

Species	Class of compounds	Reference
	Oxygenated sesquiterpenes α-Cadinol, α-humulene oxide, alloaromadendrene, β-basibolol, β-basibolol isomer, β-eudesmol, cubenol, caryophyllene oxide, 1,10-di-epi-cubenol, dihydroactinidiolide, isospathulenol, muurolol, spathulenol, T-cadinol, viridiflorol, (Z)-nerolidol	[19, 21]
	Triterpene Alphitolic acid, betulin, betulinic acid, 3-epimaslinic acid, euscaphic acids, oleonolic acid, pomolic acid, ursolic acid, basilol, ocimol	[27]
	Aromatic compounds 4-Allylphenol, anethole, anisaldehyde, benzyl alcohol, cuminaldehyde, estragole, ethyl cinnamate, methyl benzoate, methyl cinnamate, methyl eugenol, methyl salicylate, *p*-methoxycinnamaldehyde, phenethyl alcohol, phenyl acetaldehyde, safrole, benzaldehyde, *cis*-hex-3-enyl acetate	[21]
O. kilimandscharicum Guerke	**Monoterpene hydrocarbons** α-Phellandrene, α-pinene, α-terpinene, γ-terpinene, β-pinene, camphene, limonene, D-limonene, β-ocimene, (E)-β-ocimene, myrcene, ρ-cymene, terpinolene, thujene, α-terpinolene, isosylvestrene, γ-himachalene	[28, 29, 34]
	Oxygenated monoterpene α-Citral, α-terpineol, borneol, bornyl acetate, camphor, linalool, citronellol, geraniol, myrtenol, 1,8-cineole, eugenol, terpinen-4-ol, *trans*-sabinene hydrate, (Z)-sabinene hydrate, α-campholenal, isoborneol, endo-borneol, globulol	[29–31, 34]
	Sesquiterpene hydrocarbons α-Copaene, α-gurjunene, α-humulene, β-caryophyllene, β-copaene, β-cubebene, β-elemene, δ-cadinene, γ-muurolene, *trans*-caryophyllene, germacrene-D, germacrene-B, β-selinene	[31, 32, 34]
	Oxygenated sesquiterpenes Cubenol, caryophyllene oxide, spathulenol, α-cadinol, viridiflorol	[28, 32]
	Phenolic compounds Vanillin	[33]
O. gratissimum L.	**Monoterpene hydrocarbons** α-Pinene, *cis*-ocimene, *trans*-ocimene, β-pinene, α-terpinene, p-cymene, myrcene, α-phellandrene, A^3-carene, sabinene, limonene, γ-terpinene, terpinolene, cis-sabinene hydrate	[35–39]
	Oxygenated monoterpene Camphor, eugenol, methyl eugenol, 1,8-cineole, trans-sabinene hydrate, linalool, δ-terpineol, terpinen-4-ol, α-terpineol, thymol, carvacrol, eugenol, methyleugenol	[36, 39–41]
	Sesquiterpene hydrocarbons *trans*-Caryophyllene, germacrene-D, α-farnese, β-bisabolene, *cis*-β-ocimene, *trans*-β-ocimene, α-copaene, β-elemene, β-caryophyllene, α-humulene, germacrene-D, β-selinene, trans-β-Farnesene, β-bisabolene, γ-cadinene, δ-cadinene, γ-muurolene, β-cubebene, α-cubebene	[33, 36, 40, 41]
	Sesquiterpenes oxygenated β-Caryophyllene epoxide	[36]
	Aromatic compounds Methyl cinnamate	[36]
O. canum Sims.	**Terpenoids** Cyclosativen	[42]

Species	Class of compounds	Reference
	Monoterpenes hydrocarbon α-Thujene, α-pinene, camphene, β-pinene, limonene, γ-terpinene, terpinolene, sabinene, myrcene, α-terpinene, *p*-cymene, (Z)-β-ocimene, (E)-β-ocimene, perillene, (E)-β-epoxyocimene, α-phellandrene, α-terpinene, *cis*-β-ocimene, *cis*-sabinene hydrate, carene, tricyclene	[43, 45, 48]
	Oxygeneated monoterpenes Eugenol, linalool, 1,8-cineole, 5-isopropyl-2-methylbicyclo[3.1.0]hexan-2-ol, fenchone, *trans*-linalool oxide (furanoid), camphor, δ-terpineol, terpinen-4-ol, α-terpineol, fenchyl acetate (endo), isobornyl acetate, borneol, thymol, menthone, geranial, α-fenchyl acetate, bornyl acetate, exo-2-hydroxycineole acetate, verbenone, camphenol, myrtenyl acetate	[43, 46, 48]
	Sesquiterpenes hydrocarbon α-Copaene, β-elemene, β-caryophyllene, germacrene D, β-bisabolene, E, E-α-farnesene, δ-cadinene, α-cadinene, *trans*-α-bergamotene, aromadendrene, α-humulene, *epi*-bicyclosesquiphellandrene, bicyclogermacrene, δ-guaiene, calamenene, (E)-α-bisabolene, valencene, *trans*-α-bergamotene, γ-muurolene, α-muurolene, epi-α-muurolol, elemol, β-selinene, α-selinene, (Z,E)-α-farnesene, *trans* β-ocimene, (E)-β-farnesene	[45, 46, 49]
	Oxygenated sesquiterpenes α-Cadinol, spathulenol, (Z)-nerolidol, caryophyllene oxide, β-eudesmol, viridiflorol	[42, 46]
	Aromatic compounds Methyl eugenol, estragole, benzyl benzoate	[45, 47]
	Esters 1-Octen-3-yl acetate	[44]
	Others Naphthalene	[46]
O. tenuiflorum L. *syn. O. sanctum* L (purple type)	**Monoterpenes hydrocarbon** α-Pinene, β-pinene, β-terpinene, D-limonene, camphene, sabinene, myrcene, *p*-cymene, limonene, α-terpinene, *α*-thujene, *α*-myrcene, α-terpinolene, terpinolene, γ-terpinene, (E)-β-ocimene, β-myrcene, *α*-camphene, β-terpinolene, β-*cis*-ocimene	[50–52]
	Oxygeneated monoterpenes Linalool, menthol, methyl chavicol, α-citral, carvone, lavandulol, hotrienol, eugenol, 1,8-cineole, globulol, borneol, bornyl acetate, camphor, thymol, geranial, citronellol, E-linalool, β-citral, carvacrol, methyl chavicol, α-fenchyl acetate, myrtenol, terpinen-4-ol, *trans*-sabinene hydrate, *cis*-geraniol, *cis*-*α*-terpineol, *cis*-linalool oxide, eucalyptol, *cis*-linalool oxide (furanoid), *trans*-linaool oxide (furanoid), δ-terpineol	[52–54]
	Sesquiterpenes hydrocarbon Caryophyllene, β-farnesene, germacrene-D, isoledene, β-selinen, β-cubebene, β-elemene, β-caryophyllene, β-bourbonene, α-humulene, γ-muurolene, bicyclogermacrene, δ-cadinene, α-copaene, *trans*-caryophyllene, selinene, β-elemene, *β*-guaiene, β-bisabolene, α-guaiene, germacrene-B, valencene, (E)-β-farnesene, *trans*-α-bergamotene, α-cubebene, β-germacrene, α-farnesene, α-caryophyllene, α-selinene, (Z)-β-farnesene	[50, 52–54]

Species	Class of compounds	Reference
	Sesquiterpenes oxygenated α-Cadinol, alloaromadendrene, caryophyllene oxide, cubenol, t-cadinol, spathulenol, viridiflorol	[53, 54]
	Aromatic compounds *p*-Methoxycinnamaldehyde, estragole, benzaldehyde	[51, 54]
O. tenuiflorum (white type)	**Monoterpene hydrocarbons** α-pinene, camphene, β-pinene, limonene, (E)-β-ocimene, *p*-cymene, γ-terpinene, camphene hydrate, carene, terpinolene, sabinene hydrate, terpinene, ocimene, limonene, terpinene, phellandrene, myrcene, sabinene, camphene, thujene, tricyclene	[55–57]
	Oxygeneated monoterpenes Linalool, borneol, eugenol, methyl eugenol, 1,8-cineole, α-terpineol, geraniol, *trans*-linalool oxide (furanoid), δ-terpineol, terpineol, terpinen-4-ol, δ-terpineol, camphor, fenchone, *trans*-sabinene hydrate, eucalyptol	[56–59]
	Sesquiterpenes hydrocarbon β-Elemene, β-caryophyllene, α-humulene, germacrene D, β-selinene, α-selinene, α-cubebene, δ-cadinene, elemol, bicyclogermacrene, α-cadinene, copaene, β-elemen, α-guaiene, γ-muurolene, δ-cadinene, amorphene, cubebene, α-bisabolene, cadinene, β-bisabolene, muurolene, germacrene, humulene, farnesene, sesquiphellandrene, bergamotene, guaiene, elemene, bourbonene, zingiberene	[55–59]
	Sesquiterpenes oxygenated Spathulenol, caryophyllene oxide, viridiflorol, β-eudesmol, γ-eudesmol	[60, 61]
	Aromatic compounds Estragole	[55, 60]

Table 1.
Compositions of species-wise distribution of bioactive compounds in Ocimum species.

Chemical diversification is of special significance if at the genus or species level both terpenes and phenylpropenes can be formed in the essential oil. Most Lamiaceae preferentially accumulate mono-(and sesqui-)terpenes in their erratic oils but some genera show oils too rich in phenylpropenes [17].

The action of essential oils begins by entering the human body via three possible different ways including direct absorption through inhalation, ingestion or diffusion through the skin tissue.

Table 1 presents published compositions of species-wise distribution of bioactive compounds in *Ocimum* species.

6. Biological activities of *Ocimum* species

The genus *Ocimum* (family Lamiaceae), collectively known as basil, is composed of a diverse and rich source of essential oil containing plants. The main issues of concern with the use of herbal drugs remain safety, validation of claims and standardization of product. Different species and forms of *Ocimum* spp. vary in growth habit, color, and aromatic composition, making the true botanical identity of basil difficult (**Figure 2**). There exist the problems of significant variation in the content of *Ocimum* plants across and within species, with the implication of varied

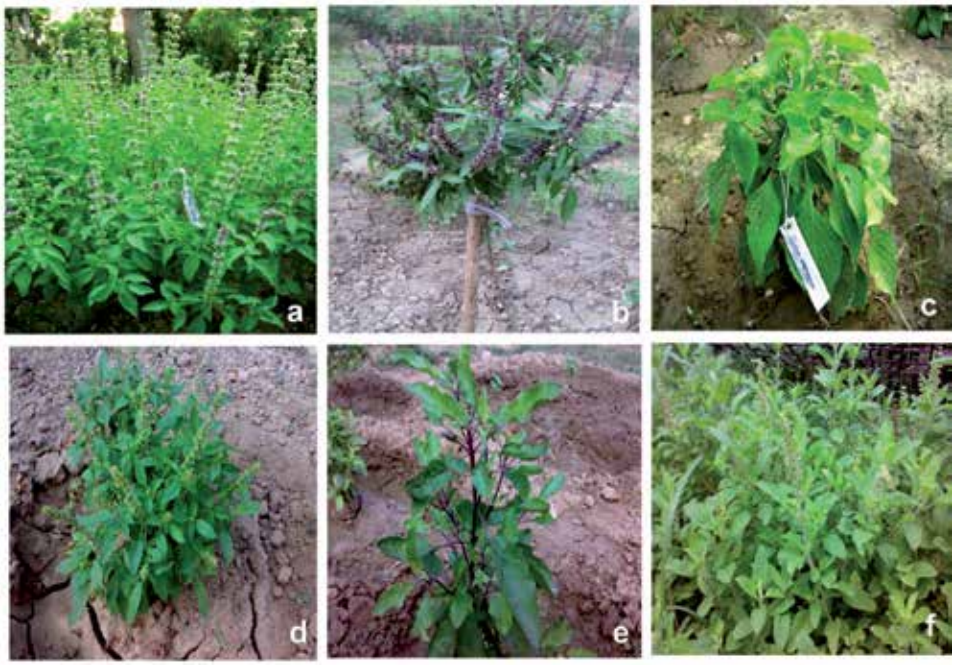

Figure 2.
Different species of Ocimum (a) Ocimum basilicum L.; (b) Ocimum kilimandscharicum Guerke; (c) Ocimum gratissimum L.; (d) Ocimum canum Sims.; (e) Ocimum tenuiflorum L. syn. O. sanctum L. (purple type); (f) Ocimum tenuiflorum (white type).

biological activities. During the last two decades, it has been shown that *Ocimum* oil and its constituents possess different biological activities including antioxidant, antimicrobial, anticancer, and anti-inflammatory properties.

6.1 Oxidant activity

Anti-oxidants play an important function in protecting the body against free radicals. They achieve this by stopping the formation of new free radicals species, converting older ones to free radicals, less toxic molecules that can be easily mopped up and preventing radical chain reaction [62]. The principal function of anti-oxidants is in suspending the oxidation of other molecules, by inhibiting the initiation or propagation of oxidizing chain reactions by free radicals and thereby reducing oxidative damage to the human body [63]. Two great mechanisms of procedure have been proposed for antioxidants [64]. The initial is a chain breaking method by which the initial antioxidant provides an electron to the free radical stage in the systems. The second technique involves the destruction of ROS/reactive nitrogen species initiators (secondary antioxidants) by cutting off chain starting catalysts (**Figure 2**). The potential role in the food industry and human health, antioxidants are getting acceptance all across the globe. Antioxidants are defined as a substance that easily in small amounts, can inhibit or prohibit the oxidation of readily oxidizable elements. The antioxidant is also defined as a substance qualified of inhibiting special oxidizing stimulants or a substance that serves with oxidizing agents prior to creating damage to other fragments or a substance that sequesters metal ions or even a substance efficient of the recovering system such as iron transporting protein [65].

Natural antioxidants have been studied intensively during the past years which are mainly phenolic compounds. Moreover, oxidation is a degenerative process in

biological systems due to the endogenous reactive oxygen species (ROS). Reactive oxygen species (ROS) are chemical properties originate in the body during metabolism that is overmuch reactive and may have one or expanded unpaired electrons. Oxidative stress, i.e., an inequality between ROS and antioxidant defenses have deleterious aftereffect, such as the peroxidation of membrane lipids and the aggression on biomolecules (proteins, membrane enzymes, carbohydrates and DNA) [66].

Various *Ocimum* species and their extracts or essential oils have been determined to achieve antioxidant activity [67, 68]. Phenolic acids, hydroxycinnamates, and flavonoids are perhaps the major antioxidants [67]. Vitamin antioxidants (e.g., ascorbic acid and carotenoids) are secondary contributors to the overall antioxidant capacity [69]. In essential oils, unsaturated terpenes having a cyclohexadiene structure (e.g., terpinene) and secondary cyclic oxygenated terpenes (e.g., thymol) may lead to antioxidant capacity, while acyclic unsaturated oxygenated monoterpenes (e.g., linalool), aromatic oxygenated monoterpenes (e.g., eugenol), methylchavicol (estragole), sesquiterpene hydrocarbons (e.g., α-bergamotene, germacrene D, γ-cadinene, δ-cadinene, β-selinene, sesquiterpenes oxygenated (e.g., spathulenol) may act as pro-oxidants [68].

Different test methods have been applied, and it becomes to be understood between the complete essential oil and individual components [70–73]. A strong chelating effect on metals as iron or copper has been reported to diminish the presence of ROS obtained from reactions bar with these metals [74]. In addition, the antioxidant actions of *Ocimum* essential oils have also been found employing metal-independent oxidative processes [75] or controlling stable free radicals, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [71, 76]. The DPPH test, a test widely used to measure the ability to donate hydrogen atoms [77], was applied to measure the antioxidant capacities of *Ocimum* species extracted by different solvent systems; these include the methanol extracts of *O. basilicum* L., *O. canum* Sims., *O. gratissimum* L., *O. kilimandscharicum* Guerke, *O. sanctum*, *O. tenuiflorum* [67, 71, 76, 78–80]; the ethanol extracts from *O. basilicum* L., *O. gratissimum* L., *O. kilimandscharicum* Guerke, *O. sanctum* [76, 79, 81–83].

Particular attention has been focused on the scavenger ability to inhibit the process of low-density lipoprotein (LDL) cholesterol oxidation since it represents a major prevention mechanism against atherosclerosis. Various experimental evidence, using in vitro and in vivo preclinical models, showed a strong action of *Ocimum* essential oils [84]. Moreover, Aqueous extracts *Ocimum sanctum* and 70% ethanol extracts *Ocimum basilicum* L. were able to reduce 5-lipoxygenase-driven cellular recruitment of leukocytes and the damaging consequences of their ability to release ROS while leaving unimpaired the generation of prostaglandins, which promote microvascular blood flow and act as immunomodulators [85–87].

In many cases, the antioxidative activity (**Figure 3**) of essential oils cannot be attributed to the main compounds; minor compounds and synergistic effect may significantly contribute to the activity.

6.2 Antimicrobial

For thousands of years, folk medicine has used *Ocimum* leaf for the treatment of infections. Such protective properties have been confirmed by several studies performed in the last decades using *Ocimum* essential oil [29] or isolated compounds. Gram-positive and Gram-negative bacteria, as well as antiprotozoal and also *anti-Trichomonas vaginalis*, resulted sensitive to the antiproliferative action of *Ocimum oil* and its derivatives [29, 88–90]. At present, the exact mechanism responsible for the antimicrobial activity of *Ocimum* oil and its derivatives is still not completely

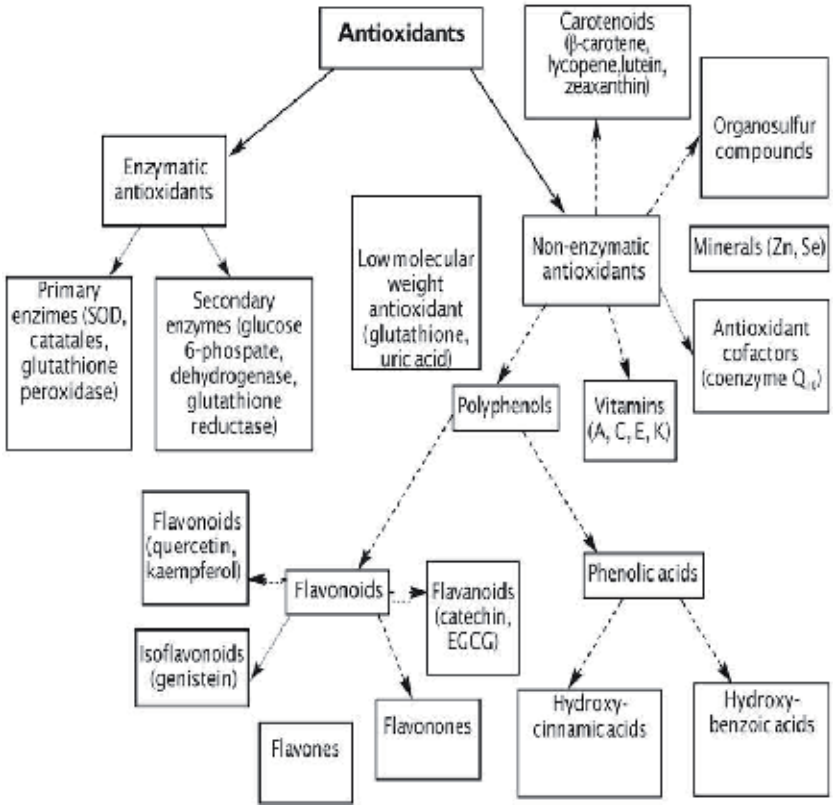

Figure 3.
Types of antioxidants.

clarified, although various modes of action in the bacterial cell have been discussed including degradation of the cell wall, damage to cytoplasmic membrane and membrane proteins, leakage of cell contents, coagulation of cytoplasm, and depletion of proton motive force [91–93].

The antibacterial and antifungal activities of *Ocimum* species have been studied on various bacteria and fungi [29, 94–96]. These studies indicate that essential oils are more efficient antifungals and antibacterials compared to the polar extracts [97–99]. *Ocimum sanctum* essential oils showed remarkable antimicrobial activity against bacteria and other microorganisms, such as periodontopathogens [100], mainly due to the presence of oxygenated monoterpenes in their chemical compositions [101].

The essential oil and methanol extracts of five *Ocimum* species have an appreciable activity against seven human pathogenic bacteria [29], essential oils of *Ocimum* species showed strong antimicrobial activity against all seven microorganisms tested. Oils of seven *Ocimum* taxa (*O. americanum* L., *O. basilicum* L., *O. campechianum* Mill., *O.* x *citriodorum* Vis., *O. kilimandscharicum* Baker ex Gürke and three botanical varieties and cultivars of *Ocimum basilicum* L.: 'Genovese', var. difforme and var. purpurascens)) showed strong antimicrobial activity against all 8 microorganisms tested by Carovic-Stanko et al. [102].

Among the antifungal activities, the *in vitro* antifungal activity of *O. basilicum* L. essential oil against Aspergillus flavus fungal growth and aflatoxin B1 production [103], essential oils of *O. basilicum* L. showed strong antifungal activity against *A. flavus*, and the main components were linalool, 1,8-cineol, eugenol, methyl cinnamate, α-cubebene, caryophyllene, β-ocimene and α-farnesene.

6.3 Anticancer activity

For a long time, the polyphenols of the *Ocimum* oil of the diet have been considered to play a role for the prevention of certain types of cancer in the Asian origin [104]. Even more than in *Ocimum* oil, constituents present in *Ocimum* leaf extract has shown strong antioxidant potency and inhibition of cancer cell proliferation, thus suggesting the protection against the genotoxic action of the ROS as one of the mechanisms explaining the anticancer effects of these compounds. Indeed, either methanol aqueous *Ocimum basilicum* L. leaf extract or the isolated constituents eugenol epoxide free radical scavenging activity and growth inhibition at low micromolar concentration on human breast cancer cell lines (MCF-7 and MDA-MB-231) [105, 106] and Human cancerous cell lines (HL60-promyelocytic blood leukemia cells) [107]. Such findings were further confirmed by other in vitro reports, testing the effects of *Ocimum basilicum* L. against four different humans cancer cell lines viz. human cervix adenocarcinoma HeLa cells, human melanoma FemX cells, human chronic myelogenous leukemia K562 cells, and human ovarian SKOV3 cells [108]. Furthermore, Karthikeyan et al. [109] demonstrated regression of tumors caused by orally administrated aqueous and ethanolic extracts of *Ocimum sanctum* in mice that developed spontaneous soft tissue sarcomas.

Monga et al. [110] studied the antimelanoma and radioprotective activity of essential oils obtained by 50% alcoholic aqueous leaf extract from five species of *Ocimum* viz. *Ocimum sanctum* (SE), *Ocimum gratissimum*, *Ocimum basilicum*, *Ocimum canum*, and *Ocimum kilimandscharicum*, were evaluated using C57BL and Swiss albino mice tumorigenesis; growth inhibition has in fact been associated with (a) reduction of tumor volume (b) blockage of messengers of pathways involved in cell proliferation was evident in all the oils but the greater was shown by that obtained from *Ocimum tenuiflorum* (syn. *O. sanctum*) compared to other *Ocimum* species. In various experiments and test systems, some mono-and sesquiterpenes showed activity, where camphor, 1,8-cineole and limonene were of greatest interest. Camphor, 1,8-cineole and limonene, the anti-inflammatory compound of *Ocimum kilimandscharicum* oil, showed a strong time-and dose-dependent cytotoxic effect on human ovarian cancer cell [111]. The potential antitumor effects of camphor have been shown previously [112, 113], and the mechanistic action of camphor against cancer included the improvement of immune function [114] and the radiosensitizing effect on transplantable mammary adenocarcinoma in mice [112]. Ursolic acid showed some potentiating effect on the anticancer activity of rosmarinic acid, cinnamic acid, caffeic acid, sinapic acid, and ferulic acid on various cell lines [106].

6.4 Cardiovascular protection

For decades, investigation on the health-promoting effects of Asian diet has been revealed that *Ocimum* oil consumption is a key factor in the cardiovascular protection found in Asian origin [115]. It is well established that the healthful properties of *Ocimum basilicum* L. oil depend largely on its Cardiac glycosides and catecholamines content [116]. But, many arguments prove that in *Ocimum* oil there are little bioactive components, much than Cardiac glycosides and catecholamines, effective for its cardiovascular protective properties: among them, the ethanolic fraction of *Ocimum* oil, and in specific omega-3 fatty acids have proved antioxidant, anti-platelet aggregation, vasodilatory, and anti-inflammatory effects, all engaged in this health beneficial action [117–119].

Oxidation of LDL cholesterol is one of the key steps in the induction of atherosclerotic lesions by increasing damage to the arterial side through several processes, including growth factor and chemotactic protein expression, inflammation, and build up local macrophages [120, 121] have indicated that ethanolic leaf extract of *Ocimum basilicum* L. oil strongly inhibits copper sulfate-induced oxidation of LDL, as a result of the step of different indicators of lipid oxidation [phospholipids (PL), cholesterol ester (CE), triacylglycerol (TG)].

Although the contraction of plasmatic cholesterol and LDL is the primary technique regulating the antiatherogenic activity of *Ocimum basilicum* L. extract, other implements are further identified [121]. It is well settled that local leukocyte and monocyte recruitment into the vessel wall is initial walk-in atherogenesis. This fact takes combined with the statement in the endothelial cells of adhesion fragments such as intercellular adhesion fragment-1 (ICAM-1) and vascular adhesion fragment-1 (VCAM-1). Aqueous extract of *Ocimum gratissimum* L. showed the capacity to reduce LPS-stimulated expression of BEAS-2B cell in human lung epithelial cells inhibiting its mRNA levels. Moreover, Li et al. [122], investigating the action of distillate and residue fractions of basil essential oil (viz. estragole, methyl eugenol, linoleic acid, α-cadinol, and α-bergamotene) in-a Raw 264.7 cells line, have demonstrated that residue fractions prevents the expression of gene and suppressed the production of cytokines (TNF-a, IL-b, IL-6) in LPS-induced Raw264.7 cells, which contribute to treating various disorders caused by extreme oxidative stress.

6.5 Anti-inflammatory

The inflammatory response involves long been compartmentalized into multiple attributes commonly termed redness, heat, pain, and edema. Inflammatory injuries lead to the discharge of a variety of fundamental mediators, cytokines, and chemokines that balance cellular infiltration that consequentially brings about resolving inflammatory response and restoration of tissue scrupulosity. However, immutable inflammatory stimuli or dysregulation of mechanisms of the resolution phase can lead to chronic inflammation [123, 124].

Ocimum extracts contain numerous constituents which could have anti-inflammatory effects. The anti-inflammatory effects of *Ocimum* oil phenolics, in RAW 264.7 macrophage cells have been described by Aye et al. [125]. When added to murine macrophages stimulated with bacterial lipopolysaccharide (LPS), *Ocimum* oil phenolics did not cause cytotoxicity in RAW 264.7 macrophage cells in vitro, as evaluated by a significant increase in the production of nitric oxide [125]. Additionally, NO is a significant inflammatory mediator generated by NOS (neuronal, inducible, and endothelial) under physiological and pathophysiological conditions [126]. It further serves as a crucial mediator during the inflammatory process. Enhanced NO production and iNOS expression contributes to the great cytotoxic function of LPS stimulated macrophages [127]. Thus, the reduction in NO production indicates the anti-inflammatory activities of the treatment in the cells. However, *Ocimum basilicum* L. ethyl acetate extract and butanol extract inhibited the growth of normal RAW 264.7 macrophage cells [128]. Also, *Ocimum basilicum* L. crude methanolic extract suppressed the induction of iNOS and the subsequent production of NO in LPS-stimulated RAW 264.7 macrophage cells [129, 130]. To test the anti-inflammatory activity of the *Ocimum basilicum* L. methanolic extracts has been determined by PBMC (peripheral blood mononuclear cells) in mitogenic lymphocyte proliferative assay, methanolic extracts enhanced the functional activity of these immune-competent cells, as evaluated by a significant inhibitory effects of methanolic leaf extracts, PHA activated PBMC proliferation could be suggestive of suppression of T cell proliferation [130]. This effect arose from its pivotal role in

immune regulation [131, 132], T cell activation provides a target for pharmacological modulation aimed at achieving clinically useful immune-suppression [133].

Złote et al. [134] studied the capacity of phenolic-rich fraction obtained from the elicited basil leaves to inhibit the activity of two enzymes of inflammatory process (LOX and COX). This research found that a higher LOX and COX inhibition efficiency was positively correlated with the increased contents of rosmarinic, benzoic and *o*-coumaric acids determined after elicitation of basil. This result partially corresponds with the study of [135]. More recently, to gain insight into the mechanism of action and pharmacological value of the anti-inflammatory activity of aqueous and methanolic extracts of *Ocimum basilicum* L. in macrophage (RAW264.7) and human chondrosarcoma (SW1353) cell lines, and human primary chondrocytes to correlate their efficacy in terms of management of osteoarthritis (OA). Raina et al. [136] evaluated aqueous extract of *O. basilicum* L. significantly accustom the production of inflammatory mediators such as NO, PGE2, LTB4, and MMPs increased than the methanolic extract. The regulation of these inflammatory intermediaries is pivotal in OA, as it would have a direct effect on (1) chondrocyte survival, (2) production of proinflammatory cytokines, prostaglandins and leukotrienes, and (3) production of extracellular matrix-degrading enzymes such as MMPs. Due to the significant side-effects related to the use of NSAIDs, the check for natural products that would regulate the inflammatory cascade related to OA, without engaging chondrocyte survival, is extremely important. To investigate the anti-inflammatory effect of *Ocimum basilicum* L. oil, Rodrigues et al. [137] investigated an acute and chronic in vivo test as paw edema, peritonitis, and vascular permeability and granulomatous inflammation model. The anti-inflammatory mechanism of action was also analyzed by the participation of histamine and arachidonic acid pathways. These researchers found that the *Ocimum basilicum* L. essential oil and estragole significantly reduced paw edema induced by carrageenan and dextran. The smallest quantities of *Ocimum basilicum* L. essential oil (50 mg/kg) and estragole (30 mg/kg) revealed effectiveness in the decrease of paw edema created by histamine and arachidonic acid, vascular permeability inhibition and leukocyte emigration in the peritoneal fluid. These dosages were carried out of decrease the assured inflammatory process. The results followed between the *Ocimum basilicum* L. essential oil and estragole determine efficacy in anti-inflammatory activity, however, the essential oil is higher efficacious in the acute and chronic anti-inflammatory action. Dextran is a high molecular weight polysaccharide, which differently to carrageenan, induces anaphylactic reactions characterized by extravasation and formation of edema due to mastocyte degranulation with release of histamine and serotonin. Carrageenan induces an inflammatory response through opinion with sulfated polysaccharides, initially encouraging the free of chemical substances which encourage multifactorial facts, mostly concerning the free of substance P, bradykinin, histamine, serotonin, cytokines, and nitric oxide and, subsequently on commodity arise from the arachidonic acid pathway [138].

6.6 Antidiabetic

Diabetes mellitus is a chronic metabolic disorder caused by an absolute or relative lack of insulin and or reduced insulin activity which results in hyperglycemia and abnormalities in carbohydrate, fat and protein metabolism [139, 140]. The hypoglycemic effect of *O. tenuiflorum* L., *O. canum* Sims. and *O. gratissimum* L. in animals with alloxan-induced diabetes was applied to potentiation of glucose-induced insulin free and parallel increased peripheral uptake of glucose [141, 142]. Successive studies have reported a greater link of antidiabetic deal with the antioxidant effects of *Ocimum* oil. The character played by OS for diabetes complications

such as retinopathy, nephropathy, and cardiovascular disease are well set up so that dietary antioxidant compounds was fixed to protect from the damages of oxidative stress and free radicals in diabetic cases [143].

In animal experimental designs of alloxan-induced diabetes, both antioxidant and hypoglycemic effects of *O. basilicum* L., *O. tenuiflorum* L., *O. canum* Sims. and *O. gratissimum* L. have been reported. By treating alloxan-diabetic rabbits [141, 142, 144], made a significant decrease in blood glucose levels as corresponded with diabetic control rabbits. Such a hypoglycemic work was related to its powerful antioxidant potentiality: in evidence, in interact, the rabbits studied with *Ocimum* oil showed further a renewal of the levels of malondialdehyde and most of the enzymatic and nonenzymatic endogenous antioxidants [141, 142, 144]. Similar results were achieved in alloxan-diabetic rats: the control, of *Ocimum tenuiflorum*-rich extracts showed significant hypoglycemic, hypolipidemic, and antioxidant effects in all the investigated diabetic rats [145, 146]. In an identical empirical design, *O. gratissimum* L. led to a decrease in the sugar level in plasma and a rise in superoxide dismutase, catalase, and glutathione peroxidase activities in liver and kidney. Furthermore, an opposed reaction against hepatic and renal toxicity in diabetic rats was also observed [147, 148]. Furthermore, the effects of *Ocimum sanctum* leaf polyphenols have been investigated also in insulin-secreting pancreatic β-cells, whose OS-induced alterations contribute to the pathogenesis of diabetes [149].

7. Future direction and conclusion

Despite the many appreciations of science and industry, present practice is filled with stress. Mobile devices and the web have vastly enhanced the pace of life so that many people feel that they are now going down in an endless-increasing ocean of data, while technical culture has overwhelmed us with growing vulnerability to unhealthy prepared and packed food and a profusion of pesticides, food container components, and many toxic modern chemicals. Urban citizens are nevertheless dealt with growing prosperity disparity, social segregation, excessive turbulence, air, water and soil pollution and disconnection from nature. Therefore, while industrialization experiences served to stronger lifespans and impressive expansions in human populations, it is now agreed that the extremest causes of death and disease on the globe are preventable lifestyle-related chronic diseases [150].

The biodiversity of essential oils containing the small molecular terpenoids remains an enchanted field of investigation, and the continuous usage of this reward in a broad field of studies suggested these demands in the consequent [151]. Screening, identifying, and dealing with this vast biodiversity will require a progressing progress of precise, rich-throughput experimental methods including new driving procedures.

The beneficial health effects of *Ocimum* oil compounds have also been proven by many randomized, crossover, controlled, human studies on biomarkers of health performed in the last decades. Several preclinical studies suggest that such beneficial effects may be mainly ascribed to the phenolic compounds. Further development of biotechnology with the genomic and metabolomic analyses and genetic engineering will advance a variety of fields involving bioactive compounds ranging from food and animal nutrition to plant protection. Although many biological activities as antimicrobial or antioxidative and other effects have been intensively studied and well documented. However, well studies are needed to further characterize the *in vivo* effects of individual *Ocimum* derivatives applied as specific agents or in a mixture, consisting of their safety analysis on mortals. Moreover, a better evidence of their molecular procedure of activity may appropriate the system to a better application in human pharmacology.

In inference, the tendency of the last moments in the treatment of herbal productions have indicated that nature prospects and trust in pure and healthful products including medicines, cosmetics, household products, since easily as foodstuffs of plant and animal origin have belonged to a vital issue. So, indeed a well-balanced risk-benefit assessment of bioactive essential oils is one of the major challenges and policymakers must be convinced that review on natural products as the volatile terpenoids in essential oils is a huge task to ensure the ultimate human and animal welfare [151].

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Chapter 10

Medicinal Properties of Selected Asparagus Species: A Review

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Abstract

Asparagus species are naturally distributed along Asia, Africa, and Europe and are known to have numerous biological properties. This review article was aimed to provide an organized summary of current studies on the traditional uses, phytochemistry, and pharmacological and toxicological studies of *Asparagus laricinus Burch*., *Asparagus africanus Lam*., *Asparagus officinalis L*., *Asparagus racemosus Willd*., and *Asparagus densiflorus (Kunth)* Jessop to attain and establish new insights for further researches. Information used in this review was obtained from electronic database including PubMed central, Google scholars, Science direct, Scopus, and Sabinet. Based on the present findings, the existing literature still presents some breaches about the mechanism of action of various constituents of these plants, and their relation to other plant compounds in poly-herbal formulations, as well as their long-term use and safety. More in-depth studies are still needed for active compounds and biological activities of *Asparagus laricinus*, *Asparagus africanus*, and *Asparagus densiflorus*. Therefore, innumerable opportunities and possibilities for investigation are still available in novel areas of these plants for future research stud¬ies. It can be concluded that all selected Asparagus species have tremendous potential to improve human health and the pharmacological activities of these plants can be attributed to bioactive phytochemicals they possess.

Keywords: Asparagaceae, *Asparagus africanus lam.*, *Asparagus densiflorus (kunth) Jessop*, *Asparagus laricinus Burch.*, *Asparagus officinalis L.*, *Asparagus racemosus Willd.*, pharmacological actions, phytochemistry

1. Introduction

Historically, plants were used for numerous purposes for mankind in general, inter alia, feeding and catering, culinary spices, medicine, various forms of cosmetics, symbols in worship and for a variety of ornamental goods. They are still being used for these purposes. The traditional medicines are sold in market places and prescribed by traditional healers at their home [1] particularly in the rural areas where herbal medicine is the main source of the healthcare system. South Africa is blessed with a vast variety of plants since it has such a large diversity of more than 20,000 types of species. The research and scientific community find this to be a great source of interest [2]. Since the 1990s, great interest is being shown in plants that can be used as important sources of new medicines and herbs, which have become mainstream throughout Africa [3].

It is estimated that three quarters of the world of mankind relies on herbal and traditional medicine as a basis for primary healthcare [4]. It was discovered that between 12 and 15 million South Africans still rely on more than 700 indigenous types of plants for the supply of their traditional herbal medicines [5]. Up to 60% of the South African population consults one of an estimated 200,000 traditional healers in rural areas [6]. These herbal medicines which are extracted from plants and used for medicinal purposes often result in acute toxicity. For example, it is estimated that between 8000 and 20,000 people die every year in South Africa due to the fact that these medicinal plants are used incorrectly [7, 8]. The Food and Drug Administration [FDA] indicates that both serious and moderate adverse events from many botanical and others traditional medicinal products are significantly underreported, and that the annual number of such cases is at least 50,000 each year [9, 10].

Different research studies to elucidate and validate the ethnobotanical value of medicinal plants have been conducted and reported by investigators world-wide, with findings that were established from the use of various methods, and also under diverse conditions.

In this review study, five native Asparagus species (namely A*sparagus laricinus Burch.*, *Asparagus africanus Lam.*, *Asparagus officinalis L.*, *Asparagus racemosus Willd.* and *Asparagus densiflorus Kunth) Jessop*) were evaluated for their historical, etymological, morphological, phytochemical and pharmacological aspects. The findings of this review study are summarized, and the medicinal properties of the chosen Asparagus species are documented in this review study.

2. Methodology

2.1 Search criteria

Original articles, research papers published in journals and in PubMed central, Google scholars on plants of interest (*Asparagus laricinus Burch.*, *Asparagus africanus Lam.*, *Asparagus officinalis L.*, *Asparagus racemosus* and *Asparagus densiflorus (Kunth) Jessop)*, and medicinal uses were studied, and related articles and papers were also taken into consideration. The two species, *Asparagus laricinus Burch.* and *Asparagus africanus Lam.* were the first choice according to the research studies at the laboratory of authors for their pharmacological activities and toxicology. The other three species of interest were randomly selected from an enormous number of Asparagus species retrieved when the key words "Asparagus species with medicinal properties" were used in the search.

2.2 Data analysis

The available literature was especially studied for historical, etymological, morphological, phytochemical and pharmacological aspects of *Asparagus laricinus Burch.*, *Asparagus africanus Lam.*, *Asparagus officinalis L.*, *Asparagus racemosus* and *Asparagus densiflorus (Kunth) Jessop*. Priority was given to ethnobotanical reports, laboratory work and clinical trials carried out on all five species. Finally, results were obtained from all collected data and literature studied.

3. Asparagus species with medicinal properties

The genus Asparagus is an herbaceous plant comprising approximately 150 species around the world which are comprised of herbs, shrubs and vines [11]. Asparagus

forms part of *Asparagaceae*, which is a monogeneric family and was formerly included in the *Liliaceae* family. Asparagus species are naturally distributed along Asia, Africa and Europe [12]. Most of these species have economic value as ornamental plants, such as *Asparagus plumosus*, and for their medicinal properties from plants such as *Asparagus laricinus Burch*. Asparagus species have numerous biological properties, such as being antioxidant, anti-inflammatory, antibacterial, antihepatotoxic, immunostimulant, and reproductive agents. Among large number of asparagus species that are used as medicine, five of them have been chosen as they have been investigated for their anticancer activity, namely: *Asparagus laricinus Burch.; Asparagus africanus Lam.; Asparagus officinalis L.; Asparagus racemosus*; and *Asparagus densiflorus (Kunth) Jessop*.

4. *Asparagus laricinus Burch.*

Vernacular names
English: Wild asparagus;
Afrikaans: Bergkatbos, Bergkatdoring, Fynkatbos, Katdoring, Langbeenkatdoring; Zulu: Ibutha, Setswana: Lesitwana [13].
Synonym: *Protasparagus laricinus (Burch*.) Oberm.

4.1 Historical aspects

Asparagus laricinus Burch. is a very hardy, evergreen, shrubby Asparagus with fine, feathery foliage and silvery, zigzag branchlets. It has myriads of tiny white, nectar-rich flowers that develop during spring and summer. These flowers are fragrant and attract insects and birds. Birds in the garden are attracted by its bright red and black berries. It may be grown in the sun or semi-shade and is a very useful plant for a security hedge as it is extremely spiny. It is fire-resistant and, if the stems burn, it shoots quickly from the base. The new shoots can be eaten as a vegetable. It grows in sun or shade and in all types of soil except water-logged soils. It can grow up to 1.5 m. *Asparagus laricinus Burch*. is native to Botswana and South Africa, Lesotho and Swaziland. They are used to treat tuberculosis, sores, red water, uterine infection, general alignments, umbilical cord inflammation, and serve as a diuretic.

4.2 Phytochemical active principals

Roots and leaves of *Asparagus laricinus Burch*. had tannins, saponins, terpenes, steroids. However, only roots showed the presence of alkaloids [14], while leaves are devoid of alkaloids [15]. The leaves further had flavonoids, glycosides, steroids and carbohydrates. The stems are rich in saponins, tannins, and flavonoids, with a lack of steroids, glycosides and carbohydrates [15]. The *Asparagus laricinus Burch*. aqueous roots extract contained 4.2 g/l GAE (Gallic acid equivalent) total phenolic content, while leaves and stem aqueous extract showed the phenolic concentration of 0.572 mg/GAE and 0.277 mg/GAE, respectively. It was apparent that leaves had more phenolic content than the stem, and this was supported by the number of active phytochemicals identified from both parts of the plant. Fuku et al. [14] isolated and identified three compounds from the *Asparagus laricinus Burch*. roots: indole-3-carbinol, α-sitosterol and ferulic acid.

4.3 Pharmacological actions

Secondary metabolites produced by plants for plant protection do not only benefit plants, but they also have health benefits for human beings. These compounds

result in antimicrobial medicines [16], anti-inflammatory drugs, anticancer drugs, and plant-based anti-oxidants. Phytochemical screening was performed on the leaves and roots of *Asparagus laricinus Burch.*, and parts had tannins, saponins, terpenes, and steroids. However, only roots showed the presence of alkaloids. It was also shown that flavonoids which are known to have an ability to inhibit microbial growth also scavenge antioxidants. The leaf extract contained steroids, these being important compounds as sex hormones. Both leaves and stem extracts showed that they contain saponins, which ultimately has a suppressive effect on inflammation [17]. This is a main reason why *Asparagus laricinus Burch.* is used in traditional medicine. *In vivo* anti-inflammatory activity studies of this plant are being conducted in the Unit of Drug discovery, CUT [18].

Tannins are generally found in most plant parts: bark, wood, leaves, fruits and roots, and can have a toxic effect on filamentous fungi, yeasts and bacteria [19]. No alkaloids were found in this study. Leaf extracts further showed positive antibacterial activity on *S. aureus*, *S. saprophyticus*, *E. cloacae* and *B. subtilis*. Inhibition of *Staphylococcus aureus* by the *Asparagus laricinus Burch.* plant extract demonstrates huge potential for using this plant to extraction in the treatment of microbial infections, especially in the light of the growing antibiotic resistance in micro-organisms. The presence of phenols correlates with the antibacterial and antioxidant activities of the leaf extract of *Asparagus laricinus Burch*., as demonstrated by Ntsoelinyane and Mashele [15].

In recent times, there has been a growing interest in finding antioxidants which occur naturally to replace synthetic antioxidants, many of which are being restricted due to their carcinogenicity [20, 21]. Free radical scavenging molecules such as flavonoids, tannins, alkaloids, quinones, amines, vitamins and other metabolites have anti-inflammatory, anti-carcinogenic, antibacterial and antiviral activities [22]. *Asparagus laricinus Burch.* aqueous extracts of roots and leaves showed positive antioxidant activity with DPPH assay [15]. Flavonoids contain anion radicals and produce membrane bound enzymes [23]. This could be the reason for the mechanisms of antioxidative action of *Asparagus laricinus Burch.* leaf extract. The antioxidant that is found in the plant extract may also be due to polyphenols as phenolics being present [24]. The aqueous leaves extract of *Asparagus laricinus Burch.* produced significant activity as an antioxidant, and this could be due to the presence of ferulic acid; and as it is a known to protect cells from oxidative stress.

Using the Ames test on *Salmonella typhimurium* strains*:* TA97, TA98, TA100 and TA102 without any metabolic activation, Mashele and Fuku [25] evaluated the mutagenic and antimutagenic properties of the aqueous root's extracts of this plant. The extract was non-mutagenic towards all strains, had moderate inhibitory effect on TA100, and had low inhibitory effects on TA102 and TA97 [7]. Root aqueous extract showed an indirect mutagenic effect toward TA102 after metabolic activation, but not in TA97, TA98 and TA100. However, it was found that the Ames test, without S9 (liver extract of a rat, hamster or human) metabolic activation, could only detect direct mutagens, while S9 metabolic activation allowed the detection of indirect mutagens which were mostly caused by conjugation reactions of metabolic oxidation systems. Cytotoxicity activity on Vero cells was also elucidated. The cytotoxicity tests indicated no cytotoxic effect below 500 μg/ml concentration of the *A. laricinus Burch.* aqueous extract [7].

The phytoconstituents detected from *Asparagus laricinus Burch.* may have caused the cytotoxic activity, although their precise mode of action is poorly understood. Only a few compounds were isolated from the roots of *Asparagus laricinus Burch.*: indole-3-carbinol, α-sitosterol and ferulic acid. β-Sitosterol have numerous therapeutic and chemo-preventive uses in the medical field [26, 27]. Prostate cancer is being treated by Indole-3-carbinol [28]. Anticancer activity on breast (MCF7), renal (TK10) and melanoma (UACC62) using roots aqueous and ethanol extracts was

shown by Mashele and Kolesnikova [29], who revealed that ethanol extracts were very active while aqueous extracts were weakly active. However, ethanol roots extract only showed the presence of tannins while the aqueous roots extract showed a number of active phytochemicals. These results should be investigated further to elucidate the aforementioned difference. It may be that the presence of other active compounds somehow affected the ability of tannins by neutralizing their activity in the aqueous root's extracts. Another possibility is that active compounds from the roots were present and these were missed during the phytochemical screening of this plant.

Mokgawa evaluated possible toxic effects of dried roots, stem and leaves of *Asparagus laricinus Burch.* extracts using Sprague Dawley rats as animal models [18]. Histological evaluation could not reveal any pathological changes in both aqueous and ethanoic extracts across all levels of dosages. Full blood count results could not point in the direction of toxicity, adverse effects or hazards as indicated by statistically similar results between the exposed and unexposed groups, using both aqueous and ethanol extracts at different concentrations [18]. According to results obtained by Mokgawa, histological assessment has proven that both aqueous and ethanolic extracts of *Asparagus laricinus Burch.* had no detrimental or adverse effects on vital organs of Sprague Dawley rats [18]. Tissue damage, lesions or inflammation were not observed on the kidney, liver or spleen of treatment groups in comparison to the control group. The pattern was observed across increasing doses of aqueous and ethanolic extracts. It was, therefore, concluded that toxicological evaluation of *Asparagus laricinus Burch.* extracts may be considered relatively free of toxicity when given orally, because it did not cause death, damage or inflammation to tissues, nor did it produce any remarkable biochemical and hematological adverse effects in both male and female Sprague Dawley rats [18]. Further studies may also be conducted to demonstrate *in vivo* efficacy against cancer as studies to date were done using cell lines *(in vitro* studies).

4.4 Reflections and future recommendations

Only preliminary screening of phytochemicals was done on crude extracts. Isolation of active pure compounds was only done on roots (3 compound identified) and not on leaves, even though leaves showed so much active compounds. This compound identification still needs to be done and testing of them has not been done either. Both leaves and roots extracts showed the presence of saponins. However, the identification and isolation of those specific saponins has not been done. Intensive work still needs to be performed regarding the mutagenicity or genotoxicity of the plant extracts for the confirmation of the safety of *Asparagus laricinus Burch.*, as the root cytotoxicity results were promising, while the safety of the leaves also needs to be investigated. The toxicological study of the roots of *Asparagus laricinus Burch.* confirmed that the plant extract did not cause any harm *in vivo* and can thus be considered as non-toxic. However, the *in vivo* anticancer activity of the root extract has not been done in order to confirm or corroborate the results obtained in the screening study that was conducted. Both *in vitro* and *in vivo* anticancer, cytotoxicity and mutagenicity studies still need to be done on the leaf extract. The ability of the crude extract of this plant as an antibacterial agent was confirmed, and findings supported the use of this plant against infections. However, not all ethnobotanical claims of this plant have been confirmed as the anti-TB activity, anti-inflammatory activity and its ability as a diuretic still needs to be elucidated.

5. *Asparagus africanus Lam.*

Vernacular names
English: Wild Asparagus climbing asparagus fern, bush asparagus;

Afrikaans: Haakdoring, Katdoring Wag-'n-bietjie, Wag-n'-bietjie, Wag-'n-bietjie-doring; Xhosa: Ubulawu Ubumhlope, Umthunzi; Zulu: Isigoba, Isigobo; Sesotho: Lelala-tau-le-leholo, Leunyeli; Banda: ngorozo; Kirundi: imburabano, umunsabe; Maasai: embere e papa; Afeen Oromo: Seriti.

Synonym: *Protasparagus africanus* (Lam.) Oberm.

Scientific classification

Kingdom: plantae; **Subfamily:** asparagoideae; **Genus:** Asparagus—asparagus; **Order:** asparagales; **Species:** *Asparagus africanus* Lam.—African Asparagus; **Family:** Liliaceae.

5.1 Historical aspects

Asparagus africanus Lam., commonly known as African Asparagus, is a monocot. *Asparagus africanus Lam* (Liliaceae) is an erect armed herb that grows to a height of up to 6 m. The plant is found in many parts of tropical Africa and can grow between 700 and 3800 m above sea level. The fruit consists of a rounded berry which has a width of about 5–6 mm and it contains only one seed. It starts off as green colored but eventually becomes orange and somewhat shriveled as it matures. They grow during most parts of the year.

Their roots are traditionally used for: the relief of pain, rheumatism and chronic gout, hematuria, hemorrhoids, headache, backache, stomach pain, sore throat and otitis. It is also used to treat malaria, central nervous system related conditions, tuberculosis, venereal diseases and as an aid during childbirth [30].

5.2 Phytochemical active principals

The main contents of the plant are carbohydrates and saponins which have small quantities of flavonoids and tannins. Three steroidal saponins were isolated from the roots of *Asparagus africanus Lam.* [31]. Two compounds namely 2 beta-, 12 alpha-dihydroxy-(25R)-spirosta-4, 7-dien-3-one, lignan (+)-nyasol, and (Z)-(+)-4,4-(3-ethenyl-1-propene-1,3-diyl) bisphenol) were isolated by Oketch-Rabah et al. [32] from *Asparagus africanus Lam* roots.

5.3 Pharmacological actions

In traditional medicine, *Asparagus africanus Lam.* is used for treating headaches, backaches, and stomach pains and also is used to assist in childbirth and for hematuria, hemorrhoids, malaria, leishmaniasis, bilharziasis, syphilis, and gonorrhea [32, 33]. External application of the root is used for the relief of pain, rheumatism and chronic gout [34]. It is further used a diuretic, for sore throats and otitis [35]. The focus of many anti-infective drugs as well as alternative sources of antimalarial agents in various parts of the world has been on the use of medicinal plants [36–38]. During *in vitro* studies on extracts from the root of *Asparagus africanus Lam.*, it has been found that they can be used as a counter activity against four different malaria schizont strains [32].

Oketch-Rabah et al. [32], were able to isolate two antiprotozoal compounds, a sapogenin (muzanzagenin) and lignan ((+) nyasol), which they reported to be responsible for the antimalarial activity. Even though this plant has displayed promising antiplasmodial activities, no remarkable in vivo studies have been found up to now which can strengthen the preclinical study profile. There has been only one report by Dikasso et al. [39], on the in vivo anti-malarial activity of extracts of hydroalcoholic from *Asparagus africanus Lam.* in mice which have been infected with *Plasmodium berghei* [39]. The extract displayed parasite suppressive effects on

P. berghei infected rats in a dose dependent manner. However, the effects on Packed Cell Volume and body temperature observed were inconclusive [39].

Asparagus species are known to have steroidal saponins as their major bioactive constituents. The presence of saponins and carbohydrates from *Asparagus africanus Lam.* showed significant analgesic and anti-inflammatory activities as reported by Hassan et al. [30]. Saponins are also known to have broad spectrum of pharmacological and antimicrobial activities [40]. According to Madikizela et al. [41] *Asparagus africanus Lam.* leaves showed very active antimycobacterial activity on *Mycobacterium aurum A+*, with moderate antibacterial activity against *Klebsiella pneumonia* [41], due to the saponins present in this plant. The methanolic extract obtained from the roots of *Asparagus africanus Lam.* were considered to be none toxic as there was no mortality caused on rats after a dose of 5000 mg/kg was administered by mouth [42], and these findings were corroborated by Kedebe et al. [43], using hydro-alcoholic extracts of *Asparagus africanus Lam.*

Three steroidal saponins, which are the most probable components of estrogen, have been separated from the roots of *Asparagus africanus Lam.* [31], and *Asparagus officinalis L.* that was reported to have uterine contractile properties. Steroidal saponins have been found to be one of the active principles of the majority of anti-fertility agents [44, 45]. *In vitro* and *in vivo* studies of the extracts of ethanol of leaves and roots *of Asparagus africanus Lam.* displayed the ability of both extracts to have a potential acetylcholine effect on uterine contraction [46]. These results suggested the possibility of interaction of the extracts with endogenous acetylcholine to induce an abortifacient effect. Thus, this plant should not be used during pregnancy because of the possibility of unintentionally abortion.

Saponins isolated from this plant have displayed anti-inflammatory activities against several experimental types of inflammation in mice and rats [47]. During the initial inflammation process, histamine and serotonin are released resulting in inflammation signs such as edema, pain, redness and heat. In a study by Kebede et al. [43], rats were injected with edemagenic agents to trigger edema (sign of inflammation), root extracts of *Asparagus africanus Lam.* were administrated to the rats, and the expected edema was not observed as the plant inhibited an antihistaminic agent. The extract activity was then found to be more pronounced in the first phase of the rat edema (within 90 min), thus making it possible for the extract to contain antihistaminic activity [43].

5.4 Reflections and future recommendations

The roots of this plant are traditionally used for the relief of pain, rheumatism and chronic gout, hematuria, hemorrhoids, headache, backache, stomach pain, sore throat and otitis. In addition, they are used to treat malaria, central nervous system related conditions, tuberculosis, venereal diseases, and aid in childbirth. *In vitro* and *in vivo* studies performed on this plant confirmed the ethnobotanical claims of this plant and active compounds were isolated and identified. However, the mechanism of action of these compounds as anti-inflammatory, antimycobacterial, antiplasmodial and anti-infertility agent has not been performed to date. Toxicological studies showed that the plant was not toxic. However, its ability to induce mutagenicity was not determined.

6. *Asparagus officinalis L.*

Vernacular name

English: Asparagus, Garden asparagus, White asparagus, Sparrow grass and Common asparagus; **Arabic:** Ehlilaj aswad, Helion, Dhagboth, Akla, al theeb;

Chinese: Shi diao baiGerman: Spargel; **French:** Asperge; **Italian:** Asparagio; **Japanese:** Oranda-kiji-kakushi; **Portuguese:** Espargo; **Spanish:** Espárrago, Esparraguera, and **Swedish:** Sparris.

Synonyms: Asparagus caspius Hohen.; Asparagus longifolius Fisch. ex Steud.; *Asparagus officinalis* var. caspius (Hohen.) Asch. & Graebn.; *Asparagus officinalis subsp. officinalis*; Asparagus polyphyllus Steven ex Ledeb.

Scientific classification

Kingdom: plantae; **Subfamily:** asparagoideae; **Clade:** Angiosperms; **Genus:** Asparagus—asparagus; **Order:** asparagales; **Species:** *Asparagus officinalis L.*—garden asparagus; **Family:** Asparagaceae.

6.1 Historical aspects

Asparagus officinalis L. is a perennial herb which grows to a height of between 60 and 150 cm. It has thick swollen root stock. Its stem is multi-branched, with 2–6 needle-like shoots in whorls. Flowers are wide (4–6 mm), 6-lobed, perianth regular with a whitish–greenish yellow. Leaves are rudimentary, scale-like and its axillary shoots are needle-like and whorled. Fruits are round, initially green and, when ripe, forms an orange, 6–10 mm wide berry [17, 49]. The plant was distributed in Central and Southern Europe, the Middle East, Western Siberia and Northern Africa. It was then cultivated in many places. It is now distributed in Eastern Africa, Asia, Europe, Northern and Southern America [48]. Asparagus stalks are commonly eaten as a vegetable. Roots and seeds have been used as a treatment for various illnesses and as a diuretic, despite the lack of clinical evidence.

6.2 Phytochemical active principals

Chemical constituents of *Asparagus officinalis L.* contain steroid saponins including asparagosides A, B, D, F, Ge3w2q H, I, the bitter steroid saponins, amino acids, fructans (asparagose and asparagosine), ferulic acid and flavonoids (quercetin, rutin, hyperoside, and isoquercitrin) [36, 49, 50]. Shao et al. [51], further isolated two oligofurostanosides *Asparagus officinalis L.* seeds, and their structures were identified as 3-O-[alpha-Lrhamnopyranosyl-(1→2)-(alpha-L-rhamnopyranosyl-(1→4))-beta-D-glucopyranosyl]-26-O-[beta-D-gluco pyranosyl]-(25R)-22 alpha-methoxyfurost-5-ene-3 beta,26-diol(methyl protodioscin), and with the corresponding 22 alpha-hydroxy analogs (protodioscin). New asparagusic acid anti-S-oxide methyl ester (a new acetylenic compound) and asparagusic acid syn-S-oxide methyl ester, 2-hydroxyasparenyn {3,4″-trans-2-hydroxy-1-methoxy-4-[5-(4-methoxyphenoxy)-3-penten-1-ynyl]-benzene}, and eleven known compounds, [asparenyn, asparenyol, (±)-1-monopalmitin, ferulic acid, 1,3-O-di-p-coumaroylglycerol, 1-O-feruloyl-3-O-p-coumaroylglycerol, blumenol C, (±)-epipinoresinol, linoleic acid, 1,3-O-diferuloylglycerol, and 1,2-O-diferuloylglycerol, were separated from an ethyl acetate-soluble fraction of the methanol extract of the aerial parts of *Asparagus officinalis L.* [48]. Two major anthocyanins (A1 and A2) were also separated from peels of the spears of *Asparagus officinalis L.*. However, A1 was identified as cyanidin 3-[3″-(O-beta-d-gluco pyranosyl)-6″-(O-alpha-l-rhamnopyranosyl)-O-beta-dglucopyranoside], while A2 was recognized to be cyanidin 3-rutinoside, which was found to be in higher plants [48].

Sun et al. [52], recognized a new steroidal saponin, yamogenin II, with a unique aglycone moiety, and a structure of (25S)-spirostan-5-ene-3β-ol-3-O-α-Lrhamnopyranosyl-(1,2)-[α-L-rhamno pyranosyl-(1,4)]-β-D-glucopyranoside from the dried stems of *Asparagus officinalis L.*. Furthermore, more saponins

were isolated from the plant included (25R)-furost-5-en-3β,22,26-triol-3-O-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside]-26-O-β-D-glucopyranoside, (25R)-furostane-3β,22,26-triol-3-O-[α-L-rhamnopyranosyl-(1→4)-β-Dglucopyranoside]-26-O-β-D-glucopy ranoside, and (25S)-furostane -3β,22,26-triol-3-O-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside]-26-O-β-D-glucopyranoside, and 3-O-[{α-L-rhamnopyranosyl-(1→2)} {α-L-rhamnopyranosyl-(1→4)}-β-Dglucopyranosyl]-(25S)-spirost-5-ene-3β-ol [53]. Nutritional analysis showed that the plant contained water 93.5%, total protein 1.91%, fat 0.16%, carbohydrates 2.04%, total dietary fiber 1.31%, and total nitrogen 0.31% [54]. The amino acid and mineral contents were found to be much higher in the leaves than the shoots [54].

6.3 Pharmacological actions

Asparagus officinalis L. is believed to have laxative, diuretic and contraceptive effects, and as a remedy for neuritis, rheumatism, cancer, toothache relieve, face acne lesion, as well as to stimulate hair growth [55]. According to research findings, the aqueous extract of *Asparagus officinalis L.* showed some antidiabetic effect after diabetic rats were treated with the extract, and their elevated blood glucose was suppressed [56]. The extract further displayed dangerous antioxidant activity in *in vitro* and *in vivo* assays. *Asparagus officinalis L.* crude saponins from the shoots (edible part) of asparagus, were found to have antitumor activity as they promoted the growth of HepG2 cells, and of human leukemia HL-60 cells in a way which caused it to become dose-dependent.

Shao et al., separated two oligofurostanosides from the seeds of *Asparagus officinalis L.* with cytotoxic activity [51]. They repressed the growth of human leukemia HL-60 cells in culture and macromolecular synthesis in a manner which promoted dose-dependence. Saponins from old stems of asparagus (SSA) exerted potential repressive activity on tumor growth and metastasis of breast, colon and pancreatic cancer cells. Sakaguchi et al. [57], found that anthocyanins A1 and A2 separated from the spears of *Asparagus officinalis L.* were found to act as antioxidants. The saponin fraction of the *Asparagus officinalis L.* exerted antifungal activity [58, 59]. The intake of asparagus also improved antioxidant status (superoxide dismutase and catalase enzymes) and prevented lipid peroxidation [60]. This corroborated with the findings by Hafizur et al. [56]. The hypolipidemic effect of n-butanol extract from asparagus by-products was evaluated in mice fed a high-fat diet, and the results were positive [56].

The antibacterial potential of the ethanolic extracts was determined against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus cereus*, and the activity was seen only on *Escherichia coli*, while there was no antimicrobial activity in the same concentration against other tested pathogenic bacteria [61]. The taking in of asparagus alleviated some clinical symptoms (stool consistency, stool blood, and spleen hypertrophy) during active colitis. Other pharmacological effects of *Asparagus officinalis L.* were anti-fatigue effects, enhanced anoxia tolerance, induced analgesia and improved memory, and decreased the prevalence of lipid peroxide in plasma, liver and brains of the rats [62].

Jang et al. examined *Asparagus officinalis L.* for its inhibitory effects against both cyclo-oxygenase-1 and -2, thus having anti-inflammatory potential [48], due to linoleic acid identified as the most active compound in the plant [48, 54]. Aqueous extract of *Asparagus officinalis L.* resulted in relaxation of spontaneous contractions of separated smooth muscle of rabbit jejunum [57]. *Asparagus officinalis L.* also induced diuretic effects [63].

6.4 Reflections and future recommendations

Roots and seeds have been used as a treatment for various illnesses and as a diuretic, despite the lack of clinical evidence. So many active chemical constituents were isolated and identified. However, not all pharmacological activity of those isolated compounds was done. *In vivo* anticancer activity is desirable to confirm the in vitro findings. Studies have claimed that *Asparagus officinalis L.* has other pharmacological effects which were not reported as ethnobotanical uses of this plant, such as anti-fatigue effects, enhanced anoxia tolerance, induced analgesia and improved memory, as well as the decreased contents of lipid peroxide in plasma, liver and brains of the rats. However, this was not linked to the active compound present from the plant that could be responsible for those pharmacological activities.

7. *Asparagus racemosus Willd.*

Vernacular names

English name: Wild Asparagus, Indian Asparagus, Hundred Roots, *Asparagus racemosus Willd*; **Hindi:** Satavar.

Synonyms: Indeevari, Sukshamapatra, Bahusuta, Shatmooli, Narayani, Bhiru, Virya, Madabhanjani, Shatpadi, Shatvirya.

Scientific classification

Kingdom: Plantae; **Sub-family:** Asparagoideae; **Clade:** Angiosperms; **Genus:** Asparagus; **Order:** Asparagales; **Species:** *Asparagus racemosus Willd*.; **Family:** Asparagaceae.

7.1 Historical aspects

Asparagus racemosus Willd. has been used traditionally for ages as a female reproductive tonic as it prevents abortion and promotes the health of the mother and growing fetus when used in antenatal care. This plant also increases lactation and is useful for the treatment of gynecological diseases when used in postnatal care [64]. Research has supported other reported ethnobotanical uses of this plant for female reproductive system-related health issues. This plant can be described as a climbing shrub which is thorny and has woody stems. The leaves become minute scales and spines. Fruits are round and are a purple black color. Roots are succulent and tuberous and taper at both ends. It is distributed throughout India, and almost commonly found in areas up to an altitude of 4000 feet in the Himalayas and in Ceylon [64]. Tuberous roots of the plant are the parts used [65]. The tubers are eaten as a sweetmeat. The root contains juice which, when fresh used with honey as a demulcent in bilious dyspepsia or diarrhea. It is used in the preparation of medicated oils for external application to sufferers of nervous and rheumatic infections, and urinary tract infections [64].

7.2 Phytochemical active principals

The main active sections of *Asparagus racemosus Willd.* are steroidal saponins (Shatavarins I–IV), which are the phytoestrogen compounds which are present in the roots of this plant [66–69]. Shatavarin IV is a glycoside of sarsasapogenin having two molecules of rhamnose and one molecule of glucose. It also contains mucilage and starch. The 8-methoxy-5,6,4′-trihydroxyisoflavone, a new isoflavone, was separated from the roots of *Asparagus racemosus Willd.* by Saxena and Chaurasia [70]. A novel oligospirostanosid 1,3-O-[α-L-3-rhamnopyronosyl-(1 → 2)-α-L-rhamnopyronosyl(1 → 4)-O-β-D-glycopyranosyl]25(S)-5β-Spirostan-3β-ol also known

as immunoside was isolated, and it was biologically evaluated as an immunomodulatory agent [71]. Wiboonpun et al. [72], isolated a new antioxidant compound named Racemofuran, together with known compounds asparagamine A and racemosol. Three steroidal saponins (Racemosides A, B and C) were also isolated from the methanolic extract of fruit of *Asparagus racemosus Willd.*. Polycyclic alkaloid like asparagamine A, and disaccharide in roots are also reported in other research studies [73–75]. *Asparagus racemosus Willd.* is also reported to have alkaloids, proteins, starch, tannin, flavonoids, glycosides of quercetin, rutin and hyperoside in roots and flowers [76]. Quercetin 3-glucuronide is present in leaves [77]. There were few trace minerals like zinc (53.15), manganese (19.98), copper (5.29), and cobalt (22.00 microgram per gram) together with calcium, magnesium, potassium, zinc, and selenium [68, 78]. The callus culture of *Asparagus racemosus Willd.* has shown synthesis of sarsasapogenin [79]. However, no report has been received on the chemistry of the contents of its fruit.

7.3 Pharmacological actions

The healing qualities of *Asparagus racemosus Willd.* are useful to a wide array of ailments. Ayurvedic (Indian traditional medicines database) literature considers it a strong drug which can improve memory intelligence and physical strength and maintain youthfulness [48, 80]. *Asparagus racemosus Willd.* can also be used as a uterine sedative. In addition, a glycoside, Shatavarin 1, separated from the roots of *Asparagus racemosus Willd.* has been found to be responsible for the competitive blocking of oxytocin-induced contractions, *in vitro* as well as *in vivo* [19, 64]. In India, it is recognized as a female tonic. In spite of being a rejuvenating herb, it is recognized as being used in female infertility, as it increases libido, is able to cure inflammation of sexual organs, and can be used to moisten dry tissues of the sexual organs. It further enhances folliculogenesis and ovulation, prepares the womb for conception, prevents miscarriages, acts as post-partum tonic by increasing lactation, normalizing uterus and changing hormones. It is also used in leucorrhoea and menorrhagia [65, 81].

The roots of *Asparagus racemosus Willd.* have been described as bitter-sweet, emollient, cooling, nervine tonics, preventing constipation, and may be used as an aphrodisiac, diuretic, carminative and antiseptic [82]. The powdered dried root exhibits galactagogic properties as there was an increase in milk secretion during lactation [83]. While active it has resulted in the action of released corticosteroids or an increase in prolactin. The other study also agreed with the galactagogic effect of this plant, because an alcoholic extract of *Asparagus racemosus Willd.*, increased the prolactin levels in female rats (Kumar et al., 2008). It served as a potential stimulator for early restoration of milk production without any adverse effects [84]. The juice of fresh roots of *Asparagus racemosus Willd.* is recommended for duodenal ulcers [85]. The plant can also be used to treat skin diseases, wounds and as a demulcent in dyspepsia [64]. The aqueous root extract possesses immunoadjuvant potential [37]. In the roots of *Asparagus racemosus Willd.* antioxidant and anti-ADH (Antidiuretic hormone) activity were found to be present [72, 86], and there was antitumor and anticancer activity [87, 88].

Asparagus racemosus Willd. displayed a preventative action on DMBA (-7,12-Dimethylbenz[a]anthracene) induced mammary carcinogenesis in rats. Rats which were fed on *Asparagus racemosus Willd.* diet displayed a decline in both tumor incidence and mean number of tumors per tumor bearing animal [76]. Studies also showed that the plant has anti-ulcerogenic activity [89], anti-inflammatory activity and antimicrobial activity [90]. Antimicrobial activity was used against *Escherichia coli*, *Shigella dysenteriae*, *Shigella sonnei*, *Shigella flexneri*, *Vibrio cholerae*, *Salmonella typhi*, *Salmonella typhimurium*, *Pseudomonas pectida*, *Bacillus subtilis* and *Staphylococcus aureus*, and sensitivity was observed in all strains under study [90]. As *Asparagus racemosus Willd.* is believed to have no antibacterial action, protection

offered by *Asparagus racemosus Willd.* against sepsis by altering function of macrophages, shows its potential immunomodulatory property [91, 92].

Methanolic extract of roots displayed important antitussive activity on sulfur dioxide-induced coughs in mice [93]. An aqueous solution of the crude alcoholic extract of the roots displayed significant antiprotozoal activity against *Entamoeba histolytica in vitro* [94]. An examination was made on rat liver mitochondria for the possible antioxidant effects of crude extract and purified aqueous fraction of *Asparagus racemosus Willd.* against member damage induced by the free radicals generated during gamma radiation [86, 95]. It also raised the urinary concentration of magnesium, which is considered as one of the suppressors of crystallization [96]. Aqueous and butanol fractions displayed less prominent effects on the release of, especially at lower glucose concentration [97]. *Asparagus racemosus Willd.* further showed the potential of anti-HIV, (Human immunodeficiency virus) and its active principles are being investigated [98].

It can be concluded that *Asparagus racemosus Willd.* has immense importance in the folk medicine. In Ayurveda, *Asparagus racemosus Willd.* has been described as perfectly safe for long term use, even during pregnancy and lactation. To support this theory, an *in vivo* study was conducted and the systemic administration of higher doses of all extracts did not display any abnormality behavioral patterns in mice and rats [99], neither did it produce mortality even up to higher oral dosages of 64 g/kg [100].

7.4 Reflections and future recommendations

Intensive research has been performed on the active compounds from this plant. The plant was reported to have anticancer activity. However, cell lines used, and solvents used were not mentioned. While antitumor activity was reported on the mammary carcinogenesis only, more research using other cell lines is required to explore the antitumor and anticancer activity of this plant root's extract. The potential of this plant as cancer inducing agent has not been thoroughly elucidated as *in vivo* studies showed the safety of this plant. However, the exposure duration was not long enough. It is well known that the process of carcinogenesis is very slow, thus the adverse effects after 10 years of using *Asparagus racemosus Willd.* has not been determined scientifically. It will, therefore, be important to understand the mutagenicity of the plant before we can conclude that it is hundred percent safe. Mechanism of action of the active compounds from this plant and human trials are required, as different metabolic reactions in humans may influence the activity of the compound.

8. *Asparagus densiflorus* (kunth) jessop

Vernacular names

Sprenger's asparagus fern, bushy asparagus, asparagus fern and smilax. Inwele in Zulu.

Synonyms: Asparagopsis densiflora Kunth, Prot*asparagus densiflorus*.

Scientific classification

Kingdom: Plantae; **Subfamily:** Asparagoideae; **Clade:** Angiosperms; **Genus:** Asparagus; **Order:** Asparagales; **Species:** A. densiflorus; **Family:** Asparagaceae.

8.1 Historical aspects

South African asparagus was observed for the first time in an illustration from 1686, while plants from the 'Sprengeri' group began to be cultivated as early as 1888. Today they are grown in all parts of the word, and are hardy, drought-tolerant and quite salt-tolerant plants which are used for plants foliage and as garden plants. The South

African Asparagus species and the European Asparagus species are related, and they display a very interesting structure botanically. They do not have true leaves at all, but these are actually cladodes which may actually be modified branches, while the spines are formed from modified branches or from modified leaves. The most popular forms form part of the emerald ferns of the *Asparagus densiflorus (Kunth) Jessop* 'Sprengeri' group [101]. They form large cushions which have long, arching stems more or less densely covered with dark green, leaves which have the appearance like needles. The plant appears fernlike, but its flowers and fruit clearly place it among the angiosperms.

Asparagus densiflorus (Kunth) Jessop is a delicate, fern-like perennial plant which has arching stems which grow up to 1 m long and have a scrambling habit. A large cushion of dark green needle-like leaves is formed by the plant. It is often proved to be of use for medicinal purposes and can also be used as ground cover in partial or light shade, but it flourishes in full sun if watered regularly. It has very small, hardly observable spines which is unlike most Asparagus fern species. It is also attractive as an indoor or patio plant in large containers or hanging baskets. Unlike most Asparagus fern species, it only has very small, hardly noticeable spines. The roots of the plants are extensive which contain numerous grape sized tubers. These provide food in nature for extensive periods of drought in summer. The root system is used extensively for binding soil on slopes [101].

8.2 Phytochemical active principals

The phytochemical analysis of the aqueous and ethanolic extracts was carried out for the presence of flavonoids, tannins, phenolics, saponins, cardiac glycosides, terpenoids, quinones, amino acids, carbohydrates and alkaloids [46]. Both extracts showed a lack of amino acids, and were found to contain flavonoids, tannins, phenolics, saponins, cardiac glycosides and carbohydrates. In addition, the ethanol extract was found to contain terpenoids and alkaloids, whereas the aqueous was found to contain quinones. Estimated flavonoid and phenolic content of *Asparagus densiflorus (Kunth) Jessop* aqueous plant extracts were 900 and 380 μg/ml [102].

8.3 Pharmacological actions

An infusion of the plants' leaves may be used to treat pain in the abdomen, as a general tonic and to boost immunity. Further it may be used as a cleansing agent to rid the body of "poison" and "dirty blood". Thrust and ulcers in the mouth associated with HIV may also be treated by this plant. According to Davids et al. [103], traditional health practitioners (THPs) reported that *Asparagus densiflorus (Kunth) Jessop* is one of the "strongest" plants used for HIV. Moreover, according to Singh et al. [102], *Asparagus densiflorus (Kunth) Jessop* is considered as one of the ethnomedicinal plants. However, its ethnomedicinal actions had never been discussed. No literature was found on the correlation or link of phytochemical active compounds and pharmacological activity of this particular plant. *Asparagus densiflorus (Kunth) Jessop* aqueous and ethanoic leaf structures were screened for their antibacterial activity against *Enterobacter aerogenes*, *Clostridium perfringens* and *Salmonella typhimurium*. However, it was found that the aqueous extract showed a potential to inhibit growth of all three selected micro-organisms, while the ethanol extract inhibited only the growth of *Enterobacter aerogenes* [46].

8.4 Reflections and future recommendations

Only the preliminary phytochemical screening was performed on the crude extract of this plant, but no deeper research has been done on the activity of this plant in order to confirm its ethnobotanical claims as a plant used to treat thrush,

ulcers in the mouth as well as for HIV. Thrush results from an overgrowth of normal flora in the mouth. The anti-fungal activity of this plant has not been studied yet, together with its anti-HIV activity, as the plant has been reported to be used ethnobotanically for HIV. Further studies to isolate the active compounds, elucidate the safety of this plant and to fully confirm its pharmacological activity are needed.

9. Conclusions

The genus Asparagus is an herbaceous plant comprising approximately 150 species around the world, and consisting of herbs, shrubs and vines. Asparagus species possess bioactive properties, such as: antioxidant, anti-inflammatory, antibacterial, antihepatotoxic, immunostimulant, and reproductive agents. In the present review study, five native Asparagus species (namely: *Asparagus laricinus*

Plant species	Active phytochemicals	Mode of Action	Beneficial role in human health
Asparagus laricinus Burch	*Asparagus laricinus Burch* roots and leaves have tannins, saponins, terpenes and steroids. The stems are rich in saponins, tannins, and flavonoids, with a lack of steroids, glycosides and carbohydrates. Only roots have alkaloids and indole-3-carbinol, α-sitosterol and ferulic acid were isolated from the roots	*Asparagus laricinus Burch*. has flavonoids that inhibited microbial growth and scavenged antioxidants. Both leaves and stem extracts showed that they contain saponins, which ultimately has a suppressive effect on inflammation. This plant was not toxic when administered orally to rats, it was non-mutagenic, however, root aqueous extract showed an indirect mutagenic effect toward *Salmonella typhimurium* TA102 strain after metabolic activation, but not in TA97, TA98 and TA100 strains. β-Sitosterol isolated from this plant is known for its therapeutic and chemo-preventive uses in the medical field, while prostate cancer is being treated by Indole-3-carbinol. This plant had anticancer activity on breast (MCF7), renal (TK10) and melanoma (UACC62)	*Asparagus laricinus Burch*. is used to treat tuberculosis, sores, red water, uterine infection, general alignments, umbilical cord inflammation, and serve as a diuretic
Asparagus africanus Lam.	The main contents of the plant are carbohydrates, saponins, flavonoids and tannins. Two compounds namely; 2 beta-, 12 alpha-dihydroxy-(25R)-spirosta-4,7-dien-3-one, lignan (+)-nyasol, and (Z)-(+)-4,4-(3-ethenyl-1-propene-1,3-diyl) bisphenol) were isolated from *Asparagus africanus Lam* roots, but their mode of actions has not been documented	This plant has acetylcholine effect on uterine contraction and antiplasmodial activity. Two antiprotozoal compounds; a sapogenin (muzanzagenin) and lignan ((+) nyasol), which were reported to be responsible for the antimalarial activity, have been isolated from *Asparagus africanus Lam*. The presence of saponins and carbohydrates from *Asparagus africanus Lam.* showed significant analgesic, anti-inflammatory activities and antimicrobial activities	*Asparagus Africanus Lam.* is used for treating headaches, backaches, stomach pains, malaria, to treat sexual transmitted infections and also used to assist in childbirth. It is also used to treat central nervous system related conditions, tuberculosis, venereal diseases and as an aid during childbirth. External application of the root is used for rheumatism and chronic gout. It is further used a diuretic, for sore throats and otitis

Plant species	Active phytochemicals	Mode of Action	Beneficial role in human health
Asparagus officinalis L.	*Asparagus officinalis L.* contain steroid saponins, amino acids, fructans, ferulic acid and flavonoids (quercetin, rutin hyperoside, and isoquercitrin), oligofurostanosides, new isolated asparagusic acid, two major anthocyanins. Carbohydrates, proteins, dietary fiber and nitrogen where found in this plant.	*Asparagus officinalis L.* showed antidiabetic activity through the suppression of blood glucose levels. The saponins from this plant inhibits the growth of leukemic cells, had antifungal activity, and scavenged antioxidants. The extracts of this plant further displayed antibacterial, anti-inflammatory potential *in vitro* also induced diuretic effects *in vivo*	*Asparagus officinalis L.* is believed to have laxative, diuretic and contraceptive effects, and as a remedy for neuritis, rheumatism, cancer, toothache relieve, face acne lesion, as well as to stimulate hair growth
Asparagus racemosus Willd.	*Asparagus racemosus Willd.* is reported to have alkaloids, proteins, starch, tannin, flavonoids, glycosides of quercetin, rutin and hyperoside in roots and flowers. There were few trace minerals identified from this plant species such as zinc, manganese, copper, cobalt, calcium, magnesium, potassium, zinc and selenium. The main active sections of *Asparagus racemosus Willd.* are steroidal saponins, racemofuran, polycyclic alkaloid, together with known compounds asparagamine A and racemosol	The glycoside from *Asparagus racemosus Willd.* blocks the oxytocin-induced contractions and have galactagogic properties that leads to increase in milk secretion during lactation. The roots of *Asparagus racemosus Willd.* showed significant antioxidant, anticancer, anti-inflammatory, anti-ADH and antimicrobial activities Methanolic extract of roots displayed important antitussive activity and antiprotozoal activity against *Entamoeba histolytica in vitro*	*Asparagus racemosus Willd.* is used to improve memory intelligence, physical strength, as a uterine sedative, and to maintain youthfulness. It is used as a female tonic to prevent abortion, to promote the health of the mother and growing fetus when used in antenatal care and acts as post-partum tonic by normalizing uterus and changing hormones. Despite being a rejuvenating herb, it is used in female infertility, as it increases libido, ovulation, can be used to moisten dry tissues of the sexual organs and it's able to cure inflammation of sexual organs. This plant also increases lactation and is useful for the treatment of gynecological diseases when used in postnatal care. The juice of fresh roots is recommended for duodenal ulcers. The plant can also be used to treat urinary tract infections, skin diseases, and wounds, in preventing constipation, and as an aphrodisiac, diuretic, carminative, and antiseptic. It is also used for nervous and rheumatic infections

Plant species	Active phytochemicals	Mode of Action	Beneficial role in human health
Asparagus densiflorus (Kunth) Jessop	This plant has flavonoids, tannins, phenolics, saponins, cardiac glycosides, terpenoids, quinones, amino acids, carbohydrates and alkaloids. In addition, the aqueous extracts have quinones	*Asparagus densiflorus (Kunth) Jessop* aqueous and ethanolic leaves extracts can inhibit antibacterial activity against *Enterobacter aerogenes*, *Clostridium perfringens* and *Salmonella typhimurium*. However, other pharmacological activities of this plant have not been scientifically investigated and documented	An infusion of the plant leaves is used to treat thrush and ulcers in the mouth, for abdominal pains, as a tonic to boost immunity, as a cleansing agent to rid the body of "poison" and "dirty blood"

Table 1.
Summary of phytochemicals, mode of action and the role of selected asparagus species in human health.

Burch., *Asparagus africanus Lam*., *Asparagus officinalis L.*, *Asparagus racemosus Willd*. and *Asparagus densiflorus (Kunth)* Jessop) were evaluated for their historical, etymological, morphological, phytochemical and pharmacological aspects. The phytochemicals, mode of action and the role of selected Asparagus species in human health have been summarized in **Table 1**.

Conflict of interest

"The authors declare no conflict of interest."

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Chapter 11

Phytochemicals and Their Antifungal Potential against Pathogenic Yeasts

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Abstract

The rate of fungal infections is increasing rapidly, and pathogenesis of their species is poorly understood. Among fungi, *Candida* species are a major cause of morbidity and mortality worldwide and thus represent a serious threat to public health. In addition, *Cryptococcus* spp. are yeasts responsible for serious lung infections and meningitis. Polyenes, fluoropyrimidines, echinocandins, and azoles are used as commercial antifungal agents to treat fungal infections. However, the presence of intrinsic and developed resistance against azole antifungals has been extensively documented. The re-emergence of classical fungal diseases has occurred because of the increment of the antifungal resistance phenomenon. In this way, the development of new satisfactory therapy for fungal diseases persists as a major challenge of present-day medicine. The urgent need includes the development of alternative drugs that are more efficient and tolerant than those traditional already in use. The identification of new substances with potential antifungal effect at low concentrations or in combination is also a possibility. This chapter briefly examines the infections caused by *Candida* and *Cryptococcus* species and focuses on describing some of the promising alternative molecules and/or substances that could be used as antifungal agents, their mechanisms of action, and their use in combination with traditional drugs.

Keywords: medicinal plants, yeast infections, antifungal agents, antifungal activity, phytochemicals

1. Introduction

Fungal infections are considered a serious health problem, especially in people with weakened immune systems, and are a main cause of morbidity and mortality worldwide [1].

However, the impact of these "opportunistic" diseases on human health is not widely highlighted [2]. Due to this, research related to fungi occurs slowly compared to those caused by other pathogens.

Among the different mycotic infections, those caused by *Candida* and *Cryptococcus* are the most threatening due to severity of the disease and higher worldwide occurrence [3]. The pathogenicity of fungal infections proceeds in well-organized steps. For example, *Candida* cell surface adhesion factors first promote its

adherence to host surface, followed by releasing of various hydrolytic enzymes and other virulence factors for invasion and damage of the host tissues [4].

Candida species can cause a variety of infections from the mildest to the most severe being candidemia the most frequent hospital infection accounting for up to 15% of bloodstream infections. *Candida* species are the main causative agents in 50–70% of systemic fungal infections [5].

Cryptococcus species are other yeasts of medical importance, with more than 39 species, among which *Cryptococcus gattii* and *Cryptococcus neoformans* are the most clinically relevant [6–8]. However, other species such as *Cryptococcus albidus* and *Cryptococcus laurentii* are emerging pathogens involved in several types of infections [6, 9–11].

These yeasts are present in several environmental niches, such as woody sites (decomposing tree trunks, mainly eucalyptus, and soil), vegetable remains, domestic dust, and bird excrement, more precisely in *Columba livia* [12–14]. The source of the infection is exogenous and occurs primarily by inhalation or by direct inoculation into the tissue after trauma of desiccated spores or yeasts. It is believed that the only source of infection is environmental, since there are no reports of transmission between animals and humans or between humans [15].

The main virulence factors of *Cryptococcus* species are growth capacity at 37°C, polysaccharide capsule, melanin synthesis, and production of urease and antioxidant enzymes, causing primary or opportunistic cryptococcosis, such as pulmonary, cutaneous, and meningitis diseases [6, 8, 13, 16–19]. Cryptococcosis is the third opportunistic infection associated with AIDS [20].

In addition to delays in yeast diagnosis, there is currently a limited antifungal armamentarium in use against yeast diseases including only four chemical classes: polyenes, triazoles, echinocandins, and flucytosine. Antifungals act by binding specific components of fungal plasma membrane or its biosynthetic pathways or even cell wall components [21]. However, most of the antifungal agents used in the clinic is fungistatic and often led to the development of resistance by fungal species. Modern early antifungal treatment strategies, such as prophylaxis and empirical and preemptive therapy, result in long-term exposure to antifungal agents, which is a major driving force for the development of resistance.

Among the available antifungal agents, azoles are the preferred and most frequently used drugs for treatment of *Candida* and *Cryptococcus* infections. Fluconazole (FLZ), a type of azole, is often preferred in treatments of *Candida* infections because of its low cost and toxicity, in addition to availability in varied formulations [22]. However, there are many reports that described resistance development among *Candida* species, especially in relation to azoles.

Infectious Diseases Society of America recommends the treatment of cryptococcosis through FLZ and amphotericin B (AMB) with or without combination with 5-flucytosine (5-FC), followed by prolonged maintenance with fluconazole. Other azole compounds such as itraconazole (ITC), voriconazole, and posaconazole may be used as an alternative to FLZ in cases of contraindication or inefficacy of the latter [23, 24]. However, there has been a progressive increase in isolates of *Cryptococcus* spp. resistant to FLZ, which complicates the management of cryptococcal meningitis [25]. On the other hand, AMB and 5-FC are not available in all countries and are, respectively, nephrotoxic and hepatotoxic, limiting the anti-cryptococcal therapeutic [24].

Considering the limited availability of antifungals in use and the emergence of resistance, the control of *Candida* and *Cryptococcus* infections is a challenge in the modern clinic. In this way there is a continuous need for the search for new substances with new mechanisms of action with the aim of developing novel broad spectrum antifungal drugs with better efficacy.

In this way, plants stand out as the major producers of promising substances, the phytochemicals. Identification of new molecules with antifungal potential for the manufacture of new drugs, more effective and less toxic, is essential to facing the challenge. The use of phytochemicals alone or in combination with traditional drugs represents an important alternative to conventional therapy. The combination of drugs usually requires lower doses of antimicrobials. This reduction might lead to a toxicity decrease, which results in a higher tolerance to the antimicrobial by the patient.

2. Pathogenic yeast infections: a serious health problem

In the last two decades, fungal infections have shown a significant increment. In addition to the increase in the number of patients with compromised immune system, factors such as increasing number of patients using catheters, the use of broad-spectrum antibiotics, the rising number of patients requiring organ transplantations, as well as those with hematological malignancies and diabetes also contribute to this phenomenon [26, 27].

Even though fungal infections cause significant amount of human morbidity and mortality, the impact of these "opportunistic" diseases on human health is not widely highlighted [2]. Due to this, the research into the pathophysiology of human fungal infections is slow in comparison to other disease-causing pathogens. Recently, an editorial published in the journal *Nature Microbiology* [28] ratified the importance of not neglecting fungi. The call proposed a reflection on fungi and how these microorganisms have been neglected, even with studies already consolidated showing their medical relevance.

The most frequent fungal diseases affecting populations in the world are candidiasis [29–34] and cryptococcosis [8, 20, 25]. There are several types of candidiasis as mucosal candidiasis, cutaneous candidiasis, onychomycosis, systemic candidiasis [35, 36], and pulmonary candidiasis. An important fact is that candidiasis is an infection that can affect both immunocompromised and healthy people [37, 38]. Candidemia is the most relevant and prevalent nosocomial fungal infection associated with a high mortality rate (up to 49%) in patients with a compromised immune system [39, 40]. The association of *Candida* with bloodstream infections depends on patient's condition, age, and geographic region. Candidemia is such an important infection that in 10–40% of cases, it is associated with sepsis or septic shock [41].

Candida albicans continues to be the most prevalent species isolated from fungal infections [27, 42–44]. However, the prevalence of other *Candida* species has increase substantially. These species are *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. glabrata*, *C. guilliermondii*, *C. orthopsilosis*, *C. metapsilosis*, *C. famata*, and *C. lusitaniae* [44–46].

Candida species presents high degree of flexibility, being able to grow in extremely different environments regarding to the availability of nutrients, temperature variation, pH, osmolarity, and amount of available oxygen [47]. This fact associated with the high resistance capacity of species to antifungals, their virulent features, and capability of forming biofilms with other species [48, 49] makes the genus *Candida* a serious risk to human health [50]. Thus, *Candida* species are highly adaptable and possess numerous strategies to survive in conditions that can affect their overgrowth and alter their susceptibility profiles.

Cryptococcus spp. may remain latent in the lungs, leading to asymptomatic infection, or may cause multifocal lung disease. The latency period of *Cryptococcus* can range from 6 weeks to more than 1 year after inhalation [51]. The fungus

presents neurotrophism and can migrate to the central nervous system (CNS) through hematogenous dissemination and, when crossing the blood-brain barrier, can cause meningoencephalitis [13, 18]. Episodes of mental confusion in patients with cryptococcosis have been described [52, 53]. Neurocryptococcosis is the most severe form of the disease with high mortality rates in the absence of adequate treatment [18, 23]. The mortality due to cryptococcosis is higher than the mortality caused by tuberculosis and similar to that caused by malaria [54].

Another clinical manifestation is cutaneous cryptococcosis, which is rare and usually secondary to hematogenous dissemination. Cutaneous lesions are characterized by an infiltrative plaque of a solid tumor mass that can present ulcerative and necrotic lesion [17]. Pulmonary and cutaneous lesions due to nodular features may be misdiagnosed as tumor lesions [55]. In addition to the respiratory tract, CNS and skin, other sites may be affected: prostate, eyes, adrenal glands, lymph nodes, bone marrow, and liver [51].

Until now, there are three proposals to explain fungal neurotropism. The first is that neuronal substrates present in the basal ganglia promote cryptococcal growth and survival, and, thus, perivascular spaces may serve as a niche for *Cryptococcus*, as described by [56] in a healthy female patient who had evidence of *Cryptococcus* infection within the perivascular spaces of the parenchyma. The second proposal describes that it is possible that there are specific neuronal receptors that can attract *Cryptococcus* to the CNS [57]. The third hypothesis, one of the most widespread, is that the fungus uses neurotransmitters such as dopamine that aids in the synthesis of melanin [19, 57, 58].

Besides the clinical importance of fungal infections caused by theses pathogenic yeasts, interestingly, climatic abnormalities due to phenomena such as La Niña and El Niño have recently been described as important in the distribution and occurrence of mycoses in countries influenced by them [59].

3. Traditional antifungal agents against yeasts

In the last two decades, there has been an increasing, but limited, discovery of antifungal agents [47]. These include azoles, such as fluconazole, itraconazole, ketoconazole (KTC), miconazole, and clotrimazole, polyenes (amphotericin B [AMB] and nystatin), allylamines, thiocarbamates, morpholines, 5-fluorocytosine, and echinocandins (for instance, caspofungins) [21]. However, fungal cells and human cells are eukaryotic, so antifungal compounds target both cell types, resulting in considerable side effects in patients and fewer available targets for drug action. Antifungals target three cellular components of fungi (**Figure 1**). Azoles inhibit ergosterol biosynthesis by interfering with the enzyme lanosterol 14-α-demethylase in endoplasmic reticulum of the fungal cell. This enzyme is involved in the transformation of lanosterol into ergosterol, a component that is part of the plasma membrane structure of the fungus (**Figures 1** and **2**). Thus, as the concentration of ergosterol is reduced, the cell membrane structure is altered, thereby inhibiting fungal growth [60].

Azoles comprise a five-member azole ring containing two (imidazole) or three nitrogen atoms (triazole) attached to a complex side chain [61, 62]. Imidazoles include KTC, miconazole, econazole, and clotrimazole, and triazoles include FLZ, ITC, voriconazole (synthetic triazole derivative of FLZ of second generation), and posaconazole (hydroxylated analog of itraconazole) [63].

AMB and nystatin bind to ergosterol causing the disruption of the membrane structure and promoting extravasation of intracellular constituents such as ions and sugars and, consequently, cell death [21] (**Figure 1**).

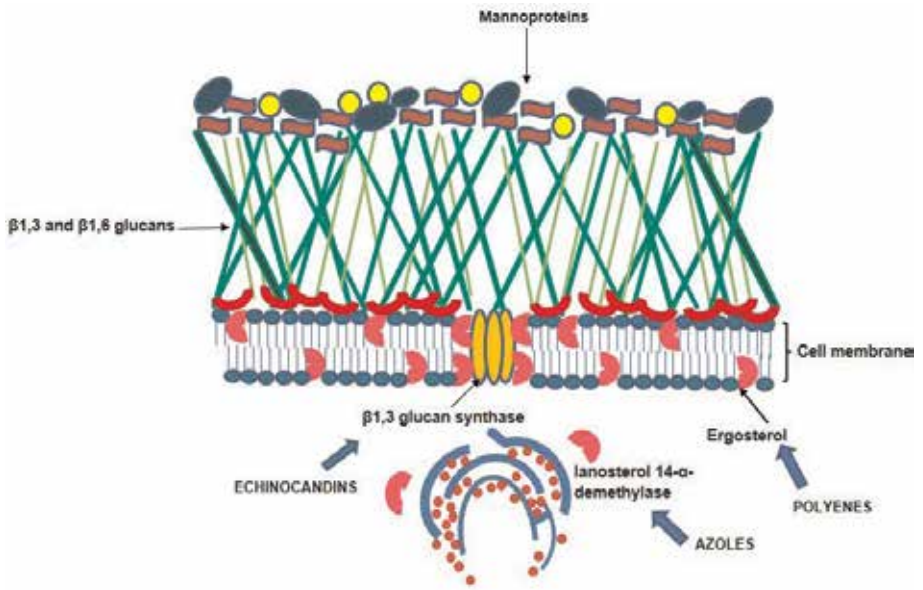

Figure 1.
Mechanisms of action of some traditional antifungal agents on cellular targets. Azoles inhibit the ergosterol synthesis in the endoplasmic reticulum of the fungal cell by interfering with the enzyme lanosterol 14-α-demethylase. Polyenes act by binding to ergosterol present at the cell membrane. Echinocandins inhibit (1,3) β-D-glucan synthase, thereby preventing glucan synthesis.

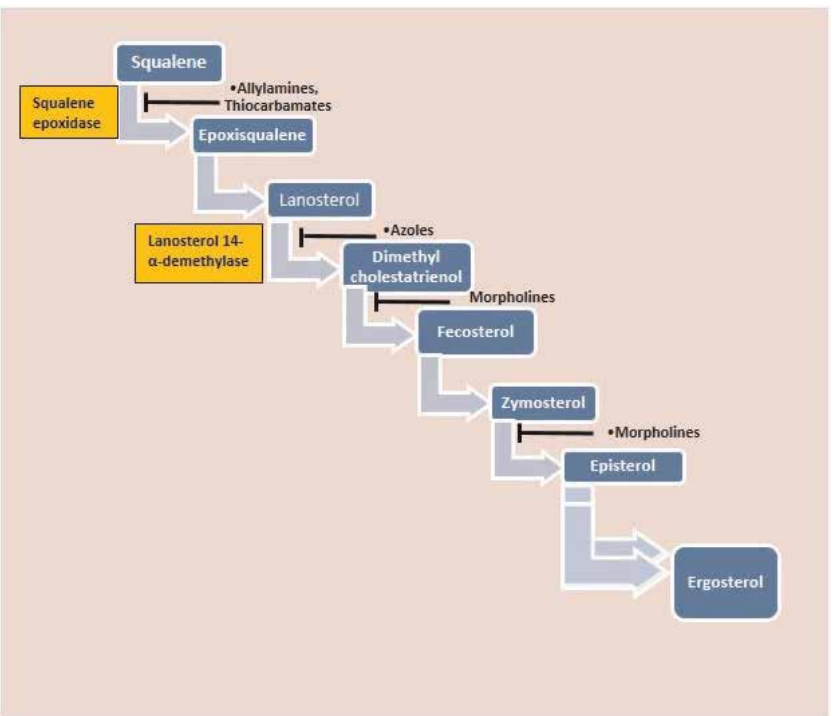

Figure 2.
Specific point of action of antifungal drugs in the ergosterol biosynthesis pathway.

Pyrimidine analogs include 5-fluorocytosine and 5-fluorouracil (5FU). The first has fungistatic properties and enters the fungal cell through cytosine permease, inhibiting the thymidylate-synthetase enzyme and interfering with DNA. 5-fluorouracil, which in turn can be phosphorylated to 5-fluorodeoxyuridine monophosphate, can be incorporated into RNA molecules [63]. Due to toxicity [64]; stronger side effects, such as hepatic impairment; interference with bone marrow function; and rapid occurrence of resistance especially among *Candida* species, the clinical use of 5-FC is preferred in association with AMB [65, 66]. In addition, the nephrotoxicity and hepatotoxicity of AMB and 5-FC, respectively, and the unavailability of these antifungals in many countries have limited their use in cryptococcal therapeutic [24].

Host's immunity, type of infection, site of origin of the samples, toxicity, bioavailability of the drug, and the sensitivity/resistance profile of the isolates interfere in the choice of the type of agent to be used [22]. AMB is considered the gold standard drug for most mycoses that affect patients at risk [67], although it has high toxicity. Azoles have fungistatic properties that affect cell growth and proliferation [65]. Among azoles, KTC was one of the firsts to emerge and was the first alternative to AMB [68]. Currently, FLZ is the drug of choice for most *Candida* and *Cryptococcus* infections [64] and is the most recommended antifungal agent for use in invasive candidiasis [47, 49].

For cryptococcosis, the choice of treatment depends on the patient's immunological status and mainly on the clinical of the disease, if it is just a pulmonary manifestation or if the infection is systemic. Fluconazole is recommended in cases of lung disease with mild to moderate symptoms. Amphotericin B with or without combination with 5-flucytosine is the recommended therapy for more serious infections such as meningoencephalitis, followed by prolonged maintenance with fluconazole [23, 24].

Although azoles are generally well-tolerated, they have limitations such as hepatotoxicity and the emergence of resistance among fungal isolates [69] which provide motivation for improving this class of antifungal agents [68]. For instance, alterations in triazole molecule gave rise to voriconazole (structurally related to FLZ) and posaconazole (related to ITC), both available for systemic therapy [66].

Echinocandins, which include caspofungin, micafungin, and anidulafungin, are a new class of antifungals and have fungicidal effects in all *Candida* species [66]. They inhibit (1,3) β-d-glucan synthase, thereby preventing glucan synthesis, which is present in the cell membrane of fungi (**Figure 1**). As this drug acts on the wall structure of the fungus, it has the advantage of a lower side effect in animal cells [47].

Allylamines (terbinafine and naftifine) and thiocarbamates inhibit the enzyme squalene epoxidase, which participates in the synthesis of ergosterol and is encoded by the *ERG1* gene (**Figure 2**). This activity leads to membrane rupture and accumulation of squalene. Allylamine effects can also prevent the production of other sterol derivatives.

To minimize toxicity and resistance, some pharmacological strategies were developed. The preparation and use of new antifungal formulas (liposomal AMB (Ambisome®), AMB lipid complexes (Abelcet®), AMB colloidal dispersions (Amphocil®/Amphotech®), and AMB lipid nanosphere formulations and β-cyclodextrin itraconazole) are one strategy [68]. Others include combination therapies of antifungal compounds (e.g., AMB + 5-FC, FLZ + 5-FC, AMB + FLZ, caspofungin + liposomal AMB, and caspofungin + FLZ) and nanostructuring of conventional antifungal agents [70–73].

However, all traditional antimycotic drugs have at least one restriction related to their use. Some do not have a broad spectrum of action or are fungistatic. Others have high toxicity and low bioavailability with significant side effects [74].

Therefore, limitations of treatment and drug resistance associated with pathogenicity of the clinical isolates support the urgent need to identify substances that are more effective, with new mechanisms of action in the fight against *Candida* and *Cryptococcus* infections.

4. Resistance in pathogenic yeasts: a significant problem

Most antifungals target sterols or the enzymes that synthesize them. However, the fungistatic nature of many of these antifungals and emergence of clinical drug resistance limits their success. Increased drug resistance in fungi is a problem that cannot be avoided, particularly for FLZ, which is the preferred antifungal for treating yeast infections [75].

The number of people at risk for fungal infections has been increasing, resulting in an increased use of antifungal agents, even as prophylaxis. Thus, besides the existence of some non-*albicans Candida* (NAC) species presenting inherent resistance to azoles, higher minimum inhibitory concentrations (MICs) for antifungals against *C. albicans* strains have been observed [76]. The World Health Organization (2014) categorizes antimicrobial resistance as that developed by the microorganism to an antimicrobial drug, which was initially effective in treatment of such infections. Low-dose prophylactic administration of azole derivatives, such as FLZ, for prolonged periods to prevent the occurrence of opportunistic infections in immunosuppressed patients also results in resistant phenotypes [27, 75]. Therapeutic failures and empiric treatment are facts which are likely to collaborate to the increased incidence of fungal infections.

In the last decade, a number of new clinical problems have arisen, requiring new guidelines regarding the treatment of cryptococcosis, mainly because clinical data have suggested that cryptococcal strains have become more resistant to drugs [23, 25]. Some relates say that clinical *Cryptococcus* isolates are frequently less susceptible to fluconazole than environmental isolates. However, Chowdhary et al. [77] evaluated the susceptibility profile of environmental and clinical strains of *C. gattii* and observed that environmental samples were less susceptible to fluconazole, itraconazole, and voriconazole in comparison to clinical isolates.

Heteroresistance is also a worrying phenomenon. It consists of the ability of a subpopulation of microorganism to adapt to high concentrations of the drug, resulting in resistant homogenous populations. However, heteroresistant strains return to the initial phenotype when the stimulus with the drug is withdrawn [78].

Some mechanisms for cellular and molecular resistance to FLZ in yeasts are described. In *Candida* and *Cryptococcus*, the first is related to the induction of multidrug pumps, which decrease the concentration of drug available in the intracellular compartment of yeast cells. Various genes belonging to the ATP-binding cassette superfamily or to the major facilitator superfamily encode efflux pumps were identified in *C. albicans*. Overexpression of some transporter genes or of their regulated genes can confer cross-resistance to various azoles [21]. In *C. gattii* and *C. neoformans*, *AFR*1, *MDR*1, and *AFR*2 genes encode ABC transporters that expel the azole out of the fungal cell, thereby causing resistance to these drugs [79].

A second mechanism of resistance involves modification of the target enzyme encoded by the *ERG11* gene, also known as cytochrome P_{450} lanosterol 14-α-demethylase (Cyp51). Mutations in this gene prevent azoles from binding to enzyme sites. Another mechanism of resistance is related to mutations in the *ERG3* gene which does not convert 14-α-methylfecosterol into 14-α-methyl-3,6-diol in the ergosterol synthesis pathway. This substitution causes azoles to have no fungistatic effects on the fungal cell membrane [21].

Transcriptional regulation is also important for the development of resistance mechanisms. YAP1, a protein, is important for the mechanism of *C. neoformans* heteroresistance to fluconazole and oxidative stress. Mutant strains of *C. neoformans* that lost protein YAP1 became hypersensitive to a variety of oxidizing agents and mainly to fluconazole [80].

Resistance to polyenes (AMB) in fungus is less common and in *C. albicans* is associated with the substitution of ergosterol with a precursor molecule or a general reduction of sterols in the plasma membrane [81]. Reduction of membrane ergosterol renders *Cryptococcus neoformans* and *Aspergillus* spp. less susceptible to amphotericin B [82]. Enzymes encoded by *ERG3* and *ERG2* genes participate in ergosterol biosynthesis and have the main alterations related to AMB resistance because mutations in their genes modify ergosterol content required for the action of polyenes [83].

The main resistance mechanism to echinocandins is related with point mutations in gene that encodes the major subunit of the glucan synthase enzyme (Fks subunit) (**Figure 1**) and can provide resistance to all echinocandin [84]. Other *Candida* species also present this resistance mechanism such as *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, *C. guilliermondii*, and *C. dubliniensis* [85, 86].

Resistance to 5-FC can be of two types: primary, occurring via cytosine permease (encoded by the *FCY*2 gene) whose mutation decreases drug uptake [87], and secondary, related to alterations in cytosine deaminase (encoded by *FCY1*) or uracil phosphoribosyltransferase (encoded by *FUR1*) activities. Cytosine permease is responsible by conversion of 5-FC to 5-fluorouridine or to 5-fluorouridine monophosphate (5-FUMP) [88]. Resistance is easily developed in fungal isolates from patients who are receiving the drug. However, other molecular mechanisms related to resistance to 5-FC must exist because most of them have not been observed in *C. albicans* [89].

The increase in the drug-resistant *Candida* and *Cryptococcus* strains to commercial antifungals has caught the attention of clinicians and researchers to medicinal plant products (commonly referred as phytochemicals). The use of phytochemicals with greater antifungal potential and different mechanisms of action may be useful in reducing the phenomenon of resistance. Lately, they have become a significant alternative for discovery of commercially viable, economically cheaper, and safe phytomedicines.

5. Medicinal plants as a source of antifungal agents

Global Action Fund for Fungal Infections (GAFFI), an international organization working to reduce infections and deaths associated with fungi, has reported that approximately 300 million people in the world suffer from a serious fungal infection every year and that among them over 1.35 million deaths are registered [90].

Despite the introduction of new and novel antifungal drugs, their production and impact are slow, and the development of antifungal resistance has forced the attention of researchers toward herbal products, mainly phytochemicals, in search of development of safe and economically viable antifungals.

Populations around the world have used folk medicine as an alternative therapy for various disorders. Currently, many species have been extensively studied in an attempt to discover new biologically active compounds with novel structures and mechanism of action for the development of new drugs.

Medicinal plants are commonly preferred because of their wide level of functional chemical groups with comparatively poor toxic substances, low-cost extracts,

fewer side effects, and easy accessibility to people. Various bioactive compounds have been abundantly found such as phytochemicals.

Leaves, as well as the seeds and fruits of plants, have higher levels of phenolic compounds. The concentration of these compounds also depends on the nature of the chemical used as solvent in the extraction process as well as on the growth and storage conditions [91].

The biological activity of plant products has been evaluated against fungi. The ethanol extract, *Lonicera japonica* aerial parts, a medicinal plant of folk medicine of China that used to treat some diseases, showed a very strong antimicrobial activity against *Candida* species and potent wound healing capacity [92]. Methanolic extract of *Lannea welwitschii* leaves was antimicrobial against clinical yeasts. A preliminary phytochemical screening of extracts revealed tannins, flavonoids, alkaloids, and glycosides as compounds [93]. *Pyrostegia venusta* crude flower extracts, fractions, and pure compounds showed an effective broad spectrum antifungal activity [94].

An extract of *Piper betle* leaves inhibited the growth of *Candida* species [95], and four different extracts of *Strychnos spinosa* showed anti-*Candida* activity [96]. Hydro-methanolic extracts of leaves from *Juglans regia* and *Eucalyptus globulus* and methanol extract of *Cynomorium coccineum* demonstrated excellent antimycotic property against *Candida* strains [91, 97]. Akroum [98] showed antifungal activity in an acetylic extract of *Vicia faba* against *C. albicans* in vitro and reduced mortality rates in *Candida*-infected mice that were treated with the extract.

Berberine, a protoberberine-type isoquinoline alkaloid isolated from the roots, rhizomes, and stem bark of natural herbs, such as *Berberis aquifolium*, *Berberis vulgaris, Berberis aristata*, *Hydrastis canadensis*, *Phellodendron amurense, Coptis chinensis,* and *Tinospora cordifolia*, was described as powerful reducer of the viability of in vitro biofilms formed by fluconazole-resistant *Candida tropicalis* cells [99].

Ethanolic and aqueous extracts from different plants from Brazilian Cerrado commonly used in folk medicine such as *Eugenia dysenterica* and *Pouteria ramiflora* were promising against *C. tropicalis*, *C. famata*, *C. krusei*, *C. guilliermondii*, and *C. parapsilosis*. A phytochemical screening of active extracts from these plants disclosed as main components flavonoids and catechins [100]. Crude extract and fractions (n-butanolic and ethyl acetate ones) from *Terminalia catappa* leaves showed antifungal properties against *Candida* spp.; hydrolysable tannins (punicalin, punicalagin), gallic acid (GA), and flavonoid C-glycosides were the active components found in butanolic fraction [101].

Bottari et al. [102] determined the antimicrobial activity of the aqueous and ethanolic leaf extracts of *Carya illinoensis*. Both extracts had MIC values against seven *Candida* reference strains between 25 and 6.25 mg/mL. Phenolic acids (gallic acid and ellagic acid), flavonoids (rutin), and tannins (catechins and epicatechins) were likely responsible, in part, for the activity against *Candida* strains. Further, the extracts inhibited the production of *C. albicans* germ tubes.

5.1 Phytochemicals: polyphenols as substances most found in plants

Several woody plant produce medicinal phytochemicals such as polyphenols that are low molecular weight naturally occurring organic compounds containing one or more phenolic groups [103]. Further, polyphenols perform various substantial functions in plant physiology and, therefore, can be found, in lesser or greater quantity, in all of them.

Phenolic acids, flavonoids, tannins, and coumarins are some examples of phenolic compounds found in and extracted from medicinal plants [104] (**Table 1**). Research has shown that polyphenols have potentially healthy effects in

Phytochemicals	Bioactive compounds	Properties	Plant sources
Flavonoids	Flavan-3-ol	Against *Candida*	*Syzygium cordatum*
	Baicalein, gallotannin	Against *Candida*	*Scutellaria baicalensis*
Coumarins	Ulopterol	Against *M. canis*	*Skimmia laureola*
	Prenyletin; prenyletin-methyl-ether	Against *T. rubrum*; *T mentagrophytes*	—
	Osthenol	*C. albicans*, *Fusarium solani*, *A. fumigatus*	—
	5,8-Dihydroxyumbelliprenin	*T. interdigitale*, *M. gypseum*	*Ferula foetida*
Saponins	Colchiside	Phytopathogenic fungi	*Dipsacus asper* roots
Terpenes or terpenoids	Triterpenes	Against dermatophytes	Ethyl acetate leaf extract of *Satureja khuzestanica*
Lectins	Lectins	*Fusarium oxysporum*	Seed from native Amazon species
Tannins	Punicalagin Punicalin	Against *Candida* spp.	*Terminalia catappa*
	Punicalagin	*T. mentagrophytes*; *T. rubrum*; *M. canis*; *M. gypseum*	*Punica granatum*
	Ellagic acid, gallagic acid, punicalins, punicalagin	*C. albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*	*Punica granatum*
	Lambertianin C, sanguiin H-6	*Geotrichum candidum*	*Rubus idaeus*

Table 1.
Phytochemicals with antifungal compounds derived from plants.

humans, working primarily as anticancer, antihypertensive, anti-allergen, anti-inflammatory, antioxidant, and antimicrobial agents. The antimicrobial activity of polyphenols has been extensively investigated mainly against bacteria [104]. Nevertheless, the antifungal activity of most of the phenolic compounds remains unknown. There are few studies on the mechanism of action of the substance, cytotoxicity, the synergism with traditional antifungals drugs, and their anti-virulence activities.

Those with the most promising antifungal activity isolated from natural sources include flavonoids, tannins, coumarins, quinones, lignans, and neolignans [105] (**Table 1**).

Flavan-3-ols, flavonols, and tannins have received the most attention among the known polyphenols, attributable to their large spectrum of efficacy and high antimicrobial property. Structurally, flavonoids are aromatic compounds with 15 carbon atoms (C15) on their basic skeleton; they consist in tricyclic phenolic compounds with two aromatic rings on their structure (C6–C3–C6) [105]. Flavonoids are a class of natural compounds with several known protective activities, including antifungal activity. The flavonoids include subclasses such as chalcones, flavones, isoflavones, flavonols, flavanols (flavan-3-ol), and anthocyanidins [106].

The activity of flavonols such as quercetin, myricetin, and kaempferol has been described in *C. albicans*. For instance, quercetin, myricetin, and kaempferol from propolis have showed activity against *Candida* species [107]. The flavanol subclass (flavan-3-ol) and gallotannin, extracted from *Syzygium cordatum*, also

showed inhibitory properties on the growth of *C. albicans* [108]. Serpa et al. [109] isolated baicalein, belonging to a subclass of flavones, from *Scutellaria baicalensis*, and induced apoptosis in *C. albicans* (**Table 1**), and apigenin, a flavone isolated from propolis, showed antifungal potential. Flavonoids as much as coumarins and lignans have shown an antifungal potential against several species of dermatophytes [105].

Other important groups of polyphenolic compounds present in various plant parts, such as the roots, flowers, leaves, fruits, and seeds, are tannins. They are divided into hydrolyzable (ellagitannins) and condensed tannins (proanthocyanidins) and gallotannins [110]. They have the ability to precipitate macromolecules such as proteins [111] as well as have antimicrobial properties. However, the mechanisms underlying the antimicrobial action of tannins in different microorganisms are still under investigation [111].

Ellagitannins constitute a complex class of polyphenols characterized by one or more hexahydroxydiphenoyl (HHDP) which can be linked in various ways to the glucose molecule [112]. Ellagic acid, gallagic acid, punicalins, and punicalagins isolated from ethyl acetate and butanolic fractions of *Punica granatum* revealed antifungal activity against *C. albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* [113] (**Table 1**).

Ellagitannins isolated from *Ocotea odorifera*, a plant commonly used in Brazil in folk medicine, have a potential against *C. parapsilosis* [114]. Two ellagitannins isolated from raspberry (*Rubus idaeus* L.) fruit, lambertianin C and sanguiin H-6, showed fungistatic activity both in vitro and in situ against *Geotrichum candidum* [115]. Dos Santos et al. [111] verified that encapsulated tannins from *Acacia mearnsii* have moderate activity against *Aspergillus niger* (ATCC 9642) and *C. albicans* (ATCC 34147).

Coumarins have a C6-C3 skeleton, possessing an oxygen heterocycle as part of the C3 unit [105]. These compounds are known to play a role in disease and pest resistance, as well as UV tolerance. The antifungal activity of 40 coumarins was tested against reference strains of *Candida albicans*, *Aspergillus fumigatus*, and *Fusarium solani*, but among them only osthenol showed the most effective antifungal activity (Table 1). The authors argue that the action of osthenol can be related to the presence of an alkyl group at C-8 position [116].

Another coumarin derivative, 4-acetetatecoumarin, was effective in inhibiting *Aspergillus* spp., acting on the factors of virulence and affecting the structure of the fungal wall. Diversinin, a coumarin isolated from the petroleum ether extract of *Baccharis darwinii*, demonstrated antifungal activity against *T. rubrum*, *T. mentagrophytes*, and *M. gypseum*, being fungicidal. Another coumarin derivative, 5,8-dihydroxyumbelliprenin, isolated from *Ferula foetida*, was active against *M. gypseum* and *Trichophyton interdigitale* [105] (**Table 1**).

Phenylpropanoids are other naturally occurring compounds categorized as coumarins, phenylpropanoic acid, and lignans frequently studied for their anti-*Candida* properties [117]. Navarro-Garcia et al. [118] and Raut et al. [119] found that a coumarin (scopoletin) and two phenylpropanoic acids (salicylaldehyde and anisyl alcohol) have antifungal property against *C. albicans*, with MICs of 25, 31, and 31 μg/mL, respectively.

Shahzad et al. [103] observed the effectiveness of pyrogallol and curcumin (CUR) against various *C. albicans* clinical isolates. In addition, curcumin inhibited the adhesion capability of cells and demonstrated anti-biofilm activity. Curcumin is a flavonoid found in turmeric (Curcuma longa L.). Pure curcumin had potential activity against *Cryptococcus gattii* both in vitro and in vivo [120]. According to Ferreira et al. [121], the essential oil from *Curcuma longa* L. can reduce the colony diameter, germination, and sporulation of *Aspergillus flavus*.

Alalwan et al. [122] undertook a series of adsorption experiments with varying concentrations of curcumin and showed that 50 μg/mL could prevent adhesion of *C. albicans* SC5314 to denture materials. Curcumin-silver nanoparticles also showed potential anticandidal activity against fluconazole-resistant *Candida* species isolated from HIV patients with MIC range of 31.2–250 μg/mL [123].

Gallic acid is a polyphenol natural compound found in many medicinal plant species that has been shown to have anti-inflammatory and antibacterial properties. GA was found to have a broad spectrum of antifungal activity against dermatophyte and *Candida* strains. Authors verified that GA reduced the activity of sterol 14-α-demethylase P450 (CYP51) and squalene epoxidase in the *T. rubrum* membrane.

Teodoro et al. [124] demonstrated that acetone fraction from *Buchenavia tomentosa* aqueous extract and its major compound gallic acid had the ability to inhibit reference strains *C. albicans* ATCC 18804 and *Candida albicans* SC 5314 adherence and to disrupt 48 h-biofilm.

5.2 Essential oils as potential antifungal

In the eagerness to research and develop new substances to suppress the development of pathogenic fungi from natural plant substances, knowledge about the biological activities of essential oils has been growing. Essential oils' pharmacological activities, mainly related to their complex chemical composition and high concentrations of phenols, make these compounds particularly interesting for both the treatment and the prevention of fungal infections. Natural phenolic substances are among the most antifungal active substances present in essential oils, generally showing low toxic effects in animals [125]. They consist in a complex mixture of monoterpene and sesquiterpene hydrocarbons and oxygenated derivatives such as alcohols, aldehydes, ketones, and phenylpropanoids.

Essential oils are also called volatile oils or ethereal oils, as they have a high degree of evaporation when exposed to air. The presence of terpenes contributes to the complex constitution with the action against microorganisms being directly related to this characteristic [126]. Since ancient times, Mondello et al. [127] proposed that tea tree oil could be used in antifungal therapy, because it showed efficacy against multidrug-resistant *Candida* species in vitro and against mucosal candidiasis in vivo; they have also showed that terpinen-4-ol was the main substance presented in the oil which contribute to the anticandidal activity.

Several oils have demonstrated activity against *Candida* species. Sharifzadeh et al. [128] observed that essential oils from *Trachyspermum ammi* have anticandidal effects against isolates resistant to FLZ. Herbal essences from *Foeniculum vulgare*, *Satureja hortensis*, *C. cyminum*, and *Zataria multiflora* were tested against *C. albicans*. Essential oils from *Z. multiflora* showed the best anticandidal activity [129].

Carica papaya essential oils have inhibitory effects against *Candida* species, detected by agar diffusion and microdilution assays [130]. Minooeianhaghighi et al. [131] verified that a combination of essential oils from *Cuminum cyminum* and *Lavandula binaludensis* showed growth inhibition of *C. albicans* isolates, at very low concentrations (between 3.90 and 11.71 μg/mL). Essential oils from *Cymbopogon nardus* have also shown antimicrobial potential against *Candida* species, with inhibition of hyphal growth in *C. albicans* at concentrations between 15.8 and 1000 μg/mL. This oil also inhibited growth of filamentous fungus from the environment. Main compounds of *C. nardus* essential oil were the oxygen-containing monoterpenes: citronellal, geranial, geraniol, citronellol, and neral [126]. In addition to inhibiting biofilm formation [132], essential oils from *Artemisia judaica* have been shown to inhibit the formation of germination tubes in *C. albicans* and have shown

that at a very low concentration (0.16 μL/mL), it inhibited 80% of *Candida* filamentation. Kose et al. [133] demonstrated the fungicidal potential of essential oils from *Centaurea baseri* against *Candida* species, with an MIC of 60 μg/mL.

Among the monoterpenes there is thymol (2-isopropyl-5-methylphenol) [134]. It is the most abundant constituent in essential oils from *Thymus vulgaris* (thyme) [135] and the major component of essential oils from *Origanum vulgare* (oregano) [136]. Thymol showed antifungal activity, fungistatic and fungicidal one, against *Candida* strains. Authors verified an MIC of 39 μg/mL against *C. albicans* and *C. krusei* and MIC of 78 μg/mL against *C. tropicalis*. Probably thymol acts by binding to ergosterol in the plasma membrane, thereby increasing ion permeability and resulting in cell death because an eightfold increase (from 39.0 to 312.5 μg/mL) in thymol MIC values against *C. albicans* was seen in the presence of exogenous ergosterol. A combination of thymol and nystatin resulted in synergy [137].

Terpenoids have shown synergistic effects with FLZ, so it may be useful as a candidate antifungal chemotherapeutic agent. In addition, terpenoids exhibit a very good antimycotic activity of filamentous-form growth of *C. albicans* at nontoxic concentrations [138]. Further, in experiments realized by [139], rubiarbonol G, a triterpenoid from *Rubia yunnanensis*, showed potent antimicrobial activity against *C. albicans*, with an MIC of 10.5 μg/mL.

The antifungal potential of terpenes, geraniol, and citronellol has been investigated previously, with effective inhibitory activity against *C. albicans* [138] and filamentous fungi of the *Aspergillus* species [140]. In addition, Mesa-Arango et al. [67] showed that oxygenated monoterpenes in the citral chemotype, such as geraniol, citral, and citronellal, have antifungal activity against *C. parapsilosis*, *C. krusei*, *Aspergillus flavus*, and *Aspergillus fumigatus.*

Terpenes' anti-biofilm activity and the efficacy of thymol, geraniol, and carvacrol in the treatment of *Candida* infections associated with the use of hospital devices have been related [141]. Effects of carvacrol on *Candida* cells can be associated with alterations in the cytoplasmic membrane and induction of apoptosis [108].

Although the process of discovering bioactive molecules is complex and time-consuming, involving isolation, identification, and optimization of pharmacokinetic and pharmacodynamic properties, as well as the selection of lead compounds for further drug development, data related here showed that plants are a promising source of active molecules with antifungal properties. Biological assays have shown that plant extracts or essential oils and their bioactivity molecules inhibit ATCC and clinical strains of fungi species, including those with resistance to drugs employed in medical practice. In addition, some are able to inhibit and control the main virulence factors of fungi species, such as the formation and proliferation of hyphae and filamentation and, more importantly, the eradication of mature biofilms.

Eugenol (4-allyl-2-methoxyphenol) is a phenolic compound and the main constituent of the essential oil isolated from the *Eugenia caryophyllata*. There are reports of some pharmacological effects of eugenol, such as antifungal and antibacterial agent, and its anti-*Candida* action seems to be related to the generation of oxidative stress concomitantly with lipid peroxidation of the cell membrane of *Candida albicans* yeast and the generation of reactive oxygen species [142]. Eugenol also showed antifungal effects against both *Cryptococcus gattii* and *C. neoformans* cells by causing morphological alterations, changes of cellular superficial charges, and oxidative stress. Thymol and carvacrol can represent alternative, efficient, and cost effective drugs for anti-biofilm therapy for *Cryptococcus* species.

Eugenol showed activity against *Alternaria* spp. and *P. chrysogenum*, by agar diffusion method [143] and, along with other monoterpenes such as carvacrol and isoeugenol, exhibited strong antifungal activity against *Rhizopus stolonifer* and *Absidia coerulea* [144].

5.3 Synergistic action between phytochemicals and antifungals

Resistance mechanisms are developed by fungi to the treatment with conventional drugs in addition to toxic side effects to human cells showed by these drugs; researchers' efforts in developing new strategies to improve treatment effectiveness of fungal infection are growing, with an interest in plants and folklore medicine.

The knowledge about synergistic effects of plant extracts or their compounds with traditional agents is nowadays a type of study that has aroused interest. Some in vitro screening assays have evidenced that plant extracts are less toxic than existing antifungal agents and, in combination with them, could reduce toxicity and increase antifungal potential [21, 145].

Accordingly, combination antifungal therapy offers the possibility of broadening the spectrum of drug activity, reducing toxicity, and decreasing fungal resistance [146].

Although combination of medications requires a careful evaluation of the synergistic, antagonistic, and agonist properties of the drugs involved [147], the use of drug combinations in treatment of infections by fungi is a common preferred strategy clinically. In many cases of fungal infection, combination therapy has been used successfully [21]. For some examples, see **Table 2**.

There are two main hypotheses about the type of interaction resulting from the combination of fluconazole and amphotericin B, based on the mechanisms of action of these drugs. In the theory of depletion, the interaction between fluconazole and amphotericin B would result in antagonism due to pre-exposure to fluconazole, which would lead to depletion of the membrane ergosterol, and thus there would be a decrease in the available sites for amphotericin B. In the second theory, the synergism, amphotericin B would lead to the formation of pores, which would facilitate the greater access of azole to the intracellular space, which by inhibiting the enzymes involved in ergosterol biosynthesis would increase the antimicrobial

Combination of antifungals	Target	References
AMP B + posaconazole	*Candida* biofilms	[148]
AMP B + caspofungin	*Candida* biofilms	[54]
AMP B + fluconazole	Cryptococcosis in murine model	[149]
Micafungin + fluconazole	*Candida* infections	[150]
Micafungin + voriconazole		[151]
Micafungin + AMP B		[152]
Micafungin + isavuconazole		
Flucytosine + voriconazole	*Candida* infections	[148]
Minocycline + fluconazole	*Candida albicans* biofilms	[148]
Posaconazole + caspofungin	*Candida* infections	[153]
		[154]
Terbinafine + azole	*Candida* growth	[155]
		[156]
Echinocandin + azole	Invasive candidiasis	[157]
AMP B + flucytosine	Invasive candidiasis	[158]
Natamycin + 5-fluorouracil	*Fusarium* species ocular isolates	[159]

AMP B: amphotericin B.

Table 2.
Various regimes of combinatorial antifungal therapy showing better efficacy in combination than that of independent drugs (adapted from [21]).

efficacy. According to these theories, the combination of fluconazole and amphotericin B could involve different interactions [160–162].

Considering the difficulties regarding to the treatment of candidiasis and cryptococcosis, the combination of antifungals represents an important alternative to conventional therapy. The synergistic effects of drugs are primarily attributable to cell wall damage by one antifungal. Thus, this component potentiates the activity of other drugs exactly against some constituent of plasma membrane. Alternatively, compromised cell wall with an increased permeability could facilitate movement of drugs across the cell membrane to their targets. Or, the synergistic action of different drugs occurs because they act on different targets of the same pathway, which can happen, for example, with the combination of azoles and allylamines.

The objective of this strategy is to maximize the antifungal effects. Tangarife-Castaño et al. [163] reported synergy between essential oils or plant extracts associated with antifungal drugs when used as anti-*C. albicans* agents. The best synergistic effects were obtained from the combination between itraconazole and *P. bredemeyeri* extract against *C. albicans*.

Synergistic potential was observed when methanolic extract of *T. catappa* leaves was combined with nystatin or AMB against reference strains of *C. albicans*, *Candida neoformans*, *C. glabrata*, *Candida apicola*, and *Trichosporon beigelii* [164]. The combination showed maximum synergy against *C. apicola*.

Santos et al. [165] related synergistic antifungal activity of an ethanol extract of *Hyptis martiusii* in combination with metronidazole against *C. albicans*, *C. krusei*, and *C. tropicalis*. Avijgan et al. [166] reported a potent synergistic effect between an *Echinophora platyloba* ethanolic extract and itraconazole or FLZ against isolates of *C. albicans* from vaginal secretions of patients with recurrent vulvovaginitis, significantly lowering the concentrations of both substances.

A combination between thymol and nystatin was found to have synergistic effects against *Candida* species [137], reducing the MICs of both products by 87.4%. Synergism was observed between a water insoluble fraction from *U. tomentosa* bark and terbinafine, as well as between it and FLZ against seven resistant isolates of *C. glabrata* and *C. krusei* [167]. Synergistic effects led to cell damage, and authors demonstrated, through differential scanning calorimetry and infrared analysis, that intermolecular interactions between the extract components and either terbinafine or FLZ occurring outside the cell wall are likely responsible for synergistic effects observed between substances.

Subfraction combinations of *Terminalia catappa*, *Terminalia mantaly*, and *Monodora tenuifolia* showed synergistic interactions against *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. neoformans* isolates. Synergistic combination between *M. tenuifolia* and *T. mantaly* subfractions also showed fungicidal effects against most tested strains [168].

The combination therapy with curcumin and fluconazole was the most effective among the treatments tested against *Cryptococcus gattii*. The association was able to reduce the fungal burden and damage on lung tissues of infected mice and to eliminate the fungal burden in the brain, enhancing the survival of mice with *C. gattii*-induced cryptococcosis [120].

Methanolic extract of *Buchenavia tetraphylla* is a great source of antimicrobial compounds and enhanced the action of FLZ against different *C. albicans* isolates from vaginal secretions as well as azole-resistant isolates. The extract increased the action of FLZ in most strains through additive (20% of strains) or synergistic (60% of strains) effects [169].

Kumari et al. [170] investigated the effect of six essential oil compounds sourced from oregano oil (carvacrol), cinnamon oil (cinnamaldehyde), lemongrass oil (citral), clove oil (eugenol), peppermint oil (menthol), and thyme oil (thymol)

against three infectious forms: planktonic cells, biofilm formation, and preformed biofilm of *C. neoformans* and *C. laurentii*. The anti-biofilm activity of the tested compounds was in the order thymol > carvacrol > citral > eugenol = cinnamaldehyde > menthol. The three most potent compounds thymol, carvacrol, and citral showed best anti-biofilm activity at a much lower concentration against *C. laurentii*. In the presence of these potent compounds, assays revealed the absence of extracellular polymeric matrix, reduction in cellular density, and alteration in the surface morphology of biofilm cells. In addition they were the most efficient in terms of human safety in keratinocyte-*Cryptococcus* spp. co-culture infection model suggesting that thymol, carvacrol, and citral can be further exploited as cost-effective and nontoxic anti-cryptococcal drugs.

The lectin pCramoll from *Cratylia mollis*, a native forage plant endemic to the semiarid region of Brazil (caatinga biome), showed an immunomodulatory effect and a synergism in combination with fluconazole, increasing the survival of animals with cryptococcosis caused by *C. gattii* and improving aspects of morbidity present in the progression of cryptococcosis [171].

Thymol exhibited synergistic effects when combined with fluconazole against clinical species of *Candida*, enhancing the antifungal potential of the drug and decreasing the concentration required for the effect [172]. Zaidi et al. [173] found that methanolic extract of leaves of *Ocimum sanctum* in combination with fluconazole showed higher antifungal potential and synergistic activity against resistant *Candida* spp. than methanolic extract or fluconazole when used alone.

Essential oils were also recently proposed to increase drug effectiveness. *Lavandula* and *Rosmarinus* essential oils were selected as antiproliferative agents to compound lipid nanoparticles for clotrimazole delivery in treatment of *Candida* skin infections. Authors confirmed the potential anti-*Candida* activity of the selected oils due to their interaction with membrane permeabilization. In addition, in vitro studies against *Candida albicans*, *Candida krusei*, and *Candida parapsilosis* showed an increase of the antifungal activity of clotrimazole-loaded nanoparticles prepared with *Lavandula* or *Rosmarinus*, thus confirming that nanostructured lipid carriers (NLC) containing these essential oils represent a promising strategy to improve drug effectiveness against topical candidiasis [174].

A novel therapeutic strategy that has been adopted is photodynamic therapy (PDT). It is based on the interaction between a nontoxic photosensitizer and a safe source of visible light at a low intensity; the combination of these two factors in the presence of oxygen leads to the development of reactive oxygen species (ROS) which are toxic and cause oxidative damage to microorganism cells [175]. Curcumin associated with LED light was an efficient strategy against biofilms of *C. dubliniensis* isolates [176]. The uptake of CUR by yeast cells and its penetration through the biofilm were accompanied by confocal laser scanning microscopy. Daliri et al. [177] have assessed the effect of curcumin- and methyl blue-mediated PDT in combination with different laser exposure parameters on *C. albicans* colonies. They verified that the 460-nm laser in combination with CUR has the maximum antifungal efficiency against *C. albicans*.

Although we have described herein many in vitro studies examining synergistic effects among potential antifungal biomolecules and traditional antifungal agents, the mechanisms underlying these synergistic effects are poorly understood. Randomized and controlled analyses have been performed with the objective of verifying the efficacy and risks of using traditional antifungal combinations; however, the results are poor and contradictory. High cost to conduct these strategies, reduced number of clinical cases, and the existence of confusing variables are factors that contribute to the obtaining of vague and non-reproducible results.

Therefore, it is extremely relevant to examine carefully possible synergism between new phytocompounds and conventional antimycotic drugs in order to obtain more insight. Understanding the cellular action of each substance in the combination process is also a key step in inferring ways to employ strategy in the clinic. A lack of consensus in the medical clinic emphasizes the need to conduct further clinical trials using combinations of antifungals. The experiments and results addressed herein support further investigation of new plant constituents with antifungal properties and the efficacy of combination therapies involving phytocomponents and traditional antifungal agents as an important start for the development of unusual and original antifungal therapies.

6. Conclusions

The increase in *Candida* and *Cryptococcus* infections is alarming leading to high rates of morbidity and mortality worldwide. Concomitantly with the increase in fungal infections, species emerged, and the resistance phenomenon increased so that the available antifungal arsenal becomes irrelevant in the face of the problem. In addition, there are limitations manifested by some antifungal agents such as fungistatic character, severe toxicity, and renal dysfunction. Therefore, it is crucial to develop new drugs as alternative therapies that are potentially active against *Cryptococcus* and *Candida*. Plants are considered abundant and safe sources of phytochemicals endowed with many biological activities. Several polyphenols have been isolated and studied in relation to their anti-yeast and anti-virulence activities and may be useful in obtaining promising, efficient, and cost-effective drugs for the inhibition of *Candida* and *Cryptococcus* infections. Many phytosubstances are extremely effective in combination therapy with traditional or other phytochemicals, which can be further exploited to lead to novel drug therapies against recalcitrant infections.

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Conflict of interest

The authors declare no competing interests.

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Chapter 12

Modulation of Edible Plants on Hepatocellular Carcinoma Induced by Aflatoxin B_1

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Abstract

Aflatoxin B_1 (AFB_1) is one of the major causes of liver cancer especially hepatocellular carcinoma that has high incidence and mortality rate in many countries. Owing to the climate that is suitable for fungal growth, the avoidance of AFB_1 exposure from agricultural product contamination is too difficult. This up-to-date review aims to collect insight on how edible plants attenuate AFB_1-toxicity. Cruciferous vegetables, green tea, purple rice, turmeric, green vegetables, ginger, *Dialium guineense*, *Parkia biglobosa*, carotenoid-rich fruits and vegetables, Allii Fistulosi Bulbus, and rosemary have reported their capabilities to alleviate AFB_1-toxicity though several mechanisms. All these plants showed anti-genotoxic activity while some of them are able to reduce hepatotoxicity, liver cancer, and oxidative stress and modulate metabolism enzymes induced by AFB_1. Furthermore, a few edible plants could handle AFB_1 in pre-exposure phase including anti-AFB_1 biosynthesis and AFB_1 absorption. Although the detoxification mechanisms of AFB_1 activated by various edible plants have been investigated in pre-clinical study for a decade, clinical trial is still rarely clarified. Further study associating with a protective effect on AFB_1 toxicity still needs to be carried out especially in the clinical study.

Keywords: mycotoxins, liver cancer, natural products, detoxification, chemoprevention

1. Introduction

Liver cancer is the third most common cause of cancer mortality worldwide especially in developing countries. Epidemiological studies among all continents found that Asia and Africa have higher incidence rate than western world [1]. Hepatocellular carcinoma (HCC), arise due to excessive growth of abnormal liver cells, is most commonly found among all liver cancer types [2]. Four main potential causes of HCC have been identified as viral infection (chronic hepatitis B and C), metabolic syndrome (diabetes and nonalcoholic fatty liver disease), immune-related disease (autoimmune hepatitis), and toxic substances (alcohol and aflatoxins) [3].

Aflatoxin B_1 (AFB_1) is a noxious carcinogen produced by certain fungi *Aspergillus flavus* and *A. parasiticus* which mostly contaminate in agricultural

products such as rice, chili, and peanuts. AFB_1 is a Class 1 carcinogen classified by the International Agency for Research on Cancer (IARC), suggesting sufficient evidence of carcinogenicity caused by AFB_1 in both animals and human [4]. Consequently, it is considered as a serious contaminant in many foodstuffs.

Once the AFB_1 is absorbed through human body, it is metabolized at the liver site by phase I metabolizing enzymes including hydroxylation, hydration, demethylation, and epoxidation. Nontoxic metabolites are resulted from hydroxylation, hydration, and demethylation while the reactive metabolite, AFB_1-8,9-epoxide, is resulted from epoxidation [3, 4]. AFB_1-8,9-epoxide is the genotoxic form and can react efficiently with DNA at the N7 site of guanine to form AFB_1 adduct. This adduct can adversely affect DNA sequence and genetic materials. However, human defensive mechanisms are able to detoxify AFB_1 toxicity through phase II metabolism enzymes. AFB_1 can be converted into excretable forms after binding with glutathione and glucuronic acid generated by specific enzyme, glutathione S-transferase (GST) and UDP-glucuronosyltransferase (UGT), respectively [5–7]. Besides acute toxicity such as hepatic necrosis, bile drug proliferation, edema, and lethargy could also be observed after exposure to high dose of AFB_1 [8].

Regarding the current situation, there are many ways to avoid the risk of AFB_1-induced liver cancer as determined by two main periods, pre- and post-harvest period and exposure period [6]. During harvest time, several techniques are used for controlling and reducing the chance of harmful effects resulted from AFB_1: cultivation of AFB_1 tolerance plants, biocontrol using competitive fungi, irrigation, and insecticide. For exposure period, most researches aim to determine the effects of several foods or supplementary foods that are capable of decreasing AFB_1-induced toxicity. For example, oltipraz, a synthetic derivative of natural compound originated from cruciferous vegetables, is reported on its capacity to reduce AFB_1 toxicity. In addition, green tea polyphenol and chlorophyllin (a derivative of chlorophyll found in green leafy vegetables) are also stated. These natural compounds have a potential against AFB_1-induced hepatocarcinogenicity by decreasing the absorption of AFB_1, controlling metabolic pathway, and increasing AFB_1 excretion [6, 9]. To update the involvement of edible plants as chemoprevention for AFB_1, this review is aimed to emphasize the mechanistic alleviation of AFB_1-induced liver toxicity by polyphenol-containing plants.

2. Effects of edible plants on toxicity induced by AFB_1

2.1 Cruciferous vegetables

Cruciferous vegetables belong to *Brassica* genus, Brassicaceae family which are usually known as broccoli, Brussels sprouts, cabbage, cauliflower, kale, and radishes and commonly used for food consumption. They are not only rich sources of fibers, vitamins, and carotenoids as their important components, but also contain higher glucosinolate content than other vegetables [10]. Glucosinolates are secondary metabolites in cruciferous veggies and can be divided into three classes based on their structure: aliphatic glucosinolates, indole glucosinolates, and aromatic glucosinolates.

Nearly 200 types of glucosinolates have been reported in scientific literature, especially glucobrassicin and glucoraphanin. These two compounds can be transformed into hydrolysis products such as isothiocyanates, sulforaphane (SF), and indole-3-carbinol (I3C) by β-thioglucosidase (myrosinase) enzyme when plant cells are damaged. This mechanism could also be processed by bacteria in the gastrointestinal tract [11, 12].

The studies of anticancer effects of glucosinolates and their hydrolysis products revealed that numerous existing compounds also had anticancer mechanism against various types of cancers. For instance, the presence of sulforaphane could suppress carcinogen and prevent DNA adduct (a biomarker of AFB_1 exposure) directly through an inhibition of phase I metabolism enzymes. At the same time, it induces phase II metabolism enzymes which play an important role in converting carcinogens to the inactive metabolites and excreting from the body. Their hydrolysis products exhibit an ability to scavenge the free radicals, inhibit inflammation and angiogenesis, and also induce an apoptosis of cancer cells [11].

Previous studies investigated the effects of bioactive compounds such as I3C and 1-cyano-2 hydroxy-3 butene (Crambene), derivatives of glucosinolate group found in cruciferous veggies, on HCC occurrence. Glucosinolates did not only respond for abnormal liver cells, but they also enhance AFB_1 detoxification in the rat model. Pre-exposure to the high-dose combination of I3C and Crambene (0.15 and 0.165%, respectively) protected the liver cells effectively more than low-dose combinations and single exposure [13]. Risk reduction of liver cancer could also be observed in rainbow trout when pre-exposed to I3C at the dose 2000 ppm prior to AFB_1; however, the adverse effects and increase of liver cancer incidence were reported when the exposure sequence was reversed [14]. In addition, further studies revealed a dose-dependent relationship between I3C dose after exposure to AFB_1 and the incidence of liver cancer and other cancer types [15]. Thus, it could be summarized that the incidence of liver cancer is induced by AFB_1 relating to timing of I3C exposure. Pre-exposure to I3C prior to AFB_1 reduced the liver cancer incidence, but post-exposure reversely raised the liver cancer incidence [15]. Accordingly, subsequent mechanistic studies indicated an induction of I3C on phase I and II metabolism enzyme activities [16]. Continuous exposure to I3C might enhance phase II enzyme activity, so the absorbed AFB_1 would be excreted rapidly. In contrast, pre-exposure to AFB_1 triggered the adverse effects such as DNA abnormality and increase of liver cancer risk. The explanation was that pre-exposure to AFB_1 generates AFB_1-8,9-epoxide and this reactive metabolite would be more activated when treated later with I3C. In addition, I3C could be able to induce both phase I and II metabolism enzyme activities, thus AFB_1-8,9-epoxide was more generated as a result of activation of phase I metabolism. Although phase II enzyme was also stimulated, it was not enough to eliminate AFB_1.

Not only I3C is frequently reported, but other glucosinolate derivatives like SF and H-1,2-dithiole-3-thione (D3T) are also stated. For example, while rats were pre-exposed to these derivatives, AFB_1-DNA adduct in rat's liver was reduced due to an increase of GST activity, a phase II detoxification enzyme for AFB_1 [17]. Likewise, other previous studies reported that SF could competitively inhibit CYP1A2 in human liver cells [16], causing a decrease of AFB_1-DNA adduct. Remarkably, upregulation of gene expression-related tissue repairing system and number of hepatocytes were observed after induction of SF [18].

The current epidemiological and clinical studies revealed that only lung, colorectal, breast, prostate, and pancreatic cancers were given the positive response to glucosinolates while animal model showed the effective inhibition of liver cancer and other cancer types through various mechanisms. Nevertheless, randomized clinical trial of glucosinolates on liver cancer showed different results [11, 19]; comparison between broccoli sprout extract treatments and control group was studied simultaneously. After treatment, AFB_1-DNA adducts were clearly determined. The results indicated that no significant difference was observed among tested groups on AFB_1-DNA adduct level ($p = 0.68$). On the contrary, an inverse linear correlation of dithiocarbamates, a metabolite of sulforaphane, and AFB_1-DNA adduct excretion was noted ($p = 0.002$, $R = 0.31$). It can be implied that exposure to glucosinolates might decrease AFB_1-induced toxicity [20]. Besides, various compounds of

glucosinolates have the potential to increase excretion of many carcinogens through glutathione *S*-transferase stimulation. Once the GST was stimulated, carcinogenicity and risk of diseases in human were also decreased [21].

2.2 Green tea

Green tea, *Camellia sinensis,* is a beverage that contains high contents of phenolic compounds at approximately 30% of dry weight. One of the major phenolic compounds in green tea is catechin, particularly epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC) [22].

Recent studies have demonstrated the positive effects of green tea on many diseases and adverse human health conditions such as coronary artery disease, oral heath, bone integrity, thermoregulation balance, and kidney stones. Furthermore, an association between green tea consumption and the incidence of many types of cancer has also been reported such as oral and pharynx, esophageal, gastric, colorectal, bladder, prostate, breast, lung, skin, leukemia, pancreatic, and liver cancers [23, 24]. Various research methods including preclinical studies (*in vitro* and *in vivo*), epidemiology, and clinical trial were used to investigate the effect of crude green tea extract or single compound like EGCG on many types of cancer [25]. Overall, an anti-cancer mechanism of green tea extracts against cancer cells was evidently elucidated. Green tea extracts were able to induce apoptosis of cancer cells through inhibiting nuclear factor kappa light chain enhancer of activated B cells (NF-κB) activity and B-cell lymphoma extra-large (Bcl-xL) mRNA expression. Besides, the reduction of angiogenesis of cancer cells was also resulted by green tea extracts through inhibition of vascular endothelial growth factor (VEGF) expression [26].

Previous studies have been reported on several protective ways against AFB_1-induced liver cancer from the exposure to catechin compounds and green tea extracts. For example, the reduction of chromosome aberration in rat bone marrow cells was observed after pre-exposure with green tea or EGCG for 24 hours prior to AFB_1 [27]. Besides, hepatic nuclear AFB_1-DNA binding and glutathione S-transferase placental form (GST-P) positive single hepatocyte, specific markers of hepatocarcinogenic potential in the rat model, were also reduced after pre-exposure with green tea extracts for 2–4 weeks prior to AFB_1 [28]. Similarly, the levels of GST-P and γ-glutamyl transpeptidase positive hepatic foci induced by AFB_1 and carbon tetrachloride were reduced during pre- or co-treatment with green tea extracts. Furthermore, the inhibition of hepatocarcinogenesis was also observed [29].

The studies of green tea against AFB_1-induced human liver cancer are still currently limited, and most reports have been retrieved from China. As some Chinese commonly consume food contaminated with AFB_1, the risk of HCC is higher than other regions. A clinical study demonstrated a protective effect of 500 and 1000 mg/day green tea polyphenol (GTP) on hepatocarcinogenesis in 124 HCC patients who presented with HBsAg and aflatoxin-albumin adducts. Results showed that 8-hydroxydeoxyguanosine (8-OHdG) level, an oxidative DNA damage biomarker originating in urine specimens, significantly decreased ($p = 0.007$) during co-exposure with GTP for 3 months [30]. Besides, AFB_1-albumin adducts (AFB_1-AA) and AFB_1-mercapturic acid (AFB_1-NAC) level in blood and urine specimens of volunteers were compared among 500 and 1000 mg GTP treatment group and control group. This result revealed a reduction of AFB_1-AA level, an indicator of AFB_1 exposure, for both 500 and 1000 mg GTP treatment groups within 3 months. This reduction was strongly related to dose and duration of GTP exposure ($p = 0.049$). Furthermore, AFB_1-NAC, an indicator of AFB_1 elimination activated by phase II metabolism enzymes, significantly increased ($p < 0.001$) in both treatment groups related to dose and duration of GTP exposure as well

($p < 0.001$). Therefore, it could be summarized that GTP effectively modulated AFB_1 biotransformation by inhibition of phase I metabolism enzymes as can be seen from the reduction of AFB_1-AA. GTP also has an induction effect to phase II metabolism enzymes which transform AFB_1–8,9-epoxide to AFB_1-NAC [31].

Furthermore, results from a meta-analysis investigating the effect of green tea extracts on HCC and other liver diseases also showed that regular green tea drinkers had a lower incidence of HCC than nonregular drinkers approximately 26% (R = 0.74, 95% CI = 0.56–0.97, p = 0.027). Although there were some inconsistent results in this study (I^2 = 80.1%, p = 0.000), no publication bias was detected and no data from one study significantly influenced the final conclusion [25].

2.3 Purple rice

Anthocyanins, members of flavonoid groups, are mostly found in blue, purple, orange, and red vegetables. Anthocyanins in plants play a vital role in attraction of bugs for pollination and insect resistance [32]. Pharmacologically, purple corn extracts have been known for its anti-diabetic and antiadipogenic effects, anti-prostate carcinogenesis, and others [33–35] while blue butterfly pea flower has a definite potential anti-inflammatory effect [36]. Furthermore, anthocyanin-rich plants were shown to protect neurodegenerative and also cardiovascular disease [37].

Purple rice bran (*Oryza sativa* L. var. *indica*) contained flavonoids and anthocyanins approximately 53 and 2 mg/g, respectively. Both compounds were reported to reduce AFB_1-induced toxicity, and they were capable of inhibiting mutagenicity in *Salmonella typhimurium* strains TA98 and TA100 [38]. In animal model, rats were pre-treated with purple rice bran extracts for a month before exposure to AFB_1. Then, the expression of CYP450 including CYP1A2 and CYP3A was investigated; both of them have an identical role in transforming AFB_1 to AFB_1-epoxide. The results showed that the extracts could not only inhibit the expression of CYP1A2 and CYP3A, but also increase the expression of GST and UGT which encouraged AFB_1 excretion. Further in *in vivo* studies, the genotoxicity was evaluated by micronucleus assay, and the result showed lower micronucleus formation in extract-pretreated group than AFB_1 treated alone, confirming the capability of purple rice bran extract on the prevention of AFB_1-induced genotoxicity [39].

Apart from purple rice bran extract, other anthocyanin-rich plants are also studied for their effects on AFB_1-induced cytotoxicity. For instance, *Lannea microcarpa,* a tropical African plant, has been studied for its activities against hepatotoxicity, DNA fragmentation, and oxidative stress induced by AFB_1. Before exposure to AFB_1, animals were pre-exposed with *Lannea microcarpa* extracts for 6 months. Results showed that hepatotoxicity, DNA fragmentation, and oxidative stress was lower in extract-pretreated group when compared to AFB1-treated group [40].

2.4 Turmeric

Turmeric is a flowering plant widely used as a food ingredient in South Asia for a long period of time. It has been also applied in pharmacognosy field as a powerful anti-inflammatory resulting from rheumatoid arthritis, bruise, epilepsy, abdominal pain or discomfort, and asthma [41]. An *in vivo* study of turmeric clearly showed the anticancer properties of turmeric on liver, skin, and colorectal cancers. It has a strong potential to inhibit cancer cell growth through stimulating apoptosis and inhibiting phase I metabolism enzymes. It can also stimulate phase II metabolism enzyme activities which play an important role in converting reactive metabolites to excretable forms. Also, turmeric exhibits the antioxidant capacity which can effectively detoxify oxidative stress [42].

Curcumin is a major active component of turmeric. It belongs to curcuminoid group and commonly found in 2–8%. Previous *in vivo* studies investigated the effects of turmeric and curcumin on AFB_1-induced toxicity, and results showed that turmeric and curcumin decreased AFB_1-adduct formation, biomolecule damage, and hepatotoxicity [43–46], and it also inhibited acute toxicity through disturbing the lysis of erythrocytes [47]. During AFB_1 metabolism, free radicals generated by AFB_1 could be readily inhibited by turmeric and curcumin via decreasing lipid peroxidation and enhancing glutathione content. Likewise, they could activate several antioxidant enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), GST, and UGT which play a fundamental role in converting AFB_1 to excretable forms [43–46].

Turmeric is found to be capable of reducing both AFB_1-induced toxicity and HCC. Besides, it could also stimulate apoptosis of liver cancer cells through a mitochondria-dependent pathway and accumulation of calcium ions within the cells [48]. Turmeric showed the protective effect against AFB_1-induced liver cancer in animal model by inhibition of metastasis and growth factor expression related to the progression of angiogenesis [49].

2.5 Green vegetables

Chlorophyll (chla), a main component of green vegetables, consists of a porphyrin ring structure where magnesium is the central atom of the ring. Chla is important for plants' photosynthesis pathway and used as food additives. One of the characteristics of chla is almost insoluble in water while chlorophyllin (CHL), a derivative of chla, is completely soluble. CHL can be transformed into water-soluble form by saponification, a reaction that magnesium central atom is replaced with copper. *In vivo* and clinical studies in pharmacological researches of both chla and CHL revealed that they provided the therapeutic uses such as wound healing, anti-inflammation, anti-oxidation, anti-mutagenesis, and anti-carcinogenesis [50, 51].

Previous studies on the protective effects of chla and CHL on AFB_1 toxicity indicated that both compounds could reduce absorption of AFB_1 from apical to basolateral sides in Caco-2 cell line [52]. Accordingly, a crossover clinical trial demonstrated that chla and CHL exposure could reduce maximum concentration (C_{max}) and area under the curves (AUC) of AFB_1 compared to untreated group [53]. These findings suggest that chla and CHL have a strong potential to decrease AFB_1 absorption. The effects of chla and CHL co-exposure with AFB_1 have also been studied in animal model by emphasizing on antioxidant activities. Both bioactive compounds are capable of reducing AFB_1 toxicity through enhancing the expression of glutathione level and several antioxidant enzyme activities such as GPx, SOD, and CAT [54].

A recent study investigated the effects of CHL on AFB_1-induced hepatotoxicity and incidence of carcinogenesis in animal model. Exposure with CHL reduced hepatotoxicity and incidence of liver cancer [54, 55]. In a clinical study, a randomized controlled trial reported that daily exposure with CHL for 4 months decreased AFB_1-N7-guanine level in urine compared to placebo group [56].

Several studies were in agreement that chla and CHL reduce AFB_1-induced liver cancer through decreasing AFB_1 absorption in digestive tract contributing to the decrease of AFB_1 bioavailability. Besides, chla and CHL are the powerful antioxidants which effectively lower AFB_1-induced oxidative stress. These two compounds not only reduce hepatotoxicity, but also incidence of liver cancer. Thus, the consumption of green vegetables is one of the alternatives to reduce toxicity caused by consuming AFB_1-contaminated foods.

2.6 Ginger (*Zingiber officinale* Roscoe)

Ginger (*Zingiber officinale* Roscoe) contained high content of phenolic compounds in which 6-gingeerol and 6-shogaol are main constitutions [57]. Ginger plays a critical role as hepatoprotective effects through antioxidant mechanism; for example, liver injury by administration of country-made liquor (CML) and iron-induced nonalcoholic fatty liver disease (NAFLD) [58] and liver cirrhosis induced by carbon tetrachloride [59]. It was also reported to show the protective effects against AFB_1-induced toxicity.

In in vitro model of AFB_1-treated HepG2 cells, ginger extract-pretreated cells exhibited higher percent cell viability and lower intracellular ROS production and DNA strand break when compared to AFB_1 treatment alone. In Wistar rats, pretreatment with ginger extract also increased the activities of antioxidant enzymes: GPx, GST, CAT, and SOD, decreased malondialdehyde (MDA) level, and increased reduced glutathione (GSH) content. Co-incubation with ginger extract along with AFB_1 also showed a hepatoprotective effect as seen by the lower level of serum enzymes: alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH). Moreover, fat droplets and hepatocyte infiltration with macro-vesicles in liver induced by AFB_1 were normalized when pre-treated with ginger extract, clearly showing the effectiveness of ginger on AFB1-induced hepatotoxicity [57].

Mechanism of ginger extract to reduce AFB_1-induced hepatotoxicity was demonstrated by *in vivo* study. The expression of nuclear factor-E2-related factor 2 (Nrf2), a redox-responsive transcription factor, was increased when pre-treated with ginger extract. Nrf2 was translocated into the nucleus to regulate the antioxidant response element (ARE) which is the promotor of detoxification and antioxidant genes. Moreover, administration of ginger extract induced the expression of heme oxygenase 1 (HO-1) which is associated with the normalization of redox status [57]. Therefore, ginger extract could reduce AFB_1-induced hepatotoxicity in both *in vitro* and *in vivo* through antioxidant activities controlled by the function of Nrf2 and HO-1.

2.7 Plants in family Fabaceae

2.7.1 Dialium guineense

Dialium guineense is a fruit-bearing tree known as the velvet tamarind. Their bark, leaves, seeds, and fruit showed biological properties such as antimicrobial activities, anti-infectious diseases, and wound-healing [60, 61]. Extract from *Dialium guineense* showed ROS scavenging activities and could normalize the levels of enzyme biomarkers of hepatotoxicity: ALP, AST, and AST induced by AFB_1. Furthermore, treatment of velvet tamarind extract before AFB_1 exposure increased the antioxidant activities of various enzymes including SOD, GPx, GR, CAT, GSH, and oxidized glutathione (GSSH), decreased lipid peroxidation, protein carbonyl and DNA fragmentation. *In vitro* and *in vivo* experiments have also confirmed the protective effects of velvet tamarind extract against hepatotoxicity induced by AFB_1 via antioxidant properties [62].

2.7.2 Parkia biglobosa

Parkia biglobosa, known as the African locust bean tree (ALBT), is a perennial tree legume growing in West Africa. Several parts of ALBT (bark, leaves, pods,

stem, and fruit pulp) showed medicinal properties such as antimicrobial activities, antihypertensive effects, antidiabetic activity, antidiarrheal activity, and others [63]. Pulp extract of ALBT exhibited abilities against antioxidant imbalance induced by AFB_1. When pretreated in animal model, pulp extract of ALBT were capable of inducing SOD, CAT, GPx, GR, and glucose-6-phosphate dehydrogenase (G6PD) activities and increasing GSH and GSSG content. In addition, pretreatment with pulp extract of ALBT reduced lipid peroxidation products, protein carbonyl, and DNA fragmentation induced by AFB_1. AFB_1 treatment also resulted in decrease of hepatocellular enzyme activities: ALP, ALT, and AST compared to control while the pretreatment with pulp extract of ALBT increased these enzyme activities in a dose-dependent manner. Accordingly, antioxidant imbalance and hepatotoxicity induced by AFB_1 were able to be alleviated by pretreatment with pulp extract of ALBT [64].

2.8 Carotenoid-rich fruits and vegetables

Carotenoids, natural plant pigments giving the color of fruits and vegetables, are responsible for the red, orange, and yellow colors in mangoes, corns, carrots, pumpkins, tomatoes, etc. More than 700 different carotenoids have long been characterized and classified as two main groups regarding their basic functional group [65]. Xanthophylls, yellow or orange-yellow pigments, are found widely in nature and the majority of their structure consists of oxygen as the core element such as lutein and zeaxanthin. Carotenes, one of another division of carotenoids, are hydrocarbon compounds without other functional groups including α-carotene, β-carotene, and lycopene [66]. Both xanthophylls and carotenes are almost known as fat-soluble compounds dissolved well in petroleum, ether, chloroform, and hexane but carotenes seem to be more soluble in these nonpolar aliphatic solvents compared to xanthophylls; some are water-soluble [67]. Carotenoids have a potential role as a provitamin A compound which can be converted within the body to vitamin A, and they are broadly accepted as free radical antioxidants inhibiting several types of cancers [68, 69].

Several carotenoids like β-carotene, canthaxanthin, lycopene, and cryptoxanthin were studied on the mitigation of AFB_1-induced mutagenesis in bacterial mutation assay. Mutagenesis was inhibited by the addition of all carotenoids, except lycopene, and cryptoxanthin was shown to be the most potent inhibitor among all tested carotenoids [70]. The comparison of both ionone rings, α and β type of carotenoids, was observed through suspended disc culture. The α-ionone ring carotenoids, α-carotene, lutein, or α-ionone, showed more inhibition of AF biosynthesis than β-ionone ring, and the existence of hydroxyl groups on the rings seemed to lessen the inhibition capacity [71].

Previous study demonstrated the effects of antioxidants β-carotene and lycopene on AFB_1-induced hepatotoxicity. The result showed the presence of lycopene followed by the addition of AFB_1 increased cell viability at approximately 14%, while pretreatment with β-carotene had the highest increase in cell survival up to 54%. Both carotenoids recovered mitochondrial dehydrogenase (MD) activity up to 85%, upregulated *p53* gene expression in AFB_1-exposed cells, and decrease in AFB_1-N7-guanine adducts. These results clearly showed that both β-carotene and lycopene could prevent AFB_1-induced toxicity in HepG2 cells [69].

Lycopene, a strong free radical scavenger having the greatest ability to cope with the singlet oxygen compared to the other carotenoids, can alleviate AFB_1-induced oxidative stress through the conjugation of the p-electron system with several

reactive oxygen species. It can protect DNA, proteins, and lipid damages against the carcinogenesis onset contributed to its numerous conjugated double bonds, high lipophilicity, and acyclic structure [72]. Regarding several scientific publications, lycopene has been confirmed as the carotenoid that exhibited robust positive effects on AFB_1 toxicities via several pathways.

2.9 Allii Fistulosi Bulbus

Allium plants like garlic and onion are well-known in Asian countries as food ingredients and remedial foods. They have been documented as medicinal foods worldwide due to their pharmacological properties. *Allium fistulosum* (*A. fistulosum*), a perennial herb in *Allium* genus, has been commonly utilized as appetite inducer and medication against cold symptoms [73]. Also, it has ability to activate the immune response and antihypertensive effect as well as antioxidant defense system. The consumption of *A. fistulosum* extract increased estrogen level, mediated the conversion of testosterone to estrogen, and conducted hormone balance in female rats resulting in the enhancement of ovarian function [74]. The extract is able to downregulate the accumulation of lipid in HepG2 cells without cytotoxic effect and fatty acid gene synthesis. Similarly, mice fed high-fat, high-sucrose diet displayed an increase in body weight, hepatic weight, and fat accumulation in hepatocytes, but these adverse effects were attenuated by extract supplementation [75].

The effects of Allii Fistulosi Bulbus (VEAF) extract on cytotoxicity and oxidative stress caused by AFB_1 exposure were observed in HepG2 cells. Preincubation with VEAF followed by the addition of AFB_1 obviously enhanced cell viability. It inhibited oxidative stress through declining ROS level and TBAR content induced by AFB_1 and promoting GSH level. The determination of 8-OHdG, an indicator of oxidative damage on DNA, was then investigated. The result showed the inhibitory effect in VEAF treatment group up to 59.1% suppression compared to AFB_1-treated group. This evidence proved the alleviating potential of VEAF on AFB_1-induced oxidative stress resulting in cytoprotection against AFB_1 toxicity [76].

Quercetin, flavonol, is one of the major bioactive compounds in *Allium* plants. It shows the potential to scavenge free radical and improve health effects, that is, aging, allergy, angioprotective properties, anti-inflammatory, anti-cancer, anti-obesity, arthritis, asthma, diabetes, etc. [77]. For AFB_1 biosynthesis in *Aspergillus flavus*, quercetin notably decreased AFB_1 production (51%) in corn flour supplemented with quercetin at 48-hour incubation. Quercetin has an ability to inhibit the expression of necessary enzymes for AFB_1 biosynthesis such as acetyl CoA synthetase, esterase, and O-methyl transferase A and involves in the MAPK pathway which is the major pathway to form AFB_1. Quercetin, therefore, has the ability to be an anti-aflatoxigenic agent [78]. Quercetin also inhibited proliferation of *Aspergillus flavus* and its AFB1-biosynthesis through regulating the expression of development-related genes and aflatoxin production-related genes [79].

In HepG2 cells, quercetin decreased AFB1-induced cytotoxicity and ROS production and increased GSH content while *in vivo* study showed enhanced antioxidant activities and reduced lipid peroxidation [80]. After AFB_1 consumption, quercetin depicted the prevention of genotoxicity caused by AFB_1 in rat liver microsomes. Co-incubation with quercetin significantly decreased micronuclei formation compared to treated with AFB_1 alone ($p < 0.05$) [81]. Corresponding to another study, serum cytokines, procollagen III, and nitric oxide were significantly reduced during co-administration with quercetin and AFB_1 ($p < 0.05$). Quercetin also upregulated the antioxidant enzymes that may affect the decrease of DNA fragmentation and apoptosis [82]. Likewise, the administration between

AFB1-contaminated diet in rat resulted in a decrease of total proteins and RNA content and fatty acid synthase (Fas) and tumor necrosis factor (TNF) gene expression in the liver tissue caused by AFB_1 while co-administration with quercetin normalized these parameters [83].

Even though numerous studies revealed the hepatoprotective effects of quercetin against xenobiotic-induced cellular toxicity, low bioavailability of quercetin absorbed into circulation is the remarkable barrier [84]. One of the supreme strategies widely used is nanoformulation. Quercetin nanoparticles not only demonstrated a noteworthy reduction of AFB_1-induced cell death, but it also suppressed the liver toxicity caused by AFB_1 including ROS formation, lipid peroxidation, mitochondrial membrane potential collapse, and GSH depletion. In addition, both quercetin and quercetin nanoparticles significantly enhanced the function of hepatic enzymes (AST, ALT, and ALP) and hepatic antioxidant enzymes (SOD, CAT, and GPx) ($p < 0.05$). Interestingly, quercetin nanoparticles showed higher effects than quercetin [84]. These result reflexes an inhibiting ability of AFB_1 toxicity by administration of quercetin AFB_1.

AFB_1 also caused increase of cytotoxicity in a bovine mammary epithelial cell line. The pre-incubation with quercetin affected to increase cell viability, AFM_1 biosynthesis (low toxic metabolite of AFB_1), GSH content, and mRNA level of glutathione S-transferase alpha 1 (GSTA1) which are important for AFB_1 detoxification [85].

2.10 Rosemary plant (*Rosmarinus officinalis L.*)

Rosemary plant (*Rosmarinus officinalis L.*), naturally found in the western Mediterranean region, has been widely used as a food additive. As it contains high polyphenolic contents, it shows many pharmacological properties such as antioxidant activity and antimicrobial and antimycotic properties, etc. [86]. Previous study proved that the growth of *Aspergillus flavus and A. parasiticus* were significantly inhibited by 4% commercial rosemary essential oil from 28.2 to 59.5% and 41.5 to 52.4%, respectively [87]. Apart from antimycotic properties, dose-dependent exposure of carnosic acid—major polyphenolic compound in rosemary plants—clearly decreased cell death caused by 10 μM AFB_1. Pre-treatment to carnosic acid also reduced the production of ROS and the concentration of 8-OH-deoxyguanine, clearly confirming an involvement of carnosic acid in the protection of cytotoxicity induced by AFB_1 [88]. Furthermore, both rosemary extract and its active components (carnosol and carnosic acid) exhibited a potent inhibition of DNA adduct formation. They not only inhibit phase I metabolizing enzymes but also induce phase II metabolizing enzymes such as GST that promote the cellular defensive mechanism against AFB_1 [89].

3. Conclusion

Consumption of AFB1-contaminated food is the current major cause of HCC in many countries. Many studies aim to lower AFB1-induced toxicity particularly the utilization of edible plants as protective foods. This review proposed the edible plants which could alleviate AFB1-induced toxicity and concluded the possible mitigation of AFB1 toxicities through several related pathways (**Table 1** and **Figure 1**). Although the detoxification mechanism of AFB1 activated by various plants has been investigated in a pre-clinical study for a decade, clinical trial is still rarely clarified. Further investigation on a risk reduction of AFB1 still needs to be carried out especially in the clinical study.

Plants	Reference	Protective effects							
		Inhibit AFB_1 biosynthesis	**Inhibit AFB_1 absorption**	**Anti-oxidant**	**Anti-genotoxicity**	**Reduce cytotoxicity**	**Modulate metabolism enzymes**	**Inhibit hepatotoxicity**	**Decrease liver cancer**
Cruciferous vegetables	[10]			/			/		/
	[14]				/				/
	[15]								/
	[16]			/			/		
	[17]				/		/		
	[20]				/				
	[21]						/		
Green tea	[25]								/
	[27]				/				
	[28]								/
	[29]								/
	[30]			/					
	[31]*						/		
Purple rice	[38]				/				
	[39]				/		/		
	[40]			/	/			/	
Turmeric	[43]			/				/	
	[44]			/	/		/	/	
	[45]			/				/	
	[46]			/				/	
	[47]					/			
	[48]					/			/
	[49]			/			/		/

Plants	Reference	Protective effects							
		Inhibit AFB_1 biosynthesis	Inhibit AFB_1 absorption	Anti-oxidant	Anti-genotoxicity	Reduce cytotoxicity	Modulate metabolism enzymes	Inhibit hepatotoxicity	Decrease liver cancer
Green vegetables	[52]		/						
	[53]		/						
	[54]			/				/	/
	[55]			/				/	/
	[56]				/				
Ginger	[57]			/	/	/		/	
Dialium guineense	[62]			/	/			/	
Parkia biglobosa	[64]			/	/				
Carotenoid-rich fruits and vegetables	[69]				/	/			/
	[70]				/				
	[71]	/							
	[72]			/			/		
Allii Fistulosi Bulbus	[76]			/		/	/		
	[78]	/							
	[79]	/							
	[80]			/		/			
	[81]				/		/		
	[82]*			/	/				
	[83]**			/	/			/	
	[84]			/		/		/	
	[85]			/		/	/		

Plants	Reference	Protective effects							
		Inhibit AFB_1 biosynthesis	**Inhibit AFB_1 absorption**	**Anti-oxidant**	**Anti-genotoxicity**	**Reduce cytotoxicity**	**Modulate metabolism enzymes**	**Inhibit hepatotoxicity**	**Decrease liver cancer**
Rosemary	[87]	/							
	[88]			/		/			
	[89]				/		/		

**Alleviate serum cytokine and procollagen III, NO.*
***Alleviate content of nucleic acid of liver tissue.*

Table 1.
The protective effects of edible plants against AFB_1-induced toxicity.

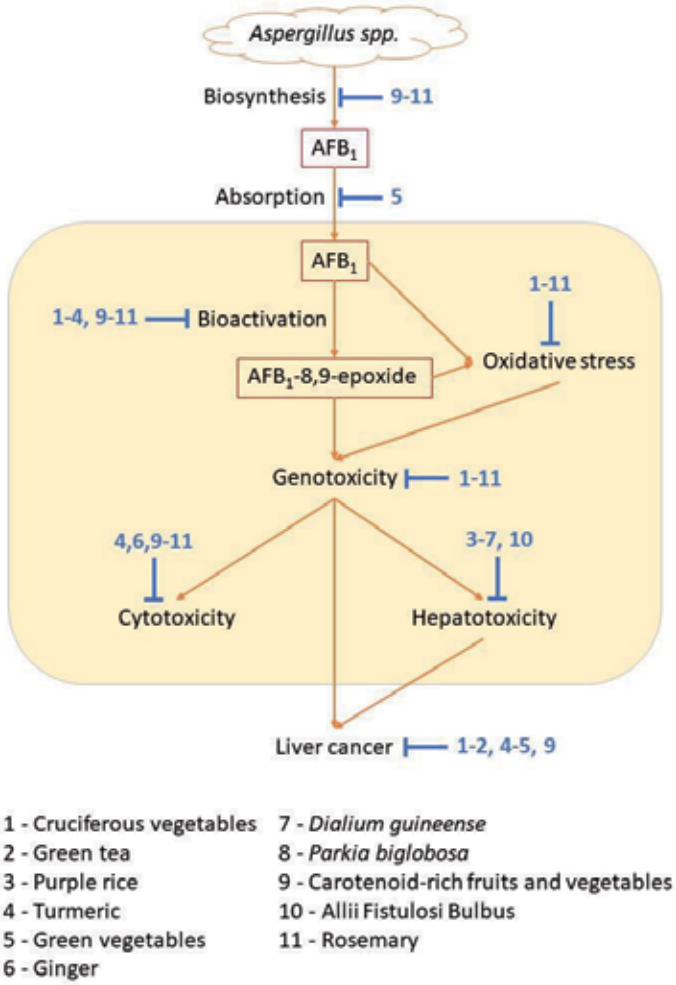

Figure 1.
Protective effects of edible plants against AFB_1-induced toxicity.

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Conflict of interest

Authors declare no conflict of interest.

Abbreviations

AFB_1	Aflatoxin B_1
AFB_1-AA	AFB1-albumin adducts
AFB_1-NAC	AFB1-mercapturic acid
ALBT	African locust bean tree
ALP	Alkaline phosphatase

ALT	Alanine aminotransferase
ARE	Antioxidant response element
AST	Aspartate transaminase
AUC	Area under the curves
Bcl-xL	B-cell lymphoma-extra large
C_{max}	Maximum concentration
CAT	Catalase
CHL	Chlorophyllin
chla	Chlorophyll
CML	Country-made liquor
D3T	H-1,2-dithiole-3-thione
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
Fas	Fatty acid synthase
G6PD	Glucose-6-phosphate dehydrogenase
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSH	Oxidized glutathione
GST	Glutathione S-transferase
GSTA1	Glutathione S-transferase alpha 1
GST-P	Glutathione S-transferase placental form
GTP	Green tea polyphenol
HCC	Hepatocellular carcinoma
HO-1	Heme oxygenase 1
I3C	Indole-3-carbinol
IARC	International Agency for Research on Cancer
LDH	Lactate dehydrogenase
MD	Mitochondrial dehydrogenase
MDA	Malondialdehyde
NAFLD	Nonalcoholic fatty liver disease
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
Nrf2	Nuclear factor-E2-related factor 2
8-OHdG	8-hydroxydeoxyguanosine
ROS	Reactive oxygen species
SF	Sulforaphane
SOD	Superoxide dismutase
TNF	Tumor necrosis factor
UGT	UDP-glucuronosyltransferase
VEAF	Allii Fistulosi Bulbus
VEGF	Vascular endothelial growth factor

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