In Vitro Production of Coumarins

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Abstract

Coumarin is a multitudinous nutraceutical compound naturally presented in various part of the plant and can be also produced by microorganisms. Additionally, coumarin can be produced at large-scale through in vitro plant cell culture systems. Coumarins can be found in simple, linear, and angular form, and the biosynthesis pathway of coumarins involves hydroxylation, glycolysis, and cyclization. The extraction process of coumarins requires using polar and nonpolar solvents, and the identification and quantification of coumarins can be performed by chromatographic techniques. Coumarins have attracted considerable attention in recent years due to their potential biological and pharmaceutical properties such as anticancer, anti-inflammatory, antimicrobial, antioxidant, and anticonvulsant properties. These medicinal properties of coumarin and its derivatives demonstrate their significance as promising nutraceutical for multifunctional applications. This chapter mainly discusses biosynthesis, in vitro production, different extractions, and detection methods of coumarins. In addition, the biological activities and the commercial applications of coumarins are also discussed.

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Anticancer · Anticonvulsant · Antioxidant · Biosynthesis · Chromatographic method · Coumarin · Extraction · Pharmaceutical

Abbreviations

bw	Body weight
DAD	Diode array detector
FTIR	Fourier transform mid-infrared
GC-MS	Chromatography-mass spectrometry
HPLC	High-performance liquid chromatography
HPLC-MS/MS	High-performance liquid chromatography-mass spectrometry
UHPLC	Ultra-high-performance liquid chromatography
UHPLC-DAD	Ultra-high-performance liquid chromatography-diode array detector
UV	Ultraviolet
V	Volt
W	Watt

7.1 Introduction

Coumarin is an essentially neutral molecule and characterized with its pleasant sweet-bitter taste (Venugopala et al. 2013; Arn and Acree 1998). It is an organic compound from a family of benzopyrones known as 1,2-benzopyrone (2H-1-benzopyran-2-one). Coumarin was first isolated from the tonka bean (*Dipteryx odorata*) in which the name coumarin comes from (Borges et al. 2005; Arn and Acree 1998).

Coumarins are formed of merged benzene and α-pyrone rings. Therefore, the prototypical compound of coumarin is known as 1,2-benzopyrone (Venugopala et al. 2013).

Coumarins naturally occur in a variety of higher plants such as cinnamon, *Angelica archangelica*, *Cinnamomum cassia* Blume, *Mikania laevigata*, *Lavandula angustifolia*, and ***Melilotus officinalis*** (Passari et al. 2014; Bourgaud et al. 2006; Miller et al. 1996). Coumarins are distributed throughout all parts of the plant including the roots, leaves, seeds, and fruits. The highest levels of coumarins have been isolated from fruits followed by seeds (Venugopala et al. 2013). It is evident that the accumulation of coumarins in plants can be triggered by various abiotic and biotic stresses (Bourgaud et al. 2006). Research on the effect of coumarin in plants have demonstrated that coumarin may possess a hormonal property (Bourgaud et al. 2006). Additionally, coumarins have been identified from fungi and bacteria (Venugopala et al. 2013; Arn and Acree 1998).

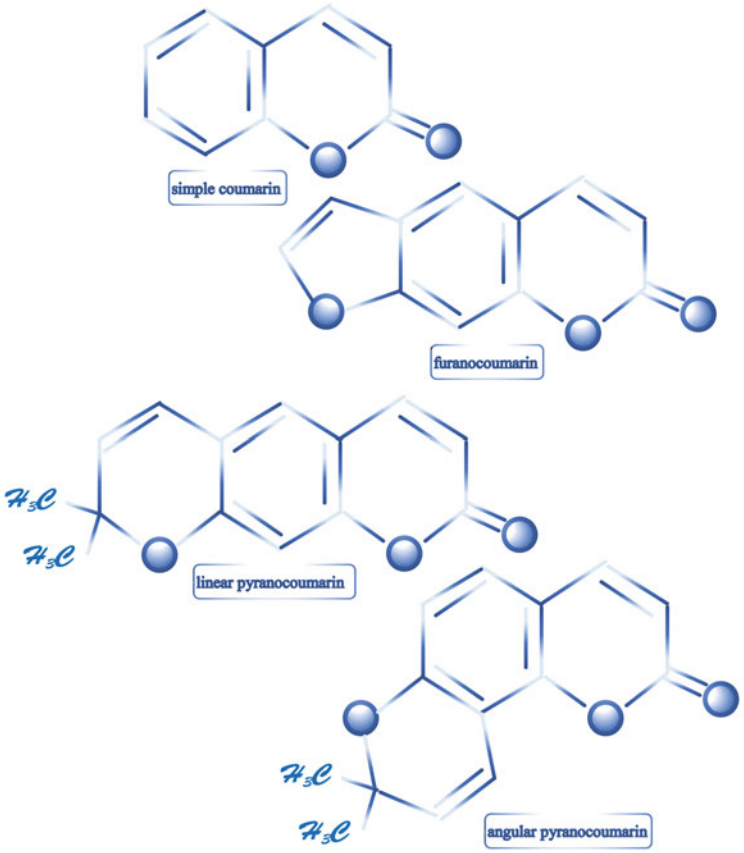

Fig. 7.1 Most common coumarin derivatives

Coumarins can be subcategorized into simple coumarins, pyranocoumarins, phenylcoumarins, and 7-oxygenated coumarin (Bourgaud et al. 2006). The most common hydroxylated coumarins are umbelliferone, herniarin and scoparone, isofraxidin, esculetin, fraxetin, isoscopoletin, daphnetin, and their corresponding glucosides (Bourgaud et al. 2006). However, the most common derivatives of coumarins are simple coumarins, lineal furocoumarins and pyranocoumarins, angular furocoumarins, and pyranocoumarins (Borges et al. 2005, 2009; Bourgaud et al. 2006; Dewick 2002). Simple coumarins are regarded as the most widespread in plants (Fig. 7.1).

Coumarins can bind to glycone by a glycosidic bond to form coumarin derivative called coumarin glycosides. This occurs when anthocyanin is converted to carbinol then into chalcone and finally cleaved into a coumarin derivative. Moreover, coumarin 3,5-diglycoside is also a common derivative that is produced from the degradation of anthocyanins (Rodriguez-Amaya 2019).

The dominant coumarin derivatives are isoimperatorin, oxypeucedanin, imperatorin, ostruthol, angelicin, bergapten, scopoletin, isopimpinellin, and xanthotoxin (Harmala and Vuorela 1990; Kumar et al. 2013).

In terms of health benefits of coumarins, they have exhibited multitudinous pharmacological activities such as anticancer, anti-inflammatory, antimicrobial, antihypertensive, anticoagulant, antioxidant, and anticonvulsant properties. However, isolated coumarin from cassia leaf exhibited cytotoxic effect (Venugopala et al. 2013).

7.2 Biosynthesis Pathway

Coumarins have been synthesized by a number of reactions such as Perkin, Knoevenagel, Reformatsky, and Pechmann reactions. The most popular example of Perkin reaction is that between acetic anhydride and salicylaldehyde (Venugopala et al. 2013; Dippy and Evans 1950). However, Knoevenagel reaction includes synthesis of coumarins by a solvent-free reaction under microwave irradiation and in the presence of piperidine through the condensation of salicylaldehyde or its derivatives with different derivatives of ethyl acetate (Bogdal 1998). The synthesis of coumarins by Pechmann reaction involves a condensation of a phenol and a carboxylic acid or ester group under acidic condition (Daru and Stirling 2011). It has been reported that the required conditions for these reactions include high temperature and strong basic or acidic reaction (Venugopala et al. 2013).

On the other hand, the biosynthesis pathway of coumarins consists of hydroxylation, glycolysis, and cyclization (Bourgaud et al. 2006; Dewick 2002). Hydroxylated coumarins are commonly stress induced in higher plant species (Bourgaud et al. 2006). In fact, coumarins biosynthesis is mostly triggered by various abiotic and biotic stresses in many plants. Additionally, coumarins and their derivative biosynthesis require specific enzymes and genes to be implicated in the biosynthesis process. It has been revealed that lactonization requires ortho-hydroxylation which is preceded by the para-hydroxylation (Bourgaud et al. 2006). The most common hydroxylated coumarins are umbelliferone, herniarin and scoparone, isofraxidin, esculetin, fraxetin, isoscopoletin, daphnetin, and their corresponding glucosides. Umbelliferone is obtained from cis-p-coumaric acid, while coumarin is derived from cis-coumaric acid (Bourgaud et al. 2006). In addition, complex coumarins could be also biosynthesized from prenylated simple coumarin (Bourgaud et al. 2006; Dewick 2002).

The key step of coumarin biosynthesis is ortho-hydroxylation. Nevertheless, coumarins, furocoumarins, and pyranocoumarins are genetically biosynthesized from cinnamic acid. Furanocoumarins can be found as linear furocoumarins and angular furocoumarins. Similarly, linear pyranocoumarins and angular pyranocoumarins have been distinguished as well. The classification is based on the location where the isopentenyl pyrophosphate is condensed to form the heterocycle. In linear type, the dihydrofuran ring is attached at C6 and C7, while it is linked at C7 and C8 in angular furocoumarins (Venugopala et al. 2013).

7.3 In Vitro Production Methodology

Several techniques have been applied for in vitro production of coumarins. In vitro plant cell culture techniques are commonly employed for production of coumarins. Conventional micropropagation approach is applied in vitro plant culture protocols. This approach involves a series of phases (Fig. 7.2). First phase is the selection of an appropriate plant when it is preferable to select insect- and disease-free plant. This first step is called pre-propagation. The second step is known as initiation of the in vitro plant culture. Explants or seeds are selected and their surface sterilization with chemicals. An appropriate culture media was used for the explants which were shortly incubated and any contaminated ones are disposed of, while the rest was used for the next step. The next step is the propagation phase when the shoot, root, or callus is proliferated. Then, the bioreactors are used for scaling up root and callus cultures. The micropropagation phase is used when the propagated shoots are transferred to culture media. Final step is called hardening when the

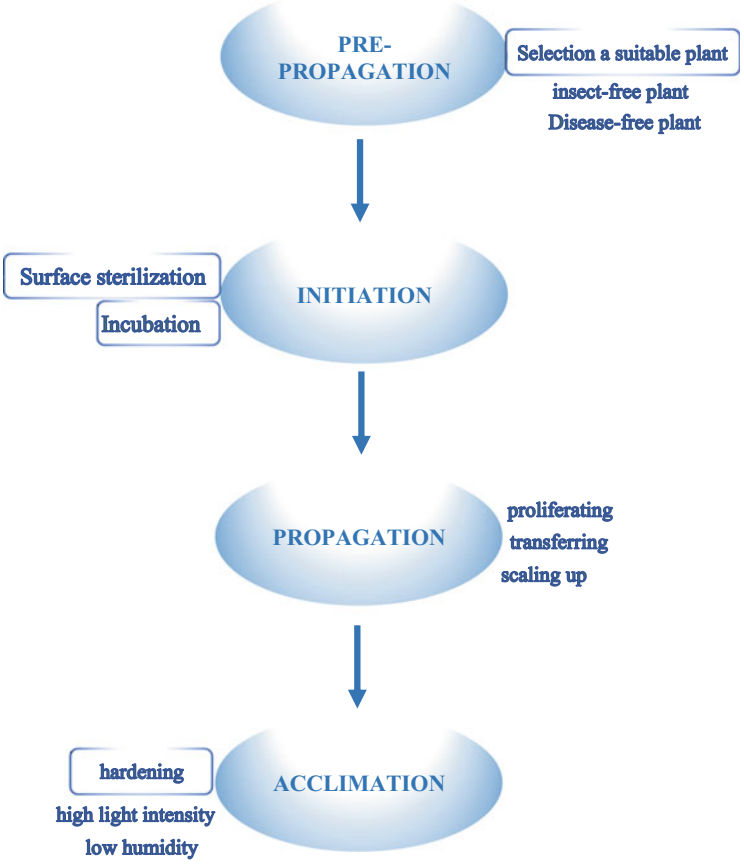

Fig. 7.2 Phases of in vitro production of coumarins

micropropagated plants are gradually hardened to allow the plants to acclimate to ex vitro conditions. In this last phase, the plants are moved from low to high light intensity and from high to low humidity (Espinosa-Leal et al. 2018).

In the unconventional micropropagation techniques, autotrophy of the explants is permitted to be developed in the last phase. An improvement in the quality of the multiplied shoots can be achieved through this autotrophic growth which in turn facilitates the acclimatization of the plantlets (Lucchesini and Mensuali-Sodi 2010).

The best in vitro plant cell culture technique for in vitro production of secondary metabolites including coumarins is cell suspension cultures which offer the most reliable and productive technique to generate phytochemical compounds (Ochoa-Villarreal et al. 2016). In this technique, the calli are first enhanced in a solid medium, and then all cells are transferred to a liquid medium allowing them for growing in shaking flasks and later transferred to a large-scale liquid-phase bioreactor (Furusaki and Takeda 2017). The in vitro production of coumarins can be enhanced by applying elicitors. Abiotic and biotic elicitors are available. Biotic elicitors can be extracted from plant cell wall molecules, plant immune signaling constituents, and microorganisms (Namdeo 2007; Ochoa-Villarreal et al. 2016). Abiotic elicitors include high pressure, high salinity, heavy metals, inorganic salts, and UV irradiation (Ochoa-Villarreal et al. 2016; Luo and He 2004). The selection of the appropriate elicitor will rely upon the metabolite being produced and the plant culture employed (Espinosa-Leal et al. 2018).

There are some factors that play an essential role on the in vitro plants production of coumarins such as the availability of nutrients, type and concentration of the growth regulators, and the type of culture media applied (Espinosa-Leal et al. 2018; Murthy et al. 2014; Fargoso Monfort et al. 2018).

Hairy root cultures are an important alternative strategy for in vitro production with high value of secondary metabolites (Ochoa-Villarreal et al. 2016). This technique is generated by transferring *Agrobacterium rhizogenes* T-DNA into the infected plant genome. To some extent, hairy root systems are genetically stable and easy to manage. A successful evidence in the production of a wide range of secondary metabolites has been achieved through hairy root cultures (Guillon et al. 2006; Ochoa-Villarreal et al. 2016). However, hairy root technique requires that the target molecule has to be synthesized within the given source plant roots. This is an essential limitation in the production of secondary metabolites by hairy root cultures (Guillon et al. 2006; Ochoa-Villarreal et al. 2016).

There is also a major limitation facing the selection of a suitable cell line because cell line is usually loose in its ability to produce the desired secondary metabolites due to genetic instability resulting from somaclonal variation (Ochoa-Villarreal et al. 2016).

7.4 Scale-Up Techniques and Bioreactors

In vitro plant production systems through applying biotechnologies are considered as an attractive approach for scaling up the production of coumarins. During the in vitro productions of coumarins, the propagated cells are scaled up using bioreactors (Ahloowalia et al., 2003; Furusaki and Takeda 2017). Scaling up plant cell cultures from laboratories to industrial large scales is an essential step for commercialization of coumarins production. Indeed, scaling up from laboratories to bioreactors is generally not straightforward as a result of modifications in the cell growth conditions in terms of rheological properties and hydrodynamic shear stresses (Sajc et al. 2000; Ochoa-Villarreal et al. 2016).

Several bioreactors have been successfully adapted for growing plant cells in vitro such as stirred tank bioreactor which is considered as the most widely exploited bioreactor. Recently, there are more improved bioreactor designs such as membrane bioreactors, wave bioreactors, and rotating drum bioreactors (Furusaki and Takeda 2017; Huang and McDonald 2012; Ochoa-Villarreal et al. 2016).

7.5 Extraction and Detection Techniques

Beside their existence in roots, leaves, seeds, and fruits of the plants, coumarins are also found in fungi and bacteria (Venugopala et al. 2013; Arn and Acree 1998). Therefore, extraction of coumarins from their natural sources requires different techniques including ultrasound-facilitated extraction, kinetic maceration, and microwaves-facilitated extraction. Three styles for these techniques are applied including serial ascendingly and descendingly ordered in polarity and non-serial. The solvents used in the extraction include n-hexane, chloroform, methanol, and water (Khalil and Mustafa 2020).

Coumarins are purified and isolated from the selected extract, and the isolated coumarins are chemically characterized to determine their biological activities (Khalil and Mustafa 2020; Mohammed and Mustafa 2020).

When serial style is applied, the coumarins are extracted from the powder of selected plant with water, methanol, chloroform, and n-hexane solvents in order. The residues are extracted by the next solvent in order, and the same methods are applied with the other solvents. Then, the three techniques of extractions were applied. Firstly, kinetic maceration extraction technique is conducted using a shaker water bath of the extracted mixture at 30 °C for 72 h. Secondly, ultrasound extraction technique is applied using ultrasonic water bath to sonicate the extracted mixture for 30 min at 30 °C (Fiorito et al. 2019; Khalil and Mustafa 2020; Mohammed and Mustafa 2020). Thirdly, microwaves-facilitated extraction technique is performed using a domestic microwave oven to irradiate the extracted mixture at 100 W for 5 min (Fiorito et al. 2019; Khalil and Mustafa 2020; Mohammed and Mustafa 2020). Finally, all the extracts from the three techniques are filtered through filter paper, concentrated by a rotary vacuum evaporator at 40 °C, and kept at 4 °C until further analysis. It has been reported that the use of the reduced pressure vacuum generated

about 2.53%, 2.81%, 6.03%, 3.89%, and 8.27% of n-hexane, chloroform, acetone, ethyl acetate, and methanol extracts, respectively (Alagesan et al. 2019). It is important to note that polar solvents are more efficient than nonpolar solvents. Methanol possesses efficient extraction yield among other solvents and low toxicity; therefore, it can be used in food and pharmaceutical industry (Fiorito et al. 2019).

Chromatographic methods are recently considered as the most effective and rapid techniques used to detect and quantify coumarins. For example, gas chromatography-mass spectrometry (GC-MS) system was used to analyze coumarins (Alagesan et al. 2019). In addition, high-performance liquid chromatography (HPLC) technique was applied to quantify coumarins in *Cinnamomum cassia* Blume (Solaiman and Al-Zehouri 2017). It is important to note that simple coumarins have similar chemical structures and polarity, and it is hard to be separated precisely. Therefore, ultra-HPLC (UHPLC) method has been developed, and it applies a less organic solvent, runs rapidly, and gives a good peak separation. Furthermore, UHPLC-DAD method can be used for coumarin detection rapidly and routinely (Lončar et al. 2020). Moreover, HPLC-MS/MS has been applied for characterization and identification of coumarins (Li et al. 2019). The HPLC conditions with C 18 column include mobile phase: 0.1% aqueous formic acid (A) and 0.1% formic acid in acetonitrile (B). The MS triple quad was operated at 3500 V positive mode. MS has a selective detector to resolve the bioactive components and shorten employed gradient. However, the MS's purchase and maintenance are very expensive and unavailable for daily use (Li et al. 2019; Lončar et al. 2020). Since some of coumarins' structures include fluorophore, UV, fluorescence, and DAD detectors can be combined with HPLC for coumarins' detections. These kinds of detectors are widely available, can be devoted routinely, and are inexpensive. Proper monitoring of wavelengths is required to achieve high sensitivity and selectivity of the detection. Suitable wavelength is 320 nm for coumarins such as esculin, daphnetin, fraxetin, umbelliferone, 4-methylumbelliferone, and herniarin, while 280 nm was proven to be proper for coumarins such as 4-hydroxycoumarin, coumarin, and scoparone (Lončar et al. 2020; Li et al. 2019).

In addition, coumarins can be analyzed using Fourier transform mid-infrared spectroscopy (MID-FITR) combined with chemometric analysis (Moreno-Ley et al. 2019; Lončar et al. 2020). This method is simple and fast and had no need for reagents or pretreatments; however, it is more expensive than HPLC and unaffordable for commercial treatments. Therefore, analyzing coumarins by combining MID-FTIR spectroscopy and the HPLC-DAD is affordable for chimerical samples (Moreno-Ley et al. 2019). Scientists have been attentive in developing new, fast, non-expensive, and less use of organic solvents technique.

All mentioned above methods have been applied for their efficacy to detect and quantify coumarins, demonstrating their benefits and boundaries including cost, time of preparation and detection, availability, and use of organic solvents. The suitable method for quantification and detection must be proper for various sample of coumarins since they have diverse structures and polarity. In addition, highly

sensitive technique is required to detect very low concentrations, selective, reproducible, accurate, non-expensive, and able to devote routinely.

7.6 Biological Activities

From chemical standpoint, coumarins are chemically represented by benzo-α-pyrone (2H-1-benzopiran-2-one), and they are organic heterocycle. Based on the chemical diversity and complexity of coumarins, they subdivided to multiple classes including simple coumarins, isocoumarins, furanocoumarins and pyranocoumarins, biscoumarins, and phenyl coumarins (Annunziata et al. 2020). Coumarins have been attracting scientists due to their simple structure, low molecular weight, and bioavailability. Moreover, they are highly soluble in polar and nonpolar organic solvents such as ethanol, methanol, water, chloroform, and n-hexane with low toxicity (Annunziata et al. 2020; Khalil and Mustafa 2020). Along with their numerous biological activities, they exhibit pharmacological and industrial potential including anticancer, antimicrobial, antioxidants, antidiabetic, anticonvulsant, anticoagulant, anti-inflammatory, neuroprotective, antiproliferative, anti-algal activity, luminescent properties, and fungicide properties (Pereira et al. 2018; Stefanachi et al. 2018; Zhu and Jiang 2018; Mark et al. 2019; Santra and Banerjee 2020; Annunziata et al. 2020).

It is well documented that human body cells are exposed to oxidative stresses through the accumulation of free radicals and highly reactive species including ions, oxygen, carbon, nitrogen, and sulfur species, which can lead to various pathologies such as inflammation, cardiovascular diseases, cancer, diabetes, and neurodegenerative disorders. It is important to maintain the radical's concentration, reduce the production of free radicals, and potentially inhibit the oxidative stress. Therefore, the natural exogenous antioxidants such as synthetic coumarins (polyhydroxy or phenolic coumarins) have been demonstrated as a potential antioxidant (Galano et al. 2016; Wahy et al. 2017; Annunziata et al. 2020). New series of coumarinyl pyrazolinyl thioamide derivatives have been designed to act as free radical scavengers and inhibitors of jack beam urease (Singh et al. 2019).

The applications of coumarin and its derivatives as anticancer agent are summarized underneath. It is well known that cancer is a term which includes various types of diseases, which can be caused by a multistep process involving different factors resulting in increasing genetic mutation and accumulation chance. Annually, it causes about nine million mortalities, and it is considered the second cause of death around the world (Zhang and Xu 2019). The effect of synthesized O-prenylated coumarin derivatives on HeLa cervical cancer has been studied, and the results showed that the substitutes 6-geranyloxycoumarin and 8-geranyloxycoumarin were the most effective anticancer activities (Maleki et al. 2020). Coumarin derivatives are highly promising anticancer drug due to their antiproliferative mechanisms of action, biodiversity, and versatility. Irosustat is a promising anticancer drug under clinical trials. Moreover, the synergic effect of combination coumarin motive with other available anticancer drugs can be a

significant approach to decrease the side effect of drugs and the emergence of drug-resistant (Zhang and Xu 2019). A series of novel isatin-coumarin hybrid molecules are synthesized and designed through triazole ring. All isatin-coumarin hybrid exhibited anti-cancer activities against various cell cancer including leukemia cancer cells and colon cancer cells (COLO-205 and HCT-116). The reason behind this significant effect might be because of the structural feature of the isatin motility substitution and the linker between isatin and triazole length (Singh et al. 2017; Singh et al. 2019).

Coumarin and its derivatives can be also applied as a drug for preventing or alleviating degenerative diseases such as Alzheimer. The main causes of Alzheimer's disease can be β-amyloids aggregation, tau proteins formation, brain's neurotransmitter (Acetylcholine) degradation, and free radicals leading to oxidative stress (Singh et al. 2019). A novel series of coumarin-N-benzyl pyridinium hybrids were designed, and it demonstrated potential effect against Alzheimer's disease by inhibiting the acetylcholinesterase (AChE) enzyme, which is responsible for acetylcholine breaking down in the brain and MAO-B enzyme (Lan et al. 2017). It has been reported that there was an effect of synthesized and organized acrine-coumarin hybrids linked to 1,2,3-triazole on Alzheimer's disease (Najafi et al. 2019).

Moreover, coumarin and its derivatives exhibit antimicrobial activity. In fact, the emergence of microbial resistant has led the scientists to develop and investigate a novel of new antimicrobial drug to protect the future of human life and health (Singh et al. 2019). For example, a series of coumarins (dithioacetals derivatives) are characterized, synthesized, and investigated (Zhao et al. 2019). These compounds have a potential role against anti-tobacco mosaic virus biological activities. Also, coumarin-pargyline hybrids and multi-target tacrine-coumarin hybrids inhibited AChE, MAO-B enzymes, and aggregation of β-amyloid (Yang et al. 2017; Xie et al. 2015). Additionally, coumarin-based antimicrobial hybrid molecules exhibited antimicrobial effect against gram-positive *S. aureus* (Chavan et al. 2018). Coumarin-theophylline hybrid series exhibited antimicrobial effect against gram-positive bacteria (*S. aureus*) and gram-negative bacteria (*E. coli*, *S. Typhi*) and fungi (*C. albicans*) (Mangasuli et al. 2018). Scaffolds containing conjugates of 4-hydroxy coumarin and phenyl glyoxal series were generated and reported for their potential role as antimicrobial agents (Gupta et al. 2016). In addition, coumarins can be a great alternative among available conventional antibiotic drugs (Reen et al. 2018) because it has been proven to block the cell-to-cell communication, which is known as quorum sensing (QS), and inhibited biofilm formation. Biofilm formation presents the most antibiotic resistance, and it is hard to control. Coumarins have been reported to have potential effect not only on human infections but also can govern plant's pathogens, aquaculture infections, preventing food spoilage, and biofilm formation (Reen et al. 2018). Coumarin derivative 7-hydroxy-6-nitro-2H-1-benzopyran-2-one have been investigated for its ability as antifungal agents of various taxonomies of fungi such as Phycomycetes, Ascomycetes, Basidiomycetes, mycelial growth and development, and conidia formation in Aspergillus spp. (Dietrich and Valio 1973; Guerra et al. 2015; Prusty and Kumar 2019).

Coumarin and its derivatives also play an important role in some chronic diseases such as diabetes mellitus. It is well known that diabetes mellitus is known as a metabolic disorder, which presents due to the resistance or deficiency of insulin, and this can cause severe damages to body' organs. There are some enzymes that play a significant role in hyperglycemia such as α-glucosidase, α-amylase, and aldose reductase (Singh et al. 2019). It has been identified a novel hybrid molecule of natural flavonoids and coumarin for their antidiabetic effect including hybrids of apigenin, chrysin, quercetin, and luteolin (Pan et al. 2016). Also, a new series of 3-thiazolylcoumarin hybrids were designed by incorporating three pharmacophores (coumarin, thiazole, hydrazide) into a single entity due to their potential role in reducing α-glucosidase activity (Salar et al. 2016).

Beside their mentioned activities, coumarin and its derivatives exhibit anti-inflammatory property. Steroidal anti-inflammatory drugs (NSAIDs) are the major anti-inflammatory agent due to their effect in preventing prostaglandins release. The existent anti-inflammatory agents are primly targeting cyclooxygenase (COX) enzyme, which presents into forms of isoforms (Singh et al. 2019). The active pharmacophores of COX-2 inhibitors (celecoxib and valdecoxib) and 5-LOX enzyme inhibitor coumarin derivatives were combined and incorporated by hybridization method into single molecule, and it demonstrated a great effect as anti- inflammatory agents by inhibiting the COX and 5-LOX enzymes (Shen et al. 2010).

Coumarin hybridization with different active pharmacophores has demonstrated a potential role in enhancing potency of the novel molecules and general pharmacological profile.

These hybrid molecules have multi-target effects, which can increase the development of small therapeutics molecule, and can act as anticancer, antioxidant, antidiabetic, antimicrobial, and anti-inflammatory effects.

7.7 Commercial Utilization and Prospects

Coumarins and its derivatives are significantly used in diverse marketing sectors including food and pharmaceutical industries due to their multifunctional pharmaceutical and biological activities including antimicrobial, antiviral, antidiabetic, anticoagulant, estrogenic, dermal photosensitizing, vasodilator, molluscicidal, antithelmintic, sedative and hypnotic, analgesic, hypothermic, anticancer, antioxidant, antiparasitic, antihelmintic, antiproliferative, anticonvulsant, anti-inflammatory, and antihypertensive activities (Hussain et al. 2019; Sharma and Katiyar 2019). New products of coumarin and its derivatives with therapeutic efficacy against metabolic disorders and various kinds of human infections have been introduced (Nadeem and Ahmad 2019; Hussain et al. 2019), and there is a huge demand on accepted extraction methods to elicit most of coumarin and its derivatives (Nadeem and Ahmad 2019). They can be extracted from their natural sources such as different parts of plants, fungi, and microorganisms in addition to the in vitro production of coumarins (Hussain et al. 2019). Recently, coumarins hybrid

molecules were newly designed and synthesized with different available pharmacophores. This strategy enhanced the biological activities because they are synergically acting through multiple modes of actions, which is more beneficial than single molecule (Peters et al. 2009; Solomon et al. 2009; Hussain et al. 2019). For example, the combination of stilbene and coumarin compound excreted significant anti-cancer properties through different mechanism of actions such as telomerase enzyme, protein kinase inhibition activities, and oncogene expression downregulating (Wu et al. 2014; Bronikowska et al. 2012; Hussain et al. 2019).

There is a potential effort to efficiently incorporate coumarins and its derivatives into dietary human intake as natural flavoring agent such as *Cinnamomum cassia*, *Anthoxanthum odoratum*, and *Dipteryx odorata* (Hussain et al. 2019; Sharma and Katiyar 2019). However, coumarins can be added only to food as naturally extracted flavoring agent from raw materials such as cinnamon, or it is naturally found in the food based on European regulations (Lončar et al. 2020). Therefore, the most exposure of coumarins in human diet comes from cinnamon since it's highly used in dessert such as cakes, cookies, and beverages. Cinnamon has been used widely in food, beverages, and pharmaceutical industry because of its culinary and medicinal characteristics. Because a great quantity of coumarins might be hepatotoxic, it is prohibited to supplement food with a pure coumarin. Due to the fact that great quantity of coumarins might be hepatotoxic, supplement of pure coumarin is prohibited. Therefore, the daily intake of coumarins from diet should not be exceeded 0.07 mg/kg bw day (Lončar et al. 2020).

7.8 Conclusions and Recommendations

Coumarins are polar heterocyclic secondary metabolites extracted from their natural sources of plants and microorganism. Additionally, coumarins can be produced in vitro through several approaches including in vitro cell suspension and hairy root cultures. However, scaling up plant cell cultures from laboratories to bioreactors is generally not straightforward due to modifications in the cell growth conditions. Several bioreactors have been successfully adapted for growing plant cells in vitro including membrane bioreactors and wave bioreactors. The extraction of coumarins from their natural sources requires different techniques including kinetic maceration and ultrasound-facilitated extraction. The most rapid and effective techniques for characterization and identification of coumarins are chromatographic approaches such as GC-MS, ultra-HPLC, HPLC-MS/MS, and MID-FITR technologies. Besides their benefit to plants as antipathogenic compounds, coumarin and its derivatives also play a crucial role as antioxidant, anticancer, anti-inflammatory, antimicrobial, antidiabetic, and anti-Alzheimer. The multifunctional bioactive compounds of coumarins and its derivatives are promising applications in food and pharmaceutical industries.

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