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BIOTECHNOLOGY APPROACHES FOR *IN VITRO* PRODUCTION OF FLAVONOIDS

Alia Amer

Address(es):
Medicinal & Aromatic Dep., Horticulture Research Institute, Agricultural research Center, Cairo, Egypt.

*Corresponding author: dr_aliaamer@yahoo.com

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ABSTRACT

Flavonoids are small molecular secondary metabolites synthesized by plants with various biological activities such as anti-inflammatory, anti-carcinogenic, antioxidant, etc. Flavonoids occur naturally in fruits, vegetables, medicinal plants and beverages such as tea and wine. Biotechnology offers different *in vitro* systems which have been developed to exploit these active ingredients such as, in callus cultures, cell suspension cultures which are the emerging fields of biotechnology to investigate and enhance the production of these products. Along with this, hairy root culture and transformation techniques have been studied widely for flavonoid production. Nano-treatment is a novel way for improvement of flavonoids production. the present review focus on describing the flavonoids biochemistry, regulation of biosynthesis, as well as the Biotechnology and Nano-biotechnology tools for *in vitro* production of flavonoids.

Keywords: Flavonoid, Biosynthesis, Medicinal Plants, *In Vitro* Production, Nanoparticles Applications

INTRODUCTION

Plants are essential for life as they supply all animals on the earth, including humans, with essential foods. Plants are being the main source of pharmacologically active compounds, with many blockbuster drugs being derived directly or indirectly from plants. Despite the current dependence on synthetic chemistry to develop and manufacture drugs, yet the contribution of plants to disease treatment and prevention is still enormous. However, several challenges have been associated with the supply of biologically active pharmaceuticals from natural sources. Alternative avenues for plant products have gained importance during the past few years among which plant biotechnology has a key role to play in plant based industries (**Veeresham and Chitti, 2013**).

Plant *in vitro* techniques becomes an important part of biotechnological tool that offers a great potential solution for the propagation of endangered and superior genotypes of medicinal plants, which could be released to their natural habitat or cultivated on a large scale for the pharmaceutical product of interest. The enhanced production of secondary metabolites from plant cell cultures through elicitation has opened up a new spot of research which could have important economic benefits for pharmaceutical industry (**Devi *et al.*, 2008**).

Flavonoids are found in most terrestrial vascular plants, which belong to a group of natural phenolic substances with variable chemical structures. They are found in fruits, vegetables, grains, tree barks, roots, stems, flowers, as well as tea and wine (**Hermann, 1976; Stafford, 1991; Harborne and Williams, 2000**). More than 6000 different flavonoids have been identified, many of which are responsible of the attractive colours of flowers, fruits and leaves (**Nijveldt *et al.*, 2001**).

The interesting biological activities of flavonoids have prompted the intensive research on the physiological properties of these compounds as well as their effects on human health (**Rusak *et al.*, 2002**). This review is entended to compile the most relevant research on flavonoids to though light on progress in using current various techniques in producing flavonoids and the need to consider more effective research methods with emphathis on nanotechnology in this context.

FLAVONOIDS

Plant flavonoids are a large group of very different compounds sharing the common feature of phenol moieties (**Harborne and Williams, 2000; Grotewold, 2006**). They are, with a few notable exceptions, plant metabolites deriving from the shikimate pathway and the phenylpropanoid metabolism (**Stafford, 1990**). Flavonoids are aromatic secondary plant metabolites, which have been recognized as important due to their physiological (**Buslig and Manthey, 2002; Forkmann, 1992; Cody *et al.*, 1988**) and pharmacological (**Di Carlo *et al.*, 1999; Wang, 1999; Tapiero *et al.*, 2002; Manach and Donovan, 2004; Sharma, 2006; Cermak and Wolffram, 2006; Ortuno *et al.*, 2006**) role and their health benefits (**Valenzuela *et al.*, 2003; Hooper and Cassidy, 2006**).

Marinova *et al.* (2005) demonstrated that the phenolics are ubiquitous secondary metabolites in plants, comprising a large group of biologically active ingredients (above 8000 compounds) from simple phenol molecules to polymeric structures with molecular mass above 30000 Da. Based on the number of phenol subunits, the modern classification forms two basic groups of phenolics, simple phenols and poly phenols. The group of simple phenols contains carboxyl group underlying the specificity of their function. Polyphenols contain at least two phenol rings, where Flavonoids belong to this group which are a subject of comprehensive studies in recent years. More than 4000 flavonoids have been identified in different higher and lower plant species. The classification of polyphenols presents a challenge, as some classes such as chalcones, flavanones, and flavan-3-ols are both intermediates as wells as end products accumulating in the plant biosynthesis process, while other classes such as flavones and flavonols are identified as end products in the biosynthesis (**Andrae-Marobela *et al.*, 2013**).

Available reports tend to show that secondary metabolites of phenolic nature, including flavonoids are responsible for the variety of pharmacological activities (**Mahomoodally *et al.*, 2005; Pandey, 2007**). Flavonoids are hydroxylated phenolic substances and are known to be synthesized by plants in response to microbial infection (**Dixon *et al.*, 1983**). Their activities are structure dependent as the chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization (**Kelly *et al.*, 2002**). Flavonoids also act as a secondary antioxidant defence system in plant tissues exposed to different abiotic and biotic stresses. Flavonoids are located in the nucleus of mesophyll cells and within centres of reactive oxygen species (ROS) generation. They also regulate growth factors in plants such as auxin (**Agati *et al.*, 2012**). Biosynthetic genes have been assembled in several bacteria and fungi for enhanced production of flavonoids (**Du *et al.*, 2011**). In order to better understand the role and mechanism of flavonoids, their chemistry and biosynthesis seems proper to be presented.

FLAVONOIDS CHEMISTRY AND BIOSYNTHESIS

Chemistry

Flavonoids are a group of natural compounds with variable phenolic structures and are found in plants. In 1930 a new substance was isolated from oranges, where at that time it was believed to be a member of a new class of vitamins and designated as vitamin P. Later on, it became clear that, this substance was a flavonoid (rutin) and till now more than 4000 varieties of flavonoids have been identified (**Middleton, 1998**).Chemically flavonoids are based upon a fifteen-carbonskeleton consisting of two benzene rings (A and B as shown in Figure 1) linked via a heterocyclic pyrane ring(C). They can be divided into a variety of classes such as flavones (e.g., flavone, apigenin, and luteolin), flavonols (e.g.,quercetin, kaempferol, myricetin, and fisetin), flavanones (e.g., flavanone, hesperetin, and naringenin), and others.Their general structures are shown in Table 1. The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings (**Middleton, 1998**).

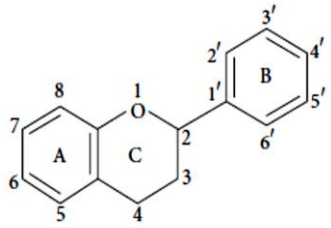
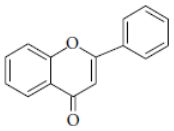
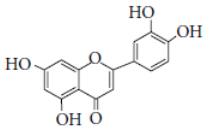
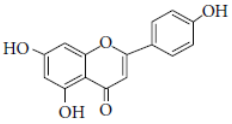
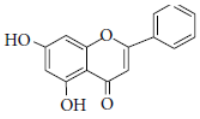
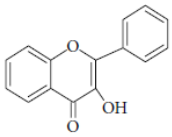
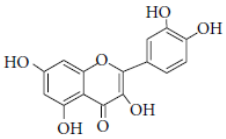
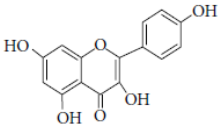
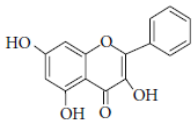
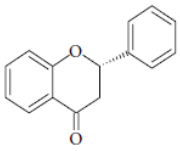
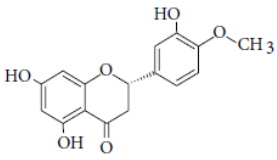
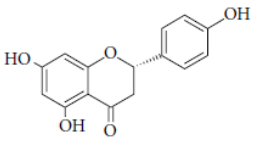
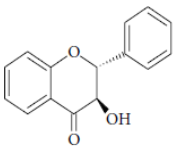
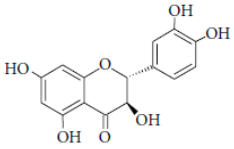
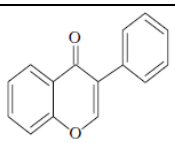
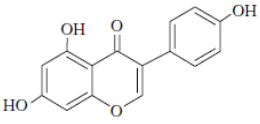
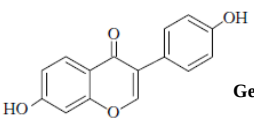


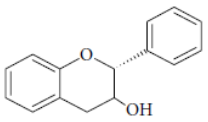
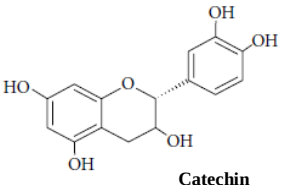
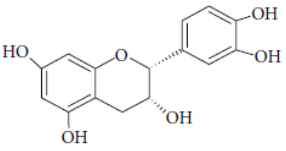
Figure 1 Basic flavonoid structure

Flavonoids occur as aglycones, glycosides, and methylated derivatives. The basic flavonoid structure is aglycone (Figure 1 and Table 1). Six-member ring condensed with the benzene ring is either a *α*-pyrone (flavonols and flavanones) or its dihydro derivative (flavonols and flavanones). The position of the benzenoid substituent divides the flavonoid class into flavonoids (2-position) and isoflavonoids (3-position). Flavonols differ from flavanones by hydroxyl group at the 3-position and a C2–C3 double bond (**Narayana *et al.*, 2001**). Flavonoids are often hydroxylated in positions 3, 5, 7, 2, 3', 4', and 5'. Methy l ethers and acetyl esters of the alcohol group are known to occur in nature. When glycosides are formed, the glycosidic linkage is normally located in positions 3 or 7 and the carbohydrate can be L-rhamnose, D-glucose, glucorhamnose, galactose, orarabinose (**Middleton, 1984**).

Studies on flavonoids by spectroscopy showed that most flavones and flavonols exhibit two major absorption bands: Band I (320–385nm) represents the B ring absorption, while Band II (250–285 nm) corresponds to the A ring absorption. Functional groups attached to the flavonoid skeleton may cause a shift in absorption such as from 367nm in kaempferol (3,5,7,4'-hydroxyl groups) to 371 nm in quercetin (3,5,7,3',4'-hydroxyl groups) and to 374 nm in myricetin (3,5,7,3',4',5'-hydroxyl groups) (**Yao *et al.*, 2004**). The absence of a 3-hydroxyl group inflavones distinguishes them from flavonols. Flavanones have a saturated heterocyclic C ring, with no conjugation between the A and B rings, as determined by their UV spectral characteristics (**Rice-Evans *et al.*, 1996**). Flavanones exhibit a very strong Band II absorption maximum between 270 and 295 nm, namely, 288 nm (naringenin) and 285 nm (taxifolin), and only ashoulder for Band I at 326 and 327 nm. Band II appears as one peak (270 nm) in compounds with a mono substituted B ring, but as two peaks or one peak (258 nm) with a shoulder (272 nm) when a di-, tri-, or *o*-substituted B ring is present. As anthocyanins show distinctive Band I peak in the 450–560 nm region due to hydroxyl cinnamoyl system of the Bring and Band II peaks in the 240–280 nm region due to the benzoyl system of the A ring, the colour of the anthocyanins varies with the number and position of the hydroxyl groups (**Wollenweber and Dietz, 1981**).

Table 1 Main flavonoids classes Structure

Groups	Structure backbone	Examples		
Flavones		 **Luteolin**	 **Apigenin**	 **Chrysin**
Flavonols		 **Quercetin**	 **Kaempferol**	 **Galangin**
Flavanones		 **Hesperetin**	 **Naringenin**	
Flavanonol		 **Taxifolin**		
Isoflavones			 **Genistein**	

Flavan-3-ols			
			
		Catechin	**Epicatechin**

Biosynthesis

Flavonoids are synthesized through the phenylpropanoid pathway, transforming phenylalanine into 4-coumaroyl- CoA, which finally enters the flavonoid biosynthesis pathway (Figure2). The first enzyme specific for the flavonoid pathway, chalcone synthase, produces chalcone scaffolds from which all flavonoids derive. Although the central pathway for flavonoid biosynthesis is conserved in plants, depending on the species, a group of enzymes, such as isomerases, reductases, hydroxylases, and several Fe^{2+}/2-oxoglutarate-dependent dioxygenases modify the basic flavonoid skeleton, leading to the different flavonoid subclasses (**Martens *et al.*, 2010**). Last, tranferases modify the flavonoid backbone with sugars, methyl groups and/or acylmoieties, modulating the physiological activity of the resulting flavonoid by altering their solubility, reactivity and interaction with cellular targets (**Bowles *et al.*, 2005; Ferrer *et al.*, 2008; Falcone Ferreyra *et al.*, 2012**).

Evidence is emerging showing that consecutive enzymes of the phenylpropanoid and flavonoid biosynthesis are organized into macromolecular complexes that can be associated with endo membranes (**Kutchan, 2005**). Metabolic channeling in plant secondary metabolism enables plants to effectively synthesize specific natural products and thus avoid metabolic interference. The existence of cytochrome P450 mono oxygenases (P450s)-related metabolons has been demonstrated: direct and indirect experimental data describe P450 enzymes in the phenylpropanoid, flavonoid, cyanogenic glucoside, and other biosynthetic pathways (**Winkel, 2004; Ralston and Yu, 2006**). Additional evidence for the channeling of intermediates between specific isoforms of phenylalanine ammonialyase and cinnamate -4- hydroxylase has been provided using transgenic tobacco plants expressing epitope-tagged versions of two phenylalanine ammonialyase isoforms (PAL1 and PAL2) and of cinnamate-4-hydroxylase (**Achnine *et al.*, 2004**). Moreover, the existence of a multi enzyme complex has been proposed for the anthocyanin pathway in rice by yeast-two hybrid experiments (**Shih *et al.*, 2008**).

Most of the flavonoid synthesizing enzymes are recovered in soluble cell fractions, immune localization experiments suggest that they are loosely bound to the endoplasmic reticulum (ER), possibly in a multi-enzyme complex, whereas the pigments themselves accumulate in the vacuole (i.e., anthocyanins and pro-anthocyanidins) or the cell wall (i.e., phlobaphenes, (**Winkel-Shirley, 2001**). Flavonol synthase1 has recently been localized in Arabidopsis nuclei (**Kuhn *et al.*, 2011**), as well as chalcone synthase and chalcone isomerase (**Saslowsky *et al.*, 2005**). Interestingly, *Antirrhinum majus* aureusidin synthase, the enzyme that catalyzes aurone biosynthesis from chalcones, was localized in the vacuole, while the chalcone 4'-*O*-glucosyltransferase is localized in the cytoplasm, indicating that chalcones 4-*O*-glucosides are transported to the vacuole and the rein converted to aurone 6-*O*-glucosides (**Ono *et al.*, 2006**). Moreover, a flavonoid- 3-hydroxylase has been recently localized in the tonoplast in the hilum region of the soybean immature seed coat (**Toda *et al.*, 2012**).

Two models have been proposed for the mechanism of anthocyanin transport from the ER to the vacuole storage sites: the ligandin transport and the vesicular transport (**Grotewold and Davis, 2008; Zhao and Dixon, 2010)**. The ligandin transport model is based on genetic evidence showing that glutathione transferase (GST)-like proteins are required for vacuolar sequestration of pigments in maize, petunia and Arabidopsis (AtTT19) (**Marrs *et al.*, 1995; Alfenito *et al.*, 1998**). The vacuolar sequestration of anthocyanins in maize requires a *m*ultidrug *r*esistance associated *p*rotein-type (MRP) transporter on the tonoplast membrane, which expression is co-regulated with the structural anthocyanin genes (**Goodman *et al.*, 2004**). MRP proteins are often referred as glutathione S-X (GS-X) pumps because they transport a variety of glutathione conjugates. However, because anthocyanin–glutathione conjugate(s) have not been found, it is proposed that these GSTs might deliver their flavonoid substrates directly to the transporter, acting as a carrier protein or ligandin (**Koes *et al.*, 2005**).

This hypothesis is supported by the fact that Arabidopsis' GST (TT19), localized both in the cytoplasm and the tonoplast, can bind to glycosylated anthocyanins and aglycones but does not conjugate these compounds with glutathione (**Sun *et al.*, 2012**). The vesicle-mediated transport model proposed is based on observations that anthocyanins and other flavonoids accumulate in the cytoplasm in discrete vesicle-like structures (anthocyanoplasts), and then they might be imported into the vacuole by an autophagic mechanism (**Pourcel *et al.*, 2010**). Nevertheless, grape vesicle-mediated transport of anthocyanins involves a GST and two multidrug and toxic compound extrusion-typetransporters (anthoMATEs). Thus, these observations point out to the coexistence of both mechanisms of transports, in which the participation of GSTs and transporters would be specific to cell and/or flavonoid-type (**Gomez *et al.*, 2011**).

Regulation of flavonoid biosynthesis

Biosynthesis genes of flavonoid regulated by the interaction of different families of transcription factors (**Falcone Ferreyra *et al.*, 2012**). Genes involved in the anthocyanin pathway are differentially regulated in monocot and dicot species by R2R3 MYB transcription factors, basic helix-loop-helix (bHLH), and WD40 proteins (**Grotewold, 2005; Petroni and Tonelli, 2011**). Thus, combinations of the R2R3- MYB, bHLH, and WD40 transcription factors and their interactions (MYB-bHLH-WD40 complex) determine the activation, and spatial and temporal expression of structural genes of anthocyanin biosynthesis. The regulation of anthocyanin biosynthesis in reproductive and other organs by MYB-bHLH-WD40 complex has been reviewed (**Petroni and Tonelli, 2011**). There are interesting differences in anthocyanin regulation between monocot and dicot species like Arabidopsis and maize. In Arabidopsis, TT2, TT8, and TTG1 form a ternary complex and activate pro-anthocyanidin biosynthesis in developing seeds, while, TTG1, aWD40 transcription factor, different bHLH (TT8, GL3, and EGL3) and MYB transcription factors (PAP1 and PAP2) interact to activate anthocyanin synthesis in vegetative tissues (Figure 2A) (**Baudry *et al.*, 2004; Feller *et al.*, 2011**).

In maize, MYB and bHLH proteins are encoded by two multi gene families (*PL*/*C1* and *B*/*R*, respectively), and each member has a tissue and developmental specific pattern, while a WD40 protein PAC1 is required by both B1or R1 proteins for full activation of anthocyanin biosynthetic genes in seeds and roots (Figure 2B) (**Carey *et al.*, 2004**). Functional Arabidopsis TTG1 is required for anthocyanin accumulation during roots and trichomes development (**Galway *et al.*, 1994**), and maize PAC1 can complement Arabidopsis *ttg1* mutants, however, maize *pac1* mutants only show a reduction in anthocyanin pigmentation in specific tissues (**Carey *et al.*, 2004**). Even more, the regulation of flavonol biosynthesis exhibit important differences between both species.

In Arabidopsis, three R2R3-MYB proteins, MYB12, MYB11, and MYB111 (PFG1-3), which exhibit differential spatial expression patterns, regulate *AtFLS1* expression in a tissue and developmental specific manner (**Stracke *et al.*, 2007**), while, *ZmFLS1/2* are regulated by both P1 (R2R3-MYB) and the anthocyanin C1/PL1 and R/B regulators (Figure2) (**Falcone Ferreyra *et al.*, 2012**). Nevertheless, flavonols are essential for pollen germination and conditional male fertility in maize (**Mo *et al.*, 1992; Taylor and Hepler, 1997**), but maize plants lacking the P1 and R/B+C1/PL1 anthocyanin regulators are fertile (**Coe and Neuffer, 1988; Dooner *et al.*, 1991; Neuffer *et al.*, 1997**). In addition, a PFG1-3-independent flavonol accumulation occurs in pollen and siliques/seeds in Arabidopsis (**Stracke *et al.*, 2010**), suggesting that, in both species, additional regulators, not yet identified, are also involved in the regulation of *FLS* expression, and consequently, in flavonol accumulation. Inaddition, the evolution of MYB and bHLH plant families has been deeply analyzed from structural and functional perspectives (**Feller *et al.*, 2011**). Interestingly, the identification of a C1-like (MBF1) regulator in the gymnosperm *Picea mariana* (black spruce) further supports the idea that the regulation of anthocyanin pathway by a C1-like class of R2R3 MYB protein precedes the evolutionary separation of angiosperms from gym- no sperms (**Xue *et al.*, 2003**).

The identification of both bHLH and MYB proteins in mosses further supports the hypothesis that the bHLH–MYB complex has evolved early during land plant evolution (**Pires and Dolan, 2010**). Many R2R3 MYB transcription factors were first identified from several model plants, such as maize, *Antirrhinum*, petunia, and Arabidopsis. These transcription factors are involved in the regulation of the flavonoid biosynthesis pathway.

Inspite of the fact that generly flavonoids are produced in plants as a secondery metabolites with varying concenterations, yet medicinal and aromatic plants are known to be more efficient in producing these substances that can be extracted in a commercial quantities using developed techniques.

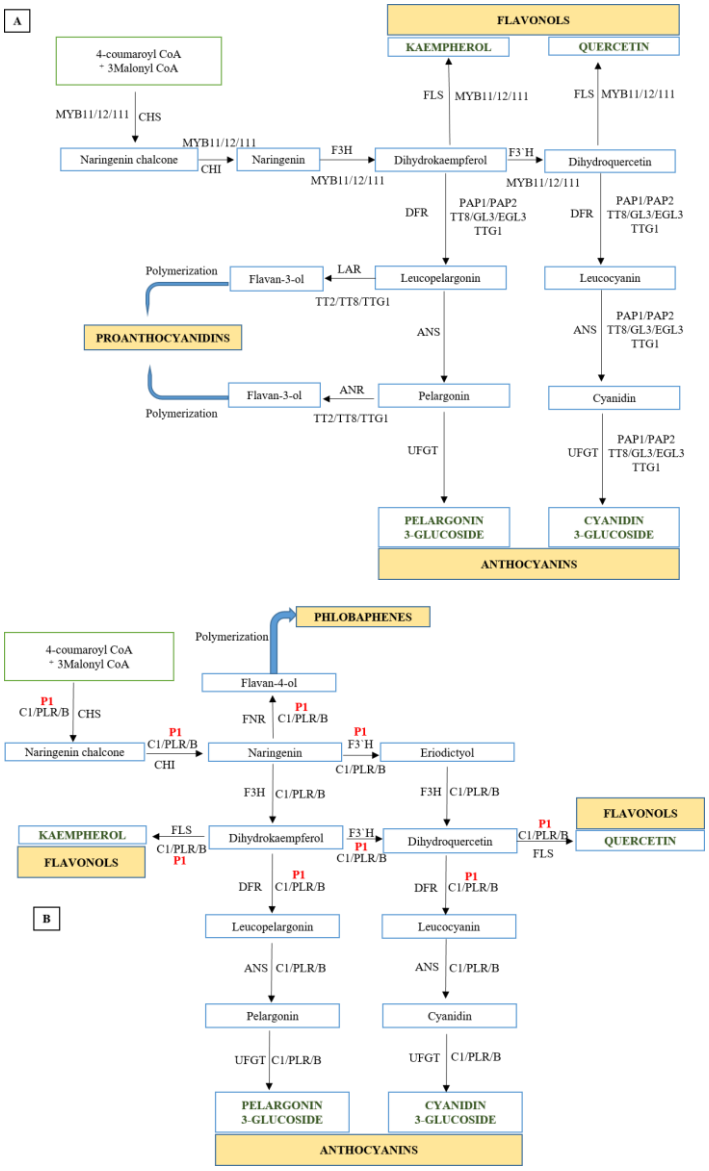


Figure 2 Regulation of the flavonoid pathway in *Arabidopsis thaliana* (A) and maize (B). Enzymes and intermediates are indicated in black and different regulators are indicated in colour. The end products are identified in capital letters. Dotted arrows indicate multiple steps. CHS, Chalcone synthase, CHI, chalcone isomerase, F3H, flavanone3-hydroxylase, F3_H, flavonoid-3_ -hydroxylase, DFR, dihydro flavonol4- reductase, FNR, flavanone4-reductase, ANS, anthocyanidin synthase, UFGT, UDP-glucose flavonoid3-Oglucosyl transferase, FLS, flavonol synthase, LAR, leuco anthocyanidin reductase, ANR, anthocyanidin reductase. (See: **Falcone Ferreyra *et al.*, 2012**)

MEDICINAL PLANTS AS A SOURCE OF FLAVONOIDS

Plants plays an important part in our everyday diet, In addition to essential primary metabolites (e.g. carbohydrates, lipids and amino acids), higher plants are also able to synthesize a wide variety of low molecular weight compounds. Plant secondary metabolites can be defined as compounds that have no recognized role in the maintenance of fundamental life processes in the plants that synthesize them, but they do have an important role in the interaction of the plant with its environment. The production of these compounds is often low (less than 1% dry weight) and depends greatly on the physiological and developmental stage of the plant (**Dixon, 2001; Oksman-Caldentey and Inzé, 2004**).

Verpoorte *et al.*, (1999) reported that the plants synthesize a remarkably diverse collection of chemicals. Quite notably, tremendous diversity is observed among "secondary" metabolites, a large group of compounds that until recently had been regarded to be not completely paramount to plant survival. These are the compounds that have emerged through evolution as the bulk of the dynamic chemical vocabulary underlying plant-environment interaction. A recent estimate has put the total number of plant secondary metabolites at 100,000 compounds, with an additional 4,000 being discovered annually.

Flavonoids are widely distributed throughout the plant kingdome and about 3000 varieties of flavonoids are known (**Kunhan, 1976**). Many have low toxicity in mammals and some of them are widely used in medicine (**Cesarone *et al.*, 1992**).

Flavonoids occurring virtually in all plant parts, particularly the photosynthesizing plant cells. They are a major colouring component of flowering plants. Flavonoids are an integral part of human and animal diet. Some food sources containing different classes of flavonoids are given in Table 2. Being phytochemicals, flavonoids cannot be synthesized by humans and animals (**Koes *et al.*, 2005**). Thus flavonoids found in animals are of plant origin rather than being biosynthesized in situ. Flavonols are the most abundant flavonoids in foods. Flavonoids in food are generally responsible for colour, taste, prevention of fat oxidation, and protection of vitamins and enzymes (**Yao *et al.*, 2004**). Flavonoids found in the highest amounts in the human diet include the soy isoflavones, flavonols, and the flavones. Although most fruits and some legumes contain catechins, the levels vary from 4.5 to 610mg/kg (Arts *et al.*, 2000). Preparation and processing of food may decrease flavonoid levels depending on the methods used. For example, in a recent study, orange juices were found to contain 81–200mg/L soluble flavanones, while the content in the cloud was 206–644 mg/L which suggest that the flavanones are concentrated in the cloud during processing and storage (**Gil-Izquierdo *et al.*, 2001**). Accurate estimation of the average dietary intake of flavonoids is difficult, because of the wide varieties of available flavonoids and the extensive distribution in various plants and also the diverse consumption in humans (**Tom´as-Barber´an and Clifford, 2000**).

Table 2 Some dietary flavonoids sources and its classification as well as structure

Class	Flavonoid	Dietary source	References
Flavanol	(+)-Catechin (−)-Epicatechin Epigallocatechin	Tea	(**Lopez *et al.*,2001**)
Flavone	Chrysin, apigenin Rutin, luteolin, and luteolin glucosides	Fruit skins, red wine, buckwheat, red pepper, and tomato skin	(**Hara *et al.*,1995; Kreft*et al.*, 1999; Stewart *et al.*, 2000; Hertog *et al.*, 1992**).
Flavonol	Kaempferol, quercetin, myricetin, and tamarixetin	Onion, red wine, olive oil, berries, and grapefruit.	(**Stewart *et al.*, 2000**)
Flavanone	Naringin, naringenin, taxifolin, and hesperidin	Citrus fruits, grapefruits, lemons, and oranges	(**Miyake *et al.*,2000; Rousseff *et al.*, 1987**)
Isoflavone	Genistin, daidzin	Soyabean	(**Reinli and Block, 1996**)
Anthocyanidin	Apigenidin, cyanidin	Cherry, easberry, and strawberry	(**Stewart *et al.*, 2000; Hertog *et al.*, 1992**).

Recently there has been an up surge of interest in the therapeutic potential of medicinal plants which might be due to their phenolic compounds, specifically to flavonoids (**Pourmorad *et al.*, 2006; Kumar and Pandey, 2012**). Flavonoids have been consumed by humans since the advent of human life on earth, that is, for about 4 million years. They have extensive biological properties that promote human health and help reduce the risk of diseases. Oxidative modification of LDL cholesterol is thought to play a key role during atherosclerosis. The isoflavan glabridin, a major polyphenolic compound found in *Glycyrrhiza glabra* (Fabaceae), inhibits LDL oxidation via a mechanism involving scavenging of free radicals (**Fuhrman *et al.*, 1997**). Several epidemiologic studies have suggested that drinking either green or black tea may lower blood cholesterol concentrations and blood pressure, thereby providing some protection against cardiovascular disease. Flavonoids are also known to influence the quality and stability of foods by acting as flavorants, colorants, and antioxidants (**Craig, 1999; Kumar *et al.*, 2012**). Flavonoids contained in berries may have a positive effect against Parkinson's disease and may help to improve memory in elderly people. Antihypertensive effect has been observed in total flavonoid fraction of *Astragalus complanatus* in hypertensive rats (**Li *et al.*, 2005**). Intake of antioxidant flavonoids has been inversely related to the risk of incidence of dementia (**Commenges *et al.*, 2000**).

Table 3 summarizes some of the medicinal plants rich in flavonoid contents. Solubility may play major role in the therapeutic efficacy of flavonoids. Low solubility of flavonoid aglycones in the water coupled with its short residence time in the intestine as well as its lower absorption does not allow humans to suffer acute toxic effects from the consumption of flavonoids, with the exception of a rare occurrence of allergy. The low solubility of the flavonoids in water often presents a problem for its medicinal applications. Hence, the development of semisynthetic, water-soluble flavonoids, for example, hydroxyethylrutosides and inositol-2-phosphatequercetin, has been implicated in the treatment of hypertension and micro bleeding (**Havsteen, 2002**).

Table 3 Some Medicinal plants as a source of flavonoids

Plant (scientific name)	Plant (Common name)	Family	Flavonoid	References
Aloe vera	Aloe	Asphodelaceae	Luteolin	(**L´azaro, 2009**)
Acalypha indica	Indian acalypha	Euphorbiaceae	Kaempferol glycosides	(**L´azaro, 2009**)
Azadirachta indica	Neem	Meliaceae	Quercetin	(**Tripoli *et al.*, 2007**)
Bacopa moneirra	water hyssop	Scrophulariaceae	Luteolin	(**L´azaro, 2009**)
Betula pendula	silver birch	Betulaceae	Quercetrin	(**Gupta *et al.*, 1983**)
Butea monospermea	flame-of-the-forest	Fabaceae	Genistein	(**Murlidhar *et al.*, 2010**)
Bauhinia monandra	orchid tree	Fabaceae	Quercetin-3-O-rutinoside	(**Murlidhar *et al.*, 2010**)
Brysonima crassa	locustberries	Malphigaceae	(+)-catechin	(**Aderogba *et al.*, 2006**)
Calendula officinalis	marigold	Compositae	isorhamnetin	(**Gupta *et al.*, 1983**)
Cannabis sativa	cannabi	Compositae	Quercetin	(**Gupta*et al.*,1983**)
Citrus medica	Lemon	Rutaceae	hesperidin	(**L´azaro, 2009**)
Clitoria ternatea	butterfly pea	Fabaceae	kaempferol-3-neohesperidoside	(**Sankaranarayanan *et al.*, 2010**)
Glyccheriza glabra	Liquorice	Leguminosae	Liquiritin,	(**Gupta *et al.*, 1983**)
Mimosa pudica	Sleepy (shy) plant	Mimosoideae	Isoquercetin	(**Sannomiya *et al.*, 2005**)
Mentha longifolia	mint	Lamiaceae	Luteolin-7-O-glycoside	(**Kogawa*et al.*, 2007**)
Momordica charantia	bitter melon	Curcurbitaceae	Luteolin	(**Ghoulami *et al.*, 2001**)
Oroxylum indicum	midnight horror,	Bignoniaceaea	Chrysin	(**Sannomiya *et al.*, 2005**)
Passiflora incarnate	Purple passionflower	Passifloraceae	Vitexin	(**Gupta *et al.*,1983**)
Pongamia pinnata	Pongame oiltree	Fabaceae	Pongaflavonol	(**Agarwal and Kamal, 2007**)
Tephrosia purpurea	Fish poison	Fabaceae	Purpurin	(**Sannomiya *et al.*, 2005**)
Tilia cordata	leaf linden-little	Tiliaceae	hyperoside	(**Gupta*et al.*,1983**)

BIOTECHNOLOGY APPROACHES FOR FLAVONOIDS PRODUCTION FROM PLANTS

Callus and cell suspension culture

Plant cell culture systems represent a potential renewable source of valuable medicinal compounds, flavors, fragrances, and colorants, which cannot be produced by chemical synthesis (**Vanisree and Hsin-Sheng, 2004**). The capability to cultivate plant callus cells and organs in liquid media has also made an important contribution to modern plant biotechnology with respect to the production of commercially valuable compounds. (**Su and Lee, 2007**). The homogeneity of an *in vitro* cell population, the large availability of material, the high rate of cell growth and the good reproducibility of conditions make suspension cultured cells suitable for the analysis of complex physiological processes at the cellular and molecular levels. Moreover, plant cell cultures provide a valuable platform for the production of high-value secondary metabolites and other substances of commercial interest. (**Moscatiello *et al.*, 2013**). Callus and cell suspension cultures have been carried out in several plants for the production of flavonoids. Agarwal and Kamal (**Agarwal and Kamal, 2007**) studied the total flavonoid content in *Momordica charantia* and observed that the maximum amount of total flavonoid (1.83 mg/l dry wt.) accumulated in 6-wk-old callus. The embryogenic callus and suspension culture of *Iphiona mucronata* showed the presence of flavonoid content but the regenerated plantlets were devoid of it. (**Al-Gendy *et al.*, 2013**) Callus culture was also studied for the isolation and detection of quercetin in *Pluchea lanceolata.* (**Arya *et al.*, 2008**)

Alfalfa callus culture was established and total flavonoids concentration was studied by (**Khalil *et al.*, 2008; Alia, 2008**), the results showed that the leaf callus produced the highest level of flavonoid with approximately (170 µg/mg fresh weight). **Cheel *et al.* (2007)** working *in vitro* culture of *Sanicula graveolens* found that on dry weight basis, total flavonoid content ranged from 1.23% to 2.23% being lower for the root culture.. **Chen *et al.* (2006)** reported that the callus growth quantity in *Cyclocarya paliurus* [Pterocarya sp.] was higher in stem than that in the leaf, but the flavonoid content in the leaf was higher than that in the stem and the optimual plant growth regulator combination promoting the callus growth and flavonoid content accumulation was 1.0 mg l^{-1} kinetin + 0.5 mg l^{-1} 2, 4- D + 0.3 mg l^{-1} NAA. These results are in agreement with (**Yamamoto *et al.*, 1986**) who mentioned that the growth and flavonoid (baicalin, baicalein, wogonin and wogonin-7-0-glucuronide) content of the St-20 line of *Scutellaria baicalensis* callus were best on a medium containing 10^{-7} to 10^{-5}M kinetin. After culture for 70 days the St-20 line had a similar flavonoid content and pattern to that of the parent plant roots. Also, (**Chen and Cao, 2007**) observed that flavonoid content in the callus of *Ginkgo biloba* from different explants was higher in root > leaf > cotyledon > stem. On the other hand, (**Saker and Kawashity, 1998**), working on Nepeta and Plantago species endemic in Egypt, found that the flavonoid contents of organized tissues, although about 3-times greater than those of unorganized tissues (callus), were still lower than those of the original plants (control). Similar results were found on *Lotus tenuis* Waldst by (Strittmatter *et al.*, 1991) who reported that flavonoids were not detected in vitro callus cultures, but flavonoid production in plantlets derived from the callus showed similar patterns to the field-grown plants. **Pasqua *et al.* (1991**) suggested that flavonoid production in *Maclura pomifera* was markedly higher from cell cultures, although the composition was similar for calluses and cell cultures. **Tadhani *et al,* (2007**) showed that the flavonoids content in *Stevia rebaudiana* was found to be 21.73 and 31.99 mg / g in the leaf and callus, respectively. **Li *et al.*, (2004**) working on *Eucommia ulmoides* plant found that flavonoid contents were highest in hypocotyl calluses.

Madhavi *et al.* (1998) studied the isolation of bioactive constituents from *Vaccinium myrtillus* fruits and cell cultures. Major fractions contained flavonoids, such as cyanidin-3- galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside and proanthocyanidins. Anthocyanin accumulation in callus was lower than in the fruit. Callus cultures accumulated proanthocyanidins were similarly present in fruit extracts (oligo- and polymeric. **Dias *et al.* (1998)** published the isolation of a new naturally occurring compound 6-C-prenyl luteolin, together with luteolin-5,3'-dimethyl ether, luteolin-5-glucoside and luteolin-3'-glucoside from the callus of *Hypericum perforatum* var. angustifolium. The total flavonoid content of callus was much lower than that found in wild growing *H. perforatum* plants.

Fedoreyev *et al.* (2000) established callus cultures from the different parts of *Maackia amurensis* and analysed for isoflavonoids. The isoflavones daidzein, retuzin, genistein and for mononetin and the pterocarpans maakiain and medicarpin were found to be produced by these cultures. The content of isoflavones and pterocarpans was essentially the same in cultures derived from leaf petioles, inflorescences and apical meristems of the plant. The maximal yield of isoflavones and pterocarpans in calluses approximately four times higher than the content of the heartwood of *M. amurensis* plants. Moreover, (**Łuczkiewicz and Głod, 2003; Luczkiewicz *et al.*, 2014**) established six callus cultures and studied the effect of plant growth regulators of Genista species with the objective to produce isoflavones of phytoestrogenic activity. The cultures were optimized for their growth and isoflavonoid production by changing various media in the presence or absence of light. The best growth and the highest isoflavone production was obtained under constant light regime on SH basal medium containing 22.6 µmol/L 2,4-dichlorophenoxyacetic acid (2,4-D), 23.2 µmol/L kinetin. Callus cultures of all species produced more isoflavones than the parent herbs.

Stable and optimized callus cultures are a logical step in the first phase of the cell culture production of plant secondary metabolites, i.e. preparing the inoculum for liquid suspension cultures. Production of flavonoids in cell suspension cultures have been widely published and it was proposed as a technology to overcome problems of variable product quantity and quality from whole plants due to the effects of different environmental factors, such as climate, diseases and pests (**Yamamoto *et al.*, 1995, Zhang *et al.*, 1997; Zhang *et al.*, 2002; Rao and Ravishankar, 2002; Yamamoto *et al.*, 2004**).

During the past decades, this technology attracted much academic and industrial interest. The approach of using plant cell suspension cultures for secondary metabolite (including flavonoids) production is based on the concept of biosynthetic totipotency of plant cells (**Rao and Ravishankar, 2002**), which means that each cell in the cultures retains the complete genetic information for production of the range of compounds found in the whole plant. Cell suspension cultures are initiated from established callus cultures by inoculating them into liquid media. The cultures are then kept in glass flasks under continual agitation on horizontal or gyratory shakers and eventually they can be transferred to a specialized bioreactor (**Bourgaud *et al.*, 2001**). There have been examples of successful production of some compounds from this group of metabolites, for instance, (**Yamamoto *et al.*, 1995**) showed the effect of polysaccharides on the production of prenylated flavanones (sophoraflavanone G and lehmanin) in Sophora flavescens callus culture. The production of these flavanones was stimulated up to 5 times by addition of 2 mg/mL yest extract. Moreover, the

production of prenylated flavanones also can be increased by 2-5 times by addition of cork pieces (**Yamamoto *et al.*, 1996**).

Another authors (**Delle Monache *et al.*, 1995**) isolated flavonoids from callus and cell cultures of *Maclura pomifera*. Among the flavonoids, flavones and flavanones were produced preferentially by suspended cells, but with the prenyl substituents exclusively on ring A, while the isoflavones did not show the 3', 4'-dihydroxyl substitution pattern found in the products isolated from fruits. The *M. pomifera* cell suspension culture showed a greater level of metabolite accumulation (0.91%) than stems (0.26%), leaves (0.32%) and fruits (0.08%) of the parent plant. (**Zhang *et al.*, 1997**) studied the temperature effect on anthocyanin production in cell suspension cultures of *Fragaria ananassa* at a temperature range of 15-35◦ C. The maximum anthocyanin production was obtained at 20◦ C. Anthocyanin production of 270 mg/L on day 9 was increased 1.8, 3 and 4-fold over that of cultures at 20, 25 and 30 ◦ C, respectively.

In addition, recent phytochemical studies have documented the presence of some phenolic acids and flavonoids in fruit extracts (**Mocan*etal.*,2016; Mocan*et al.*, 2016; Mocan*et al.*,2014).** Analyses of *S. chinensis* fruit extracts confirmed the presence of chlorogenic, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, salicylic and syringic acids (**Szopa and Ekiert, 2012**). Other authors have additionally proved the presence of gentisic acid and flavonoids: hyperoside, isoquercitrin, rutin and quercetin (**Mocan *et al.*, 2014**).

Also, there have been different interest reports showed the successful production of flavonoids as active ingredients, for instance, Rosmarinic acid in cultures *in vitro* of many Lamiaceae and Boraginaceae species **(Ekiert *et al.*, 2013),** rosmarinic and chlorogenic acids in cell and organ cultures of *Eryngium planum* **(Kikowska *et al.*, 2012**), ellagic acid in shoot cultures of *Rubus chamaemorus* (**Thiem *et al.*, 2003**), protocatechuic acid in shoot cultures of *Ruta graveolens* **(Ekiert *et al.*, 2009**), and *p*-coumaric acid in shoot-differentiating callus cultures of *Ruta graveolens* ssp. *divaricata* **(Ekiert *et al.*, 2014).** Furthermore, considerable amounts of flavonoids have been obtained in cultures *in vitro* of plant species such as *Astragalus missouriensis* **(Ionkova, 2009),** *Cyclopia genistoides* **(Kokotkiewicz *et al.*, 2014),** *Hyoscyamus muticus* **(Biondi *et al.*, 2002),** or *Dionaea muscipula* and *Drosera capensis* **(Krolicka *et al.*, 2008).** *Schisandra chinensis* **(Szopa *et al.*, 2016a,b; Szopa *et al.*, 2017).**

Hairy root culture

Transgenic hairy root cultures have revolutionized the role of plant tissue culture in secondary metabolite production. They are unique in their genetic and biosynthetic stability, faster in growth, and more easily maintained. Using this methodology, a wide range of chemical compounds has been synthesized. (**Shanks and Morgan, 1999; Giri and Narasu, 2000**) Hairy root cultures of many plant species have been widely studied for the production of secondary metabolites useful as pharmaceuticals, cosmetics, and food additives. (**Christey and Braun, 2005; Georgiev *et al.*, 2007; Srivastava and Srivastava, 2007**) Hairy root cultures represent an interesting alternative to dedifferentiated cell cultures for the production of secondary plant products. Because hairy roots originate from a single plant cell infection by *Agrobacterium rhizogenes*, they are usually considered as genetically stable, in contrast with callus lines.

Also, in contrast to dedifferentiated cells, the production of secondary metabolites is not repressed during the growth phase of the culture. Therefore, hairy roots usually produce secondary plant compounds without the loss of concentration frequently observed with callus or cell suspension cultures. (**Bourgaud *et al.*, 1997**) Therefore hairy root cultures of seven *Psoralea* species were established (**Bourgaud *et al.*, 1999**) and the flavonoid (daidzein, coumestrol), production was enhanced by using chitosan as elicitor. The effect of rare earth element Praseodymium (Pr) on flavonoids production and its biosynthesis was studied in *Scutellaria viscidula* hairy roots (**Lei *et al.*, 2011**).

Zhang *et al.* (2009) reported that over a culture period of 3 weeks, the wild-type hairy roots of *G. uralensis*, the untreated transgenic hairy roots, and the double-treated transgenic hairy roots accumulated 0.842, 1.394, and 2.838 (g/100 g DW) of total flavonoids, respectively. Moreover, the enhanced accumulation of flavonoids was correlated with the elevated level of *chi* transcripts and CHI activity, confirming the key role of *chi* in the flavonoids synthesis and they demonstrated that the combination of the metabolic engineering and PEG8000-YE elicitation treatment was an effective strategy to increase the flavonoids production in hairy roots of *G. uralensis* Fisch.

Studies showed that compared to callus cultures, hairy roots from the 7 *Psoralea* plant species (*Leguminosae*), displayed comparable concentrations of flavonoids. However, high-producing lines were more frequently found with hairy roots (4 out of 18) than with callus cultures (4 out of 217) (**Bourgaud *et al.*, 1999**). Moreover, (**Zhao *et al.*, 2014**) indicated that the *F. tataricum* hairy root culture could be an effective system for rutin and quercetin production and the maximal flavonoids yield was enhanced to 47.13 mg/L, about 3.2 fold in comparison with the control culture of 14.88 mg/L.

Hairy root cultures of many plant species have already been widely studied regarding the production of secondary metabolites which could be used as pharmaceuticals, cosmetics, and food additives (**Crane *et al.*, 2006; Georgiev *et al.*, 2007, Thiruvengadam *et al.*, 2014**). Biotechnological approaches which used hairy root culture have greatly enhanced the production of rutin by common buckwheat (**Lee *et al.*, 2007; Kim *et al.*, 2010**) and the production of phenolic compounds by tartary buckwheat (**Kim *et al.*, 2009; Thwe *et al.*, 2013**). Also rutin and quercetin biosynthesis in *Fagopyrum tataricum* Gaertn (**Huang *et al.*, 2016**).

Elicitors and elicitation for flavonoids production

Until now, various strategies have been developed to improve the production of secondary metabolites *in vitro* cultures, such as manipulating the parameters of the environment and medium, selecting high yielding cell clones, precursor feeding and elicitation (reviewed in **Collin, 2001; Rao and Ravishankar, 2002; Verpoorte *et al.*, 2002**).

This broader definition of elicitors includes both substances of pathogen origin (exogenous elicitors) and compounds released from plants by the action of the pathogen (endogenous elicitros). Elicitors are molecules of biological and nonbiological origin that stimulate secondary metabolism synthesis and could play an important role in biosynthetic pathways to enhanced production of commercially important compounds (**Dornenburg and Knorr, 1995**). Elicitiation can be used as one of the important strategies in order to get better productivity of the bioactive secondary products (**Chong *et al.*, 2005; Smetanska, 2008; Sharma *et al.*, 2011; Hussain *et al.*, 2012**) and lowering production costs. (**Miao *et al.*, 2000; Jian-Yong, 2003**) Elicitors are compounds stimulating any type of plant defense. (**Radman *et al.*, 2003**) The secondary metabolites are released due to defense responses which are triggered and activated by elicitors, the signal compound of plant defence responses. (**Patel and Krishnamurthy, 2013**) Copper sulphate as abiotic elicitor was used on the production of flavonoids in cell cultures of *Digitalis lanata*. (**Bota and Deliu, 2011**) Callus cultures of Ononis arvensis with $AgNO_3$ as an elicitor was used to enhance flavonoid production. (**Tumova and Polivkova, 2006**). In addition a wide variety of elicitors, such as fungal elicitors, methyl jasmonate, benzoic acid and arachidonic acid can induce the biosynthesis of secondary metabolites (**Yukimune *et al.*, 1996**).

Legumes such as bean, soybean, chickpea, and alfalfa *(Medicago sativa)* response to elicitation in the accumulation of antimicrobial isoflavonoid phytoalexins (**Kessmann *et al.*, 1990**). In alfalfa cell-suspension cultures elicitor-induced accumulation of the isoflavonoid phytoalexin medicarpin is preceded by increases in the extractable activities of all enzymes involved in its biosynthesis L-Phenylalanine (**Kessmann *et al.*, 1990**). Previous work on the relationship between gene transcription and subsequent metabolic events in elicitor treated alfalfa cells has demonstrated a correlation between an increased transcription rate and subsequent increases in enzymatic activity for a range of genes involved in the core phenylpropanoid pathway and the flavonoid isoflavonoid branch pathway (**Kessmann *et al.*, 1990**). Also Yeast extract (YE) elicitor treatment during the exponential growth phase showed a significant flavonoid induction than during the stationary growth phase. aslo Jasmonic acid affecting the production of flavonoids in alfalfa suspension culture (**Khalil *et al.*, 2008; Alia, 2008**). **Dixon *et al.* (1995)** showed that the isoflavonoid conjugates medicarpin-3-0-glucoside-6"- O-malonate (MGM), afrormosin-7-O-glucoside (AG), and afrormosin-7-O-glucoside-6"-O-malonate (AGM) were isolated and characterized from cell suspension cultures of alfalfa (*Medicago sativa* L.), where they were the major constitutive secondary metabolites. They were also found in alfalfa roots, but not in other parts of the plant. The phytoalexin medicarpin accumulated rapidly in suspension cultured cells treated with elicitor from *Colletotrichum lindemuthianum*, and this was subsequently accompanied by an increase in the levels of MGM.

Mizukami *et al.* (1993) and **Szabo *et al.* (1999)** reported that the jasmonates have been shown to induce rosmarinic acid and shikonin production in cell cultures of *Coleus blumei* and *Lithospermum erythrorhizon*, respectively. They have also been reported to play an important role in signal transduction processes that regulate defence genes in plants during assaults such as insect feeding (**Farmer and Ryan, 1990; Walling, 2000**). In addition, JA and methyl jasmonate increase the production of hypericin in cell suspension cultures of *H. perforatum* (**Travis *et al.*, 2002; Jing *et al.*, 2015**). Moreover **Yamamoto *et al.* (2004)** showed that some elicitors such as methyl jasmonate and yeast extract stimulated the production of sophora flavanone G (SFG) in cultured cells of *Sophora flavescens*.

Flavonoids are produced as protective substances against UV-B radiation in plant. As an effective abiotic elicitor, some studies have described the production of flavonoids by buckwheat sprouts in response to UV-B irradiation (**Kreft *et al.*, 2002; Eguchi and Sato, 2009**). Rutin (sometimes called vitamin P) displays strong antioxidant activity which could alleviate the damage from UV-B stress. **Tsurunaga *et al.* (2013)** found that rutin content and radical scavenging activity of buckwheat sprouts were enhanced under various levels of UV-B radiation.

Huang *et al.* (2016) reported that rutin and quercetin content of hairy roots and all parts of tartary buckwheat were increased under UV-B stress. The maximal increase of rutin content (from 3.19 to 29.79 mg g^{-1}, 9.35-fold) was observed in leaves. Interestingly, the next-highest increase of rutin content (from 0.93 to 4.82

mg g^{-1}, 5.18-fold) was observed in hairy roots. In a previous study of buckwheat, (**Kim *et al.*, 2010**) found that rutin content was ~2.4-fold higher in hairy roots than in WT roots. These findings are consistent with those of transformation studies on other plants, which suggested that *Agrobacterium* transfection might greatly enhance rutin content (**Fu *et al.*, 2006**). According previous work, some work indicated that biotic elicitors can also enhance rutin and quercetin production in *F. tataricum* hairy root, e.g., Yeast polysaccharide (**Zhao *et al.*, 2014**) and exogenous fungal mycelia (**Zhao *et al.*, 2014b**).

The elicitors can be biological or chemical in origin. The yeast elicitor, Saccharomyces cereviseae increased the production of berberine by 4-folds in *Thalictrum rugosum*. **Rajendran *et al.* (1994)** observed 3-fold elicitation of anthocyanin by *Aspergillus flavus* mycelial extract in cultured cells of Daucus carota. **Kang *et al.* (2006)** studied the effect of the elicitor salicylic acid (SA) on the production of bilobalide, ginkgolide A (GA), and ginkgolide B (GB) in cell suspension cultures of *Ginkgo biloba*. **Buitelaar *et al.* (1992)** reported 85% increase in thiophene production with *Aspergillus niger* elicitor whereas it was 55% with *Penicillium expansum* elicitor in the hairy roots of *Tagetes patula*. Cell suspension cultures of *Taxus chinensis*, treated with 20, 40 and 100 mg /L *Aspergillus niger* elicitor showed 5, 8 and 3-fold increase in taxol production than that of the control (**Lan *et al.*, 2003**). **Mendhulkar *et al.* (2016)** indicated that for flavonoid elicitation in *Blumea lacer*a, *Aspergillus niger* is more responsive than Salicylic acid. Also Yeast extract (YE) elicitor treatment during the exponential growth phase showed a significant flavonoid induction than during stationary growth phase. YE at 1 g l^{-1} with culture harvested on day 12 were the best treatment affecting the production of flavonoid (**Alia, 2008**).

Nano treatments for flavonoids enhancement

The phenomenal surface characteristics of Nanoparticles (NP) attribute to its extraordinary and unique properties. By increasing the number of atoms on surface, there is an increase in total free energy, resulted in the alteration of material characteristics. Nanoparticles have the potential to be used as novel effective elicitors in plant biotechnology for the elicitation of secondary metabolite production (Fakruddin *et al.,* 2012). Many researchers have studied the role of NPs as elicitors (**Aditya *et al.*, 2010; Asghari *et al.*, 2012; Sharafi *et al.*, 2013; Zhang *et al.*, 2013; Ghanati and Bakhtiarian, 2014; Raei *et al.*, 2014; Ghasemi *et al.*, 2015; Yarizade and Hosseini, 2015**). A number of studies have supported the possible role of NPs as elicitors for enhancing the expression level of genes related to the production of secondary metabolite (**Ghasemi *et al.*, 2015; Yarizade and Hosseini, 2015**). Nanoparticles have successfully offered a new strategy in enhancing the secondary metabolite production. But still an in-depth and consolidate insight in research is required to elucidate the effects of NPs in production mechanisms of secondary metabolite production in medicinal plants (**Misra *et al.*, 2016**).

Flavonoids and isoflavonoids are the most popular groups of secondary metabolites found in plants. Many legume seeds have been reported to be rich sources of these secondary metabolites (**Heiras-Palazuelos *et al.*, 2013**). **AL-Oubaidi and Kasid (2015)** demonstrated the increased production of secondary metabolite (phenolic and flavonoid compounds) in gram on exposure to TiO_2 NPs under *in vitro* condition. Secondary metabolite contents in the callus were estimated qualitatively and quantitatively using HPLC and compared with the mother plant. TiO_2 NPs at varying concentrations (0.5, 1.5, 3, 4.5, 6) mg L−1 were used for an effective increase in secondary metabolites. The results revealed that the secondary metabolite concentration from callus embryo of gram increased to a highly significant level at the concentrations of 4.5 and 6.0 mg L-1. The HPLC outcomes confirmed the elevation in the secondary metabolite level under the effect of the TiO2 NPs when compared with the mother plant. In a very recent report, **Khan *et al.*, (2016)** examined the effect of nine types of metal nanoparticles including monometallic and bimetallic alloy nanoparticles [Ag, Au, Cu, AgCu (1:3), AgCu (3:1), AuCu (1:3), AuCu (3:1), AgAu (1:3), AgAu (3:1)] on total phenolic and flavonoid contents in milk thistle plant. The sterilized seeds were soaked in NPs suspensions for 2 h and allowed to grow under in vitro condition. The experiment was conducted for 6 weeks, and samples for total phenolic and flavonoid contents were collected on a weekly interval. NPs suspensions affected total phenolic and flavonoid contents in the plant in a different way. It was observed that the amount of phenolics and flavonoids did not show any correlation with the total dry mass of the plant.

However, duration of the experiment significantly affected the amount of total flavonoids and phenolics in milk thistle. After 21 days presoaking of seeds in bimetallic alloy, enhanced whereas monometallic NPs suspensions, reduced phenolics and flavonoids content in milk thistle plantlets. After 28 days, Au and Cu NPs caused maximum total phenolic and flavonoid accumulation in milk thistle plants. Therefore, the maximum effect on secondary metabolites was recorded with monomatellic NPs. Mainly three factors (size, surface area, and composition of NPs) played a significant role either singly or in combination.

CONCLUSION

Plants are the main repository of all kinds of biochemicals which are produced as primary and secondary metabolites. Secondary metabolites are industrially important as they constitute the major chunk of pharmaceutically important drugs. As a result of their huge demand in modern market they are overexploited from their natural habitat, resulting in the disappearance of many plant species. Therefore, Biotechnology offers different in vitro systems and have been developed to exploit these active ingredients such as, in callus cultures, cell suspension cultures hairy root cultures and nanoparticles which are the emerging fields of biotechnology to investigate and enhance the production of these products.

This review briefly summarized the flavonoids chemistry and biosynthesis as one of the most important secondary compounds found in medicinal plants, as well as the possible sources of flavonoids for their perspective biotechnological production. Flavonoids are a large group of low-molecular weight polyphenolic secondary metabolites, Fruits and vegetables are natural sources of flavonoids. The basic flavonoid structure is aglycone. Flavonoids are synthesized through the phenylpropanoid pathway, transforming phenylalanine into 4-coumaroyl- CoA, which finally enters the flavonoid biosynthesis pathway which is regulated by the interaction of different families of transcription factors.

Plant tissue cultures are being potentially used as an alternative new strategy for industrial production of flavonoids, the production of flavonoids via tissue culture techniques have been reported in both callus and cell suspension cultures. The spectrum of the produced compounds and their yields depended on the proper selection of plant species, explant types and culture conditions.

Biotechnological approaches which used hairy root culture have greatly enhanced the production of many flavonoids compound which usually produce flavonoid compounds without the loss of concentration frequently observed with callus or cell suspension cultures. Therefore hairy root cultures represent an interesting alternative to dedifferentiated cell cultures for the production of plant flavonoids. Because hairy roots originate from a single plant cell infection by *Agrobacterium rhizogenes*, they are usually considered as genetically stable, in contrast with callus lines.

The most recent techniques is the use of nanoparticles for the elicitation of secondary metabolite production as novel effective elicitors in plant biotechnology. Nanoparticles have successfully offered a new strategy in enhancing the secondary metabolite production. But still an in-depth and consolidate insight multi desciblinary research research is required to elucidate the effects of NPs in production mechanisms of secondary metabolite synthsis in medicinal plants.

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