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REVIEW ON SECONDARY METABOLITES IN *Salvia* spp.

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Abstract

Salvia genus is the largest member of the Lamiaceae family with around 900 species and is widely distributed in tropical and temperate regions. These species are usually aromatic and have numerous pharmacological and therapeutic applications. They are used in traditional medicine to treat eczema, colds, bronchitis, digestive problems, sore throat, tuberculosis, haemorrhage, some cardiovascular and menstrual disorder. Modern studies suggest that various Salvia species have antibacterial, antifungal, anticancer, antioxidant, and anti-inflammatory properties. These activities are consequence of biosynthesized bioactive substances, including terpenoids, flavonoids, phenolic compounds, etc. These chemical constituents refer to as secondary metabolites and exert multiple therapeutic activities without showing important side effects. The present review summarized the information published in the scientific literature on the secondary metabolites in Salvia spp. In addition, the recent biotechnological approaches, advances in metabolic engineering strategies, successful results and potential problems were presented.

Key words*: bioactive substances, Lamiaceae*, *metabolic engineering, Salvia* sp., *Secondary metabolites.*

INTRODUCTION

The genus *Salvia*, commonly known as Sage, is the largest member of *Lamiaceae* family. Sage includes 900 species of herbs, shrubs, perennials and rarely annuals and biennials that have fragrant leaves and flowers and grow in tropical and temperate regions of the world (Nikavar et al., 2008; Itani et al., 2008). The name *Salvia* is derived from the Latin word "salor" which means "to save". This plant has been of special interest since ancient times, it is the most valuable medicinal plant of the mint family and has important therapeutic properties (Ayatollahi et al., 2009; Smidling et al., 2008; Hamidpour et al, 2014). Sage is used in traditional medicine to treat eczema, colds, bronchitis, digestive disorders, sore throat and tuberculosis. Also, today's studies indicate the antibacterial, antifungal, antitumor, antioxidant and anti-inflammatory properties of different species of this plant. These activities are consequence of biosynthesized bioactive metabolites: Sterols, Flavonoids and other Phenolics, di-and triterpenes, etc. (Topçu et al., 2017; Marchev et al., 2014). The production of secondary metabolites through common methods such as collection from nature and cultivation in agricultural fields is not an easy task for several reasons. On the other hand, secondary metabolites may be limited to a specific genus or species, or may be produced only in a specific growth stage, or seasonal conditions, stress and special nutrition. Therefore, in the last few decades, many efforts have been made to develop alternative methods of producing valuable plant compounds, among which the use of cell cultures as a source for the production of secondary metabolites has received considerable attention from researchers (D'Amelia et al., 2017).

MEDICINAL PROPERTIES OR PHARMACOLOGICAL EFFECTS OF *Salvia*

During the Middle Ages, European traditional healers have used *Salvia* for the treatment of constipation, cholera, gout, chronic rheumatism, Alzheimer's, liver disorders, epilepsy, and paralysis. In addition, Sage is a plant that is used as an anticonvulsant, febrifuge, digestive pain reliever and headaches of nervous origin or caused by indigestion, memory enhancement, lowering blood pressure and blood sugar, as well as in migraine and Parkinson's disease (Shafizadeh, 2002).

So far, several species of *Salvia* such *as S. sclarea, S. officinalis, S. hypoleuca* and *S.*

syriaca have been studied and researched (Musarurwa, 2013; Topçu et al., 2017). Almost all species of Sage are used in traditional medicine. Previous studies have shown that the *Salvia* has valuable secondary metabolites such as Terpenoids and Flavonoids in roots, leaves and flowers, which are most concentrated during flowering. Rosmarinic acid and Salvianolic acid are secondary metabolites found in Sage, which belong to the group of flavonoids and are dimer and tetramer of Caffeic acid, respectively. In the mint family, Caffeic acid plays a key role as a component in the production of various phenolic metabolites from simple monomers to various oligomeric products. These secondary metabolites have diverse biological activities such as antioxidative, antiplatelet, antitumor and antiviral (Min-Hui et al., 2008; Sharma et al., 2019). The research carried out considers the presence of some compounds in the essential oil of these plants, such as Thujone, Cineole and Camphene, to be responsible for the antimicrobial, antioxidant and possibly anticancer properties of *Salvia.* This plant also has hypoglycemic action (Zarzuelo et al., 1990). *Salvia* has sedative and hypnotic effects, hallucinogen, skeletal muscle relaxant, anti-pain and inflammation, memory booster, anticonvulsant, neuroprotective effects, anti-Parkinson activity and control of alcohol and morphine addiction syndrome. Studies have shown that a diterpene quinones isolated from the extract of *S. miotiorrhiza* binds to central benzodiazepine receptors. Miltirone, another diterpene present in the extract of this plant, binds to benzodiazepine receptors with greater affinity than GABA (cheng et al., 2010). *Salvia aethiopis* leaf extract has analgesic and anti-inflammatory effects. Aethiopinone, a diterpene compound isolated from the extract, has similar effects to Piroxicam or Ibuprofen in reducing acute inflammation (Hosseinzadeh et al., 2003) (Table 1).

PRODUCTION OF SECONDARY COMPOUNDS

Secondary metabolites are organic compounds that do not directly participate in the growth and development or reproduction of a living organism. Over the years, humanity has benefited from this feature in medicinal plants in various industries such as food, pharmaceutical, and cosmetic industries, etc. Unlike primary metabolites, the absence of secondary metabolites does not lead to the immediate death of the organism, but it may cause a long-term disturbance in the survival of the organism, its fertility or its appearance characteristics, or it may not cause any obvious changes. These compounds often play an important role in the defense system of plants (Musarurwa, 2013).

Table 1. *Salvia* species, active substances and possible mechanism

Effective substance or possible mechanism	Type of effect	*Salvia* species
Cirsiliol and caffeic acid ethyl ester	Sedative and sleep-inducing effect	*Salvia miltiorrhiza*
Neo-clerodane Diterpenes salvinorin A	Hallucinogenic effects	*Salvia divinorum*
Aethiopinone	Pain killer	*Salvia aethiopis*
α-Pinene, Camphor, 1,8-cineole	Strengthening memory	*Salvia lavandulaefolia*
Un known	Anticonvulsant effects	*Salvia haematodes*
Inhibitory effect on superoxide production	Neuroprotective effects	*Salvia leriifolia*
Amphetamine-like and dopamine release induced by k+ stimulation	Inhibition of monoamine oxidase enzyme	*Salvia miltiorrhiza*
Miltirone	Effect on morphine withdrawal syndrome	*Salvia miltiorrhiza*

As mentioned, secondary metabolites are not part of the basic molecular structure of the cell, are found in small amounts and may not play an obvious role in growth and development, and if present, are found in specific tissues, organs or at specific stages of development. Also, their production may be limited on a wide level or to a family or a specific genus or even a specific species of plants. Secondary metabolites are widely used in industrial products and are used in the manufacture of medicine, soap, essential oil, paints, gums, resin, rubber, food and beverage seasoning, etc. These compounds have important functions in the plant itself, such as the function of hormones and growth regulators, removing microbial contamination, attracting pollinating agents, and also driving away herbivores and insects, which by this means reduce the damage of animals and insects and

help the producing plants to survive in their ecosystem. Using these compounds, which belong to different chemical families including Alkaloids and Flavonoids, plants protect themselves against microbial attacks, herbivores, and ultraviolet radiation. Secondary metabolites also play a key role in the attraction of flower pollinating insects (especially Anthocyanin pigment and Terpenoid) (Karuppusamy, 2009).
Metabolites or secondary products, apart from the complex biosynthetic pathway for their production, also have a complex structure, and this has made their study slow. Meanwhile, the high value of these compounds in industries such as medicine necessitates the chemical study of these compounds. The study of secondary metabolites also increases our understanding of their biosynthesis and activity. It should be noted that the structure of these molecules is often too complex for fully efficient chemical synthesis. For this reason, despite the low concentration of these metabolites in plants, plants are still the only economically viable source of many of these valuable metabolites (Pichersky & Gang, 2000; Dewick, 2002; Musarurwa, 2013).
Recently, according to numerous reports regarding the importance and medicinal value of secondary metabolites in *Salvia*, special attention of researchers has been drawn to obtain and apply methods to increase their production capacity in this plant. Important and major medicinal metabolites in Sage plants fall into three main categories, which include: Monoterpenes (Cineole, Thujone, Camphor) and Triterpenes (Ursolic Acid and Oleanolic Acid), Diterpenes, and Phenolic acids. Monoterpenes and triterpenes are present in essential oil and together with Flavonoids, they are more abundant in the aerial parts than other compounds, while Diterpenoids and Phenolic acids are the dominant compounds in the root. The main Phenolic acids in Sage species are Caffeic acid and its derivatives. Caffeic acid usually exists as a dimer as Rosmarinic acid (RA), which is the most important Phenolic acid in *Salvia*. Trimers and tetramers of Caffeic acid are of more interest in terms of treatment. The most important Phenolic acids in *Salvia* are Caffeic acid, dihydroxyphenyl Lactic acid, Chlorogenic acid and Danshensu (Caffeic acid monomers), RA (Caffeic acid dimer), Salvianolic acid A (Caffeic acid trimer), and Salvianolic acid B (Caffeic acid tetramer or RA dimer). Of course, there are many types of Salvianolic acids (SAs), but none of them are as well known and studied as salvianolic acid A (SA-A) and salvianolic acid B (SA-B), because the amount of these two compounds in Sage is more than other types of SAs (Topçu et al., 2017) - Figure 1.
In 2017, Topçu et al. conducted a comprehensive study of bioactive compounds in Anatolian *Salvia* species. By examining about 45 species of Anatolian Sage, they succeeded to isolate 317 biochemical compounds. According to their investigation, most of the extracted compounds had diterpene structure (158 compounds) and the highest degree of diversity among diterpene structures has been seen by using spectroscopic methods. In addition, they managed to identify about 60 Triterpenoids, 15 Steroids, 5 Sesterterpenes, 9 Sesquiterpenes, 41 Flavonoids and 29 other phenolics (Topçu et al., 2017).

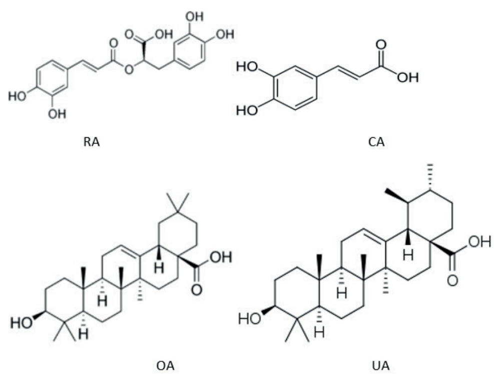

Figure 1. Some important bioactive compounds in *Salvia* species (RA: Rosmarinic Acid, CA: Caffeic Acid, UA: Ursolic Acid, OA: Oleanolic Acid) (Marchev et al., 2014)

TISSUE CULTURE AND CELL CULTURE IN THE PRODUCTION OF SECONDARY METABOLITES

The common method of producing secondary metabolites at the commercial level is extraction from the whole plant. Besides this old method, plant cell culture and tissue culture on a large scale is a significant alternative method that is possible with the help of bioreactors.

Bioreactors provide a sterile environment where environmental factors such as temperature, respiration, oxygen level, movement rate and pH can be controlled, and their use is a reliable method for the propagation of industrial plants (Paek et al., 2005).

Considering the economic importance of secondary metabolites and the limited number of plant species in their natural habitats, cell culture methods are a suitable way to produce valuable chemical compounds. One of the uses of plant cell culture is the production of products with uses such as medicine, fragrances, flavorings and fine chemicals that can limit problems such as slow growth, genetic instability, inability to maintain photoautotrophic growth, and instability of productive cells lines (Paek et al., 2005; Fulzele, 2005). The undifferentiated cells of the hairy roots are of special interest for cell culture, as they are able to continue growing at a high rate by settling in the liquid culture medium, and despite their small size and sensitivity, many bioreactors are able to culture them (Giri & Narasu, 2000). Somatic cells are also a potential source for the mass production of secondary metabolites in bioreactors and because they are small and uniform in size and do not need to be cut for cultivation, they are preferable to traditional micropropagation methods (Paek et al., 2005). The biggest limitation of *in vitro* production is the scale-up of mass production in order to commercialize the product. Hairy roots, callus and suspension cultures face many problems during accumulating. Among the hundreds of secondary metabolites that were mass-produced by undifferentiated cell culture, only Shikonin, Jebensenoside, and Berberine have been produced in high volume, and they have been the most successful samples produced at the commercial level (Paek et al., 2005).

In the *Salvia* species, only *S. officinalis* and *S. miltiorrhiza* are the most widely grown as commercial field crops (Musarurwa, 2013). Many successful studies on the tissue culture of Sage species have been widely conducted. Also, the optimization of micropropagation conditions and increasing the production levels of secondary metabolites have been studied (Santos-Gomes et al., 2003; Mirajalili et al., 2006; Bolta et al., 2013; Musarurwa, 2013).

CELL AND HAIRY ROOT CULTURES

There are some studies on cell suspensions from *Salvia* species as a source of secondary metabolites, but this is clear that due to the abundance of these compounds in the plant, more extensive studies are needed (Marchev et al., 2014). Wang and Wu (2010) investigated callus production using different explants of *Salvia* species. In this study, different explants were able to produce callus on MS medium with the help of growth regulators (Wang & Wu, 2010). One of the common methods of producing secondary metabolites that are created in the root is root inoculation using *Agrobacterium rhizogenes*. Inoculation of plants with microorganisms such as rhizosphere bacteria stimulate specific pathways of secondary metabolites. The production of secondary metabolites in inoculated roots is caused by contamination with *Agrobacterium*, and they usually have the same or higher performance than the metabolites produced in natural plants. This feature makes them suitable for biochemical studies that are difficult on normal plants due to their genetic stability and rapid general growth in a simple culture medium without plant hormones (Ghorbanpoor et al., 2011; Sevon & Oksman-Caldentey, 2002). Due to having a wide level of bioactive compounds, *Salvia* is one of the favorite options of researchers for hairy root culture, however, only limited species of this plant have been used for this purpose (Marchev et al., 2014; Yuan et al., 2009; Xiao et al., 2011; Zhao et al., 2011; Gu et al., 2012; Hao et al., 2012; Grzegorczyk & Wysokińska, 2010). As far as we know, the first successful transformation of tissues from the Sage species was carried out by Hu & Alfermann, 1993. In this study, with the help of different *Agrobacterium* strains, they succeeded in producing transgenic hairy roots in *Salvia miltiorrhiza* (Hu & Alfermann, 1993).

In 2017, Norouzi et al. investigated hairy root induction in some species of Sage. They studied two Iranian endemic species (*Salvia eremophila* and *S. reuterana*) and five non-endemic species (*S. macrosiphon*, *S. multicaulis*, *S. nemorosa*, *S. verticellata* and *S. virigata*) for this purpose. 4 *Agrobacterium* strains 1728, 2659, ATCC-15834 and A4 have been used in this study. According to the obtained results, stem and petiole explants were unable to produce hairy roots, while all leafy explants produced hairy roots. The results have shown that different strains of *Agrobacterium* and different species of Sage have a significant effect on the number and abundance of hairy roots (Norouzi et al., 2017).

It is obvious that the optimization of cell culture and hair root methods can help to produce biochemical compounds on a large scale. However, the commercialization of the protocols for the production of secondary metabolites in *Salvia* species faces many pitfalls and requires extensive research (Marchev et al., 2014).

ELICITATION

Elicitors are compounds of biological or non-biological origin that cause the biosynthesis and accumulation of secondary metabolites through the induction of defense responses. Biological elicitors include polysaccharides, proteins, glycoproteins or parts of the cell walls of fungi, plants (cellulose and pectin) and microorganisms (glucan and chitin). In general, the use of elicitors in biotechnological studies of plant metabolites pursues two main goals: 1- Obtaining information on the biosynthetic pathways that lead to the formation and regulation of secondary metabolites. 2- Increasing the production of secondary metabolites for commercial use (Zhao et al., 2005; D'Amelia et al., 2017).

Many stimulants such as polysaccharides, glycoproteins, low molecular weight organic acids, fungal cell wall materials, ultraviolet rays and heavy metal salts have been tested to increase bioactive compounds in *Salvia*. As a group of efficient non-biological stimuli, the use of metal ions including Ca^{2+}, Co^{2+}, Ag^{+}, Cd^{2+}, Cu^{2+}, Ce^{3+}, Li^{+}, Mn^{2+} and Zn^{2+} to induce the production of bioactive compounds in plants. Heavy metals have a significant effect on synthesis and secondary metabolism. Many types of heavy metals have been used as stimulants to induce the accumulation of bioactive compounds in the hairy roots of *S. miltiorrhiza*. Among metal ions, silver ion is known as an effective stimulant in improving the production of phenolic acids (Rosmarinic acid and Salvianolic acid B-B) and Tanshinones (Zhao et al., 2010a; Marchev et al., 2014). Another non-biological stimulant effective in inducing the production of phenolic acids in Sage plants is methyl jasmonate. Methyl jasmonate induces the production of secondary metabolites by activating phenylalanine ammonia-lyase (PAL). Jasmonates have been introduced as key messenger compounds in the induction process leading to the accumulation of secondary metabolites (Kuz'ma et al., 2008).

GENETIC MANIPULATION OF BIOCHEMICAL PATHWAYS TO PRODUCE MORE BIOACTIVE COMPOUNDS

Metabolic engineering is the purposeful change of metabolic pathways of an organism in order to better understand and identify cellular pathways and use this information for chemical transport and energy management and supramolecular assemblies. The use of these methods in plants provides the possibility of manipulating biochemical pathways, and transgenic plants are produced, the amount of production of their natural products has been changed according to commercial and agricultural interests and suitable characteristics after harvesting (Lessard, 1996; Kinney, 1998; D'Amelia et al., 2017). In recent decades, plant cell culture has been identified as a powerful tool for the production of commercial metabolites, but despite many efforts in the field of *in vitro* production, since the amount of production is not sufficient for their commercialization, metabolite engineering can be useful methods for Increasing production. Identifying the genes involved in the biosynthesis of bioactive compounds can help to increase the production of these compounds. The biosynthesis of plant metabolites in *Salvia* can be specifically changed through increasing the expression or silencing of genes encoding the production of secondary metabolites (Zhou et al., 2011).

D'Amelia et al. (2017) studied isolated and engineered genes involved in the biosynthesis of Polyphenolic and Terpenoids compounds from different *Salvia* species. Most of these genes have been identified and isolated from *Salvia miltiorrhiza* (D'Amelia et al., 2017) .

PLANT/MICROBE CO-CULTURES

In the production of secondary metabolites, there are three theories about the origin of the metabolism of secondary metabolites in plants. One is that both plants and microbial agents participate in the process of making these natural products. Another theory suggests the transfer of genes between plants and microbes in distant times, and the third theory believes that either plants or fungi produce these secondary metabolites and transfer them to their symbionts (Wink et al., 2005).

The combination of inducing agents in symbiotic plants and fungi increases the accumulation of secondary metabolites in both of them. Therefore, the coexistence and interaction between plants and fungi and their impact on each other during the production of important biological compounds needs further study and can be a good topic for the production of secondary metabolites using metabolic engineering and genetics (Zhang et al., 2009). The studies that have been done so far have shown that among the bacterial species, *Bacillus cereus* has the most impact in increasing the production of plant metabolites, including Tanshinones in *S.* miltiorrhiza (Zhao et al., 2010b). Although few studies in this field have been conducted on the *Salvia*, it is clear that more comprehensive and detailed studies are needed to find more results (Marchev et al., 2014).

FUTURE PROSPECTIVE

The advances made in the field of biotechnology have led to the creation of new methods such as cell culture for the production of biochemical compounds of plants and their commercialization.

The advantage of these methods over the traditional methods of making secondary metabolites under controlled conditions and independent of climate conditions is that they can create reliable renewable resources for the required natural products.

The production of medicinal compounds *in vitro* is considered a promising development in the field of plant sciences, and the use of genetic tools, the identification of the structure of secondary metabolites and their metabolic pathways can help the commercial production of these products. The growing need of the market for natural compounds for the production of medicine, in addition to the lack of raw materials and low production efficiency, helped the emergence of new biotechnological methods for the production of secondary metabolites on a mass level. Until today, there is still no sufficient knowledge about the biosynthesis pathways of chemical compounds in plants and cell cultures, therefore, providing solutions to develop information on the molecular and cellular level of these pathways is a major need.

CONCLUSIONS

Salvia is one of the most valuable medicinal plants, which can be a rich source of plant metabolites due to its wide range of bioactive compounds. The use of modern biomolecular methods and the production of transgenic crops can effectively influence the biosynthetic pathways and will be a big step towards the production of secondary metabolites on a mass and commercial level in this plant.

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