An Overview on Two Valuable Natural and Bioactive Compounds, Thymol and Carvacrol, in Medicinal Plants

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Abstract

Thymol (2-isopropyl-5-methylphenol), and carvacrol (5-isopropyl-2-methylphenol) are the main components of the essential oils of some Laminaceae,Verbenaceae and Ranunculaceae members such as oregano, thyme, savory, *etc*. The formation of thymol and carvacrol is thought to involve hydroxylation of *γ*-terpinene and *p*-cymene precursors. They are produced by the plant species as a chemical defense mechanism upon exposure to pathogens, pests, herbivores, or environmental stresses. Accordingly, the potent antimicrobial and fungi toxic properties of these compounds against various plant pathogens have been demonstrated. They have antibacterial, antifungal, insecticidal, and anti-oxidative properties which are the basis for the wide use of these compounds in the cosmetic, food, and pharmaceutical industries.

Keywords: **Thymol, Carvacrol, Biological activity, Biosynthesis pathway, Terpenes**

Introduction

Plants are sophisticated light-driven "green" factories able to synthesize an immense number of bioactive natural products [1]. These natural products are also referredto as secondary metabolites since they are not directly essential for thebasic processes of growth and development [2]. They can be divided into two major classes, the first class formed by nitrogen-containing substances, such as alkaloids, amines, cyanogenic glycosides, non-protein amino acids and glucosinolates, and the second class consisting of nitrogen-free substances which are represented by polyketides, poly-acetylenes, saponins, phenolics and terpenes [3].

The family Lamiaceae contains many aromatic plants of great scientific and economic interest such as rosemary, sage, mint, marjoram, oregano and thyme. The aroma associated with these plants arises from the essential oil found in palate glandular trichomes on the aerial parts of the plant. These glandular trichomes consist of highly specialized secretory cells which the components of the essential oil are synthesized and accumulate subsequently in subcuticular storage cavity [4, 5]. The mono and sesqui-terpenes are main compounds of essential oils of oregano, thyme and marjoram [6]. These substances are responsible for the aroma and flavor of these herbs, and the extracted essential oils are used for the manufacturing of perfumes and cosmetics as well as for medicinal purposes as antimicrobial or antiseptic agents [6, 7]. Mono- and sesqui-terpenes are also thought to help defend the plant against herbivores and pathogens [8]. Two monoterpenes of the Lamiaceae that have attracted much attention are thymol and carvacrol which are often found in thyme and oregano. These two phenolic monoterpenes are especially known for their anti-herbivore, antimicrobial, and antioxidant activities [9-13].

Biosynthesis of terpenes

Terpenes (also known as terpenoids or isoprenoids) form the largest group of natural products with more than 30,000 different structures [14]. Terpenes play important roles in almost all the basic processes, including plant growth, development, reproduction and defense [8]. The oxidized monoterpenes, thymol and carvacrol, are most likely derived from one of the initial cyclic products, *γ*-terpinene, by oxidation [15]. *γ*-terpinene formed from geranyl pyrophosphate (GPP) by a monoterpene synthase, *Ov*TPS2 [16]. Several evidences are supporting the involvement of cytochrome P450s in thymol and carvacrol biosynthesis. Eleven new cytochrome P450 gene sequences are described from oregano, thyme and marjoram which were assigned to five new cytochrome P450 genes. The expression levels of these genes, quantified by relative and absolute quantitative real-time PCR, then correlated with thymol and carvacrol biosynthesis in *Origanum vulgare* L., *Thymus vulgaris* L., and *Origanum majorana* L.

The mevalonic acid (MVA) pathway is located in the cytosol in peroxisomes and endo-plasmatic reticulum (ER). It starts and overs with three units of acetyl-CoA and farnesyl pyrophosphate (FPP) respectively, which is the precursor molecule for all sesqui-terpenes. The methyl-erythritol-phosphate (MEP) pathway is located in the plastids and the initial substrates are glyceraldehyde-3-phosphate (GA3P) and pyruvate. Geranyl pyrophosphate (GPP) is the precursor for all monoterpenes and geranyl geranyl-pyrophosphate (GGPP) is the precursor for diterpenes. Carotenoids are derived from two units of GGPP. Dimethylallyl pyrophosphate (DMAPP) is the backbone to which different numbers of the isomer isopentenyl pyrophosphate (IPP) are added to form GPP, FPP or GGPP. Ubiquinone is formed in mitochondria [17, 18]. Terpenes are formed from the universal five-carbon building blocks, isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP), which are both synthesized by the plastidic methylerythritol pathway and the cytosolic mevalonate pathway (Figure 1) [8].

The reaction starts by ionization of GPP and the resulting geranyl cation is isomerized to a linalyl intermediate capable of cyclizations. The initial cyclic *α*-terpinyl cation is the central intermediate and can be subject to further cyclizations, hydride shifts and rearrangements before the reaction is terminated by deprotonation or water capture. The number of carbon atoms in intermediates and products corresponds to GPP [19].

Within the secretory cells, the five-carbon structure blocks for terpenes, isopentenyl pyrophosphate and its isomer dimethylallyl pyrophosphate, are synthesized by the plastidial methylerythritol pathway and the cytosolic mevalonate pathway [8]. Two of these five-carbon units are fused to form GPP, the usual precursor of the monoterpenes, and three of these units are fused to form farnesyl pyrophosphate (FPP) the precursor of most sesqui-terpenes. Next, the linear carbon skeletons of GPP and FPP are converted to the basic terpene skeletons by terpene synthases, a widespread class of enzymes responsible for the huge structural diversity of monoterpenes and sesqui-terpenes [20]. Terpene synthase genes have been isolated and characterized from several Lamiaceae species, including the genus *Mentha* where a limonene synthase, an (*E*)-*β*-farnesene synthase, and a *cis*-muuroladiene synthase have been identified [21, 22]. From other Lamiaceae, terpene synthase genes are known from *Salvia officinalis*, *Salvia pomifera* and *Salvia fruticosa* [23], *Rosmarinus officinalis* [24], *Lavandula angustifolia* [25] and *Ocimum basilicum* [26]. The initial terpene synthase products (olefins and monoalcohols) are often further oxidized or conjugated. In *Mentha*, the biosynthesis of menthol and carvone involves hydroxylation steps catalyzed by cytochrome P450 monooxygenases [21, 22, 26].

Plant terpene biosynthesis is often restricted to special morphological structures like idioblasts (e.g. oil cells in *Laurus* sp.) or ducts (e.g. resin ducts in *Pinus* sp.) or trichomes (e.g. glandular trichomes in Lamiaceae and Asteraceae) that can store these lipophilic compounds in high concentrations (Figure 2) [27, 28]. Studies on Lamiaceae species such as mint (*Mentha* sp.) and sweet basil (*Ocimum basilicum*) have provided many insights about essential oil biosynthesis. The essential oil in mint and sweet basil is produced in glandular trichomes situated on the aerial parts of the plants. These glandular trichomes consist of a cluster of secretory cells covered by a subcuticular storage cavity where the essential oil accumulates [4, 5].

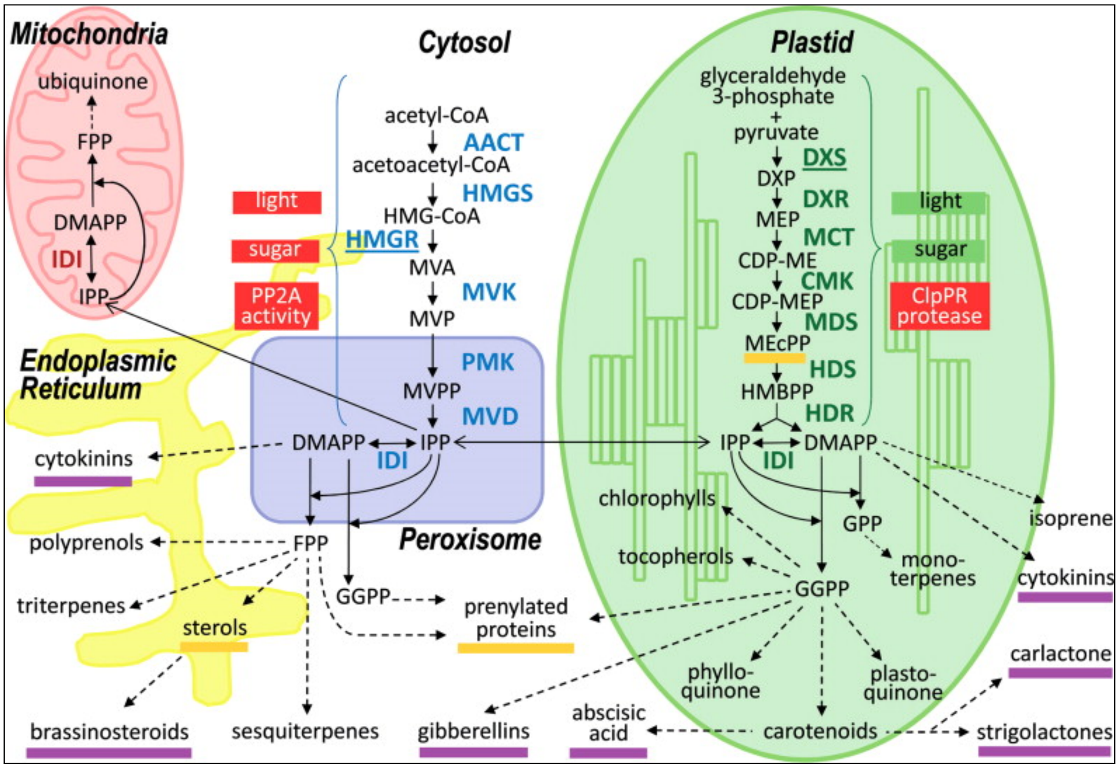

Figure 1- Biosynthesis pathway of terpenes in the plant cell. **Abbreviations: CDP-ME, 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol; CDP-MEP, CDP-ME 2-phosphate; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; HBMPP, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; IPP, isopentenyl diphosphate; MEcPP, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; MEP, 2-C- methyl-D-erythritol 4-phosphate; MVA, mevalonic acid; MVP, 5-phosphomevalonate; MVPP, 5-diphosphomevalonate [8]**

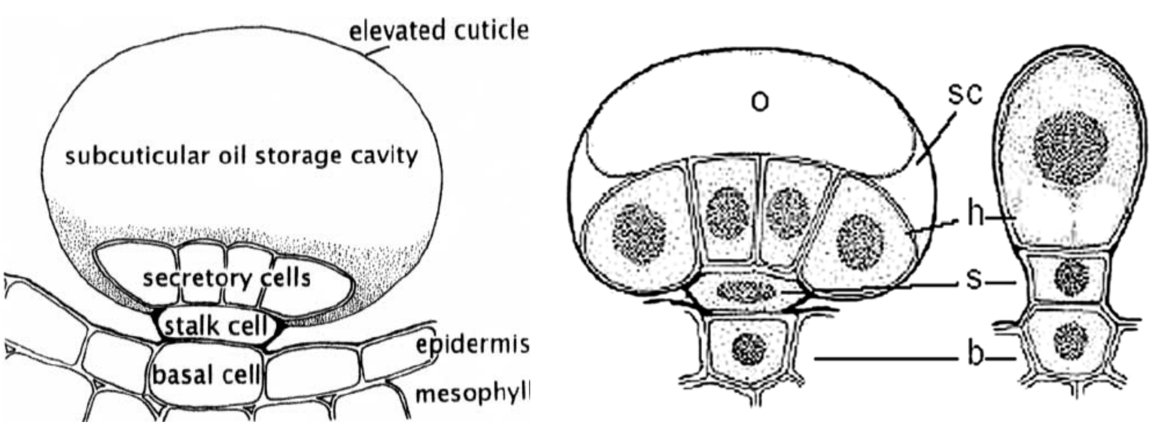

Figure 2-Schematic of peltate glandular trichomes of Lamiaceae. **Schematic drawing of a peltate glandular trichome with a basal cell (b) and the head cells (h) based on one stalk cell (s). Secreted terpenes are stored in the subcuticular cavity (sc) as an oil drop (o). Eight larger cells are arranged around four smaller inner cells [28].**

Oxidation of monoterpenes

Chemical transformation of abundant and cheap products into novel and more valuable compounds can be achieved by liquid-phase oxidation reactions using hydrogen peroxide as clean oxidant and zeolite encapsulated metal complexes as heterogeneous catalyst [29]. Hydrogen peroxide is a clean oxidant because it is easy to handle and its reaction produces only water as by-product [30]. Catalytic oxidation of aromatic monoterpenes with hydrogen peroxide is a reaction of industrial importance [31].

Activation of hydrogen peroxide can be divided into two parts: Catalytic activation by transition metal complexes and direct activation (unproductive reaction), Fenton chemistry [32]. For these activations, two general types of mechanisms have been postulated for decomposition of hydrogen peroxide. The first is Fenton reaction mechanism which is free radical mechanism (homolytic pathway) and second is the peroxide complex mechanism (heterolytic pathway) [17]. Oxidations with H_2O_2 can involve homolytic pathways via free radical intermediates and/or heterolytic oxygen transfer processes [30].

Carvacrol is used as reactant which is the major monoterpene component of essential oil. In the presence of transition metal complexes oxidation reactions with hydrogen peroxide are direct activation (unproductive oxidation) and productive oxidation reactions as seen in Figure 3, and water is only by product. Catalysts (transition metal complexes) are transferred oxygen from hydrogen peroxide to the reactant or substrate by a homolytic cleavage of the metal oxygen bond [17].

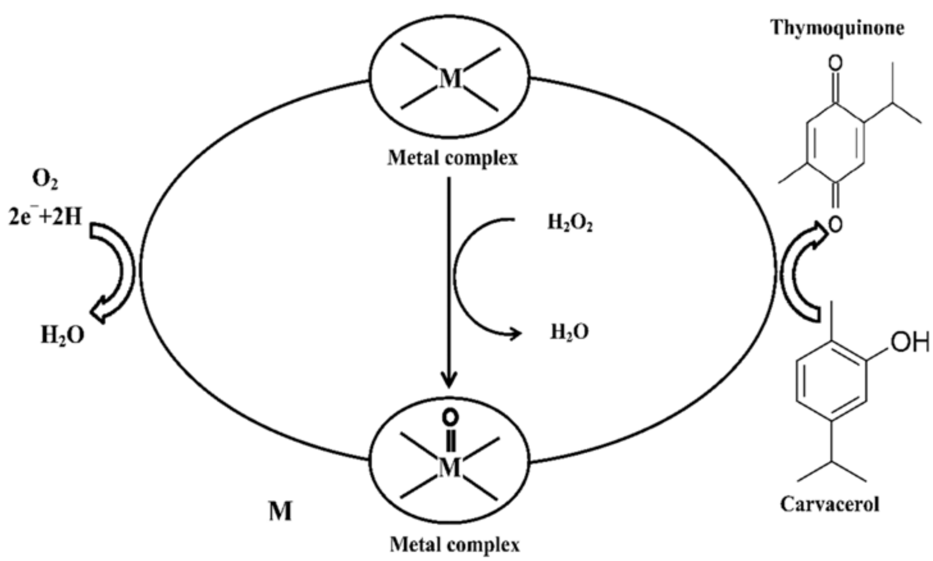

Figure 3- Oxidation of carvacrol by hydrogen peroxide by using metal complex [31].

Cytochrome P450s - a widespread enzyme family

Cytochrome P450s monooxygenases (P450s) form an ubiquitous class of enzymes known from all kinds of organisms including animals, bacteria, fungi, archaea, protists and even viruses. Cytochrome P450 monooxygenases are hemi-dependent mixed function oxidases that utilize NADH (Nicotinamide adenine dinucleotide phosphate) and/or NADPH to reductively cleave atmospheric dioxygen producing a functionalized organic product and a molecule of water [33]. All plant cytochrome P450s described to date are bound to membranes of the endoplasmic reticulum through a short hydrophobic segment of their N-terminus [34, 35]. P450s need to be coupled to an electron donating protein, a cytochrome P450 reductase or a cytochrome b5, which is also anchored to the endoplasmic reticulum by its N- or C-terminus [33, 35]. γ-terpinene is the most likely converted to thymol by the action of one or more cytochrome P450 oxidases, catalyzing a hydroxylation similar to (-)-*S*-limonene in menthol biosynthesis in *Mentha* sp. [26, 36].

Hydroxylation reactions catalyzed by cytochrome P450s [33] which is important since the position of hydroxylation can determine the downstream fate of the products in biosynthetic pathways. In mint, the oxygenation pattern of monoterpenes is determined by the region specificity of P450 mediated (-)-*S*-limonene-hydroxylation either at carbon C_3 or C_6. The structural similarity of these highly region specific limonene hydroxylases to those enzymes forming thymol and carvacrol was previously explained [37]. The biosynthesis of thymol and carvacrol is thought to involve hydroxylation of *γ*-terpinene and *p*-cymene precursors. Researcher found that an *Origanum vulgare* L. (oregano) cDNA library for sequences similar to those of known cytochrome P450 monoterpene hydroxylases [38] (Figure 4).

Production of thymoquinone from thymol and carvacrol

Thymoquinone has a commercial value considerably higher than its precursors (thymol and carvacrol) found in thyme essential oil. It can be obtained by catalytic oxidation of thymol and carvacrol. Essential oil rich in carvacrol and thymol were easily oxidized to oil containing thymoquinone as the main component in the presence of Fe (III) porphyrin and phtylocyanine complexes [39]. The carvacrol oxidation with hydrogen peroxide was also studied using Mn (III) porphyrin complexes and keggin-type tungstoborates [40, 41]. Oxidation of carvacrol yielded a mixture of benzoquinones containing a small amount of thymoquinone for keggin-type tungstoborates whereas for Mn (III) porphyrin complexes oxidation of carvacrol selectively yielded thymoquinone. Thymoquinone can be obtained in carvacrol oxidation reactions catalyzed by zeolite encapsulated metal complexes. The oxidation of carvacrol ($<25\%$ conversion) and thymol ($<18\%$ conversion) gave thymoquinone with 100% selectivity. However, leaching of the porphyrin complex from the zeolitic matrix occurred in the presence of H_2O_2 [42].

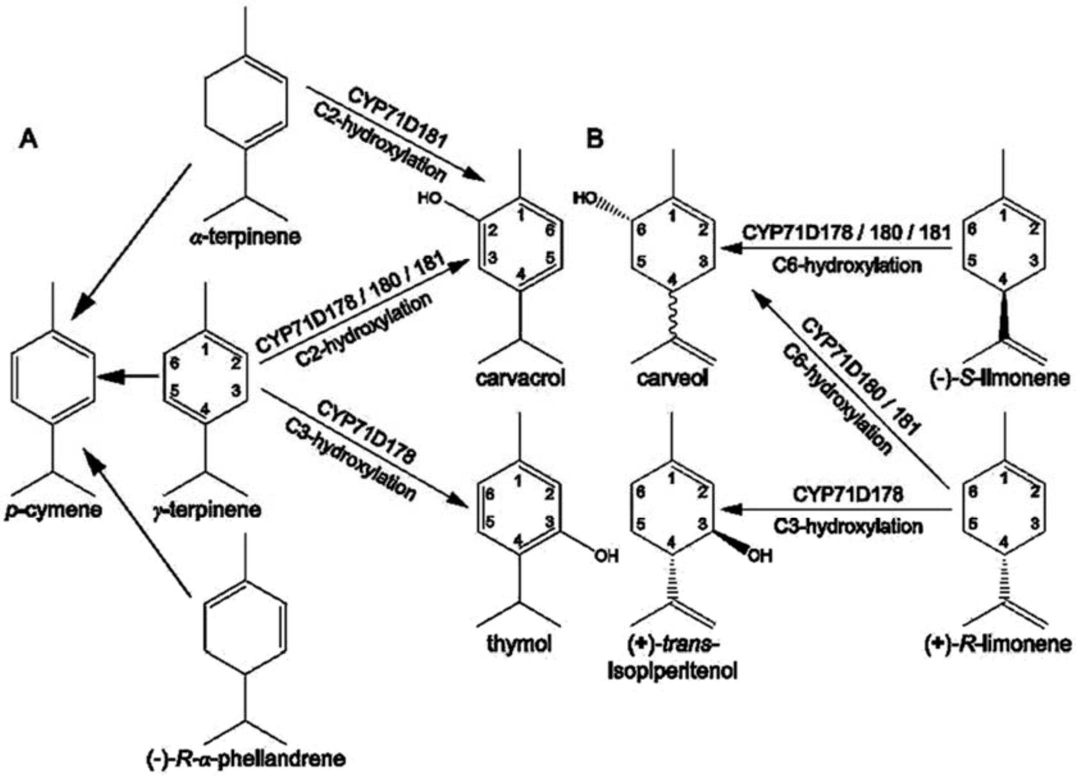

Figure 4-Chemical structures of all substrates and major products formed by CYP71D178, CYP71D180v1 and CYP71D181 in vitro. **(A) All three enzymes form mainly p-cymene from γ-terpinene, α-terpinene and (-)-R-α-phellandrene as indicated by the arrows. CYP71D178 forms thymol and carvacrol from γ-terpinene while CYP71D180v1 and CYP71D181 from only carvacrol. CYP71D181 forms carvacrol also from α-terpinene. (B) Major products formed from (-)-*S*-limonene and (+)-R-limonene. Mainly carveol is formed from (-)-*S*-limonene by all three enzymes. CYP71D180v1 and CYP71D181hydroxylate (+)-R-limonene only at carbon C6 while CYP71D178 catalyzes a hydroxylation at C3. Hydroxyl groups at carbon C2 in carvacrol corresponds to that designated as C6 in carveol. The numbering differs because of different substituent priorities in the p-cymene vs. limonene carbon skeleton [16].**

Plants containing carvacrol and thymol

Carvacrol, thymol and *p*-cymene are *p*-menthane type of aromatic monoterpenes, which can be found in the essential oils of aromatic plants [40]. These two phenolic monoterpenes are especially known for their anti-herbivore, antimicrobial, pharmaceutical and antioxidant activities [43, 10, 44, 45, 13]. Thyme volatile phenolic oil has been reported to be among the top 10 essential oils, with antibacterial, antimycotic, antioxidative, natural food preservative, and mammalian age delaying properties [46, 47]. Thymol and its chemical relative carvacrol are found not only in thyme and oregano, but also in many other plant species in different families [7, 48, 31, 49].

Table 1- List of some plants that contain thymol and carvacrol

Family	Species	Local name	Origin	Thymol (%)	Carvacrol (%)	References
Lamiaceae	*Zataria multiflora* Boiss.	Avishan Shirazi	Iran, Pakistan and Afghanistan	3.6 ± 4	68.3 ± 15.5	[49]
Lamiaceae	*Thymus vulgaris* L.	Garden Thyme	North America, Europe and North Africa	45.2-60.5	5.1 -3.3	[50, 51]
Lamiaceae	*Thymus daenensis* Celak	Avishane-Denaei	The endemic *Thymus* species in Iran	73.9	6.7	[52, 53]
Lamiaceae	*Thymus kotschyanus* Boiss. & Hohen	Avishane-Kouhi	Zagross mountain	6.8	50.4	[54, 55]
Lamiaceae	*Satureja bachtiarica* Bunge	Marze-Bakhtiyari	South-eastern in Iran	20.6	26.4	[56, 57]
Lamiaceae	*Satureja montana* L.	Winter Savory	Europe, North America, and South America	28.9	45.7	[58]
Lamiaceae	*Origanum vulgare* L.	Oregano, wild Marjoram	Eurasia and the Mediterranean	3.5	2.4	[59]
Lamiaceae	*Origanum dictamnus* L.	Dittany	Greek	-	68.9	[60]
Verbenaceae	*Lippia sidoides Cham.*	Alecrim-pimenta	Northeast of Brazil	56.7	16.7	[61]
Ranunculaceae	*Nigella sativa* L.	Nigella or Kalonji	South and South West Asia	1.67	2.53	[62]

Thymol

Thymol (also known as 2-isopropyl-5-methylphenol, IPMP) is a natural monoterpenephenol derivative of cymene, $C_{10}H_{14}O$, isomeric with carvacrol. Thymol is only slightly soluble in water at neutral pH, but it is extremely soluble in alcohols and other organic solvents. It is also soluble in strongly alkaline aqueous solutions due to deprotonation of the phenol. Thymol not directly formed by the characterized terpene synthases. It is the aromatic monoterpene which is predicted to be synthesized from γ-terpinene (a product of *Ov*TPS2) via *p*-cymene [63, 64, 65].

Analysis of the terpene content of the inbreed lines ff2 and ff7 (Figure 4) supports this hypothesis that no thymol is found in lines that lack the γ-terpinene and *p*-cymene. γ-terpinene is most likely converted to thymol by action of one or more cytochrome P450 oxidases similar to (-)-*S*-limonene in menthol biosynthesis in *Mentha* sp. [66, 22]. Conversion of γ-terpinene to thymol might proceed via a *p*-cymene intermediate which was detected in minor amounts in *in vitro* assays of the γ- terpinene synthase *Ov*TPS2. However, these low levels of *p*-cymene may be instead due to spontaneous conversion of γ-terpinene into *p*-cymene [67].

Biosynthesis pathway of thymol

The biosynthesis pathway of thymol has been studied and coworkers identified thymol as a terpenoid biosynthetic product despite the fact that it is aromatic [68]. It was demonstrated the involvement of cytochrome P450 mono oxygenases in the conversion of *γ*-terpinene to thymol and carvacrol [16]. Heterologous expression of two of them in yeast resulted in active proteins catalyzing the formation of *p*-cymene, thymol and carvacrol from *γ*-terpinene. Since *p*-cymene itself was not accepted as a substrate, it is likely that *γ*-terpinene is directly converted to thymol and carvacrol with *p*-cymene as a side product. The properties and sequence motifs of these P450s are similar to those of well-characterized monoterpene hydroxylases isolated from mint. The general outline of monoterpene biosynthesis is well known [8]. First, the ubiquitous C10 intermediate, geranyl pyrophosphate (GPP), is converted by enzymes known as monoterpene synthases to cyclic or acyclic products. Then, oxidized monoterpenes are formed from these initial cyclic or acyclic products by reactions catalyzed frequently by cytochrome P450s monooxygenases [69]. The oxidized monoterpenes, thymol and carvacrol, are most likely derived from one of the initial cyclic products, *γ*-terpinene, by oxidation [64].

The possible positions of ^{13}C atoms are highlighted as colored bars and small boxes, respectively (Figure 5); the color depends on the metabolic pathway which leads to a certain metabolite. 3-phosphoglycerate (PGA) and glyceraldehydes 3-phosphate (GAP) are built up in the Calvin cycle which generate either molecules with two ^{13}C atoms (pink bar) or molecules carrying three ^{13}C atoms (red bars). A label at position 1 exclusively is also possible. The synthesis of monoterpenes is possible via the MEP pathway (left side) based on pyruvate and GAP which leads to a labelled

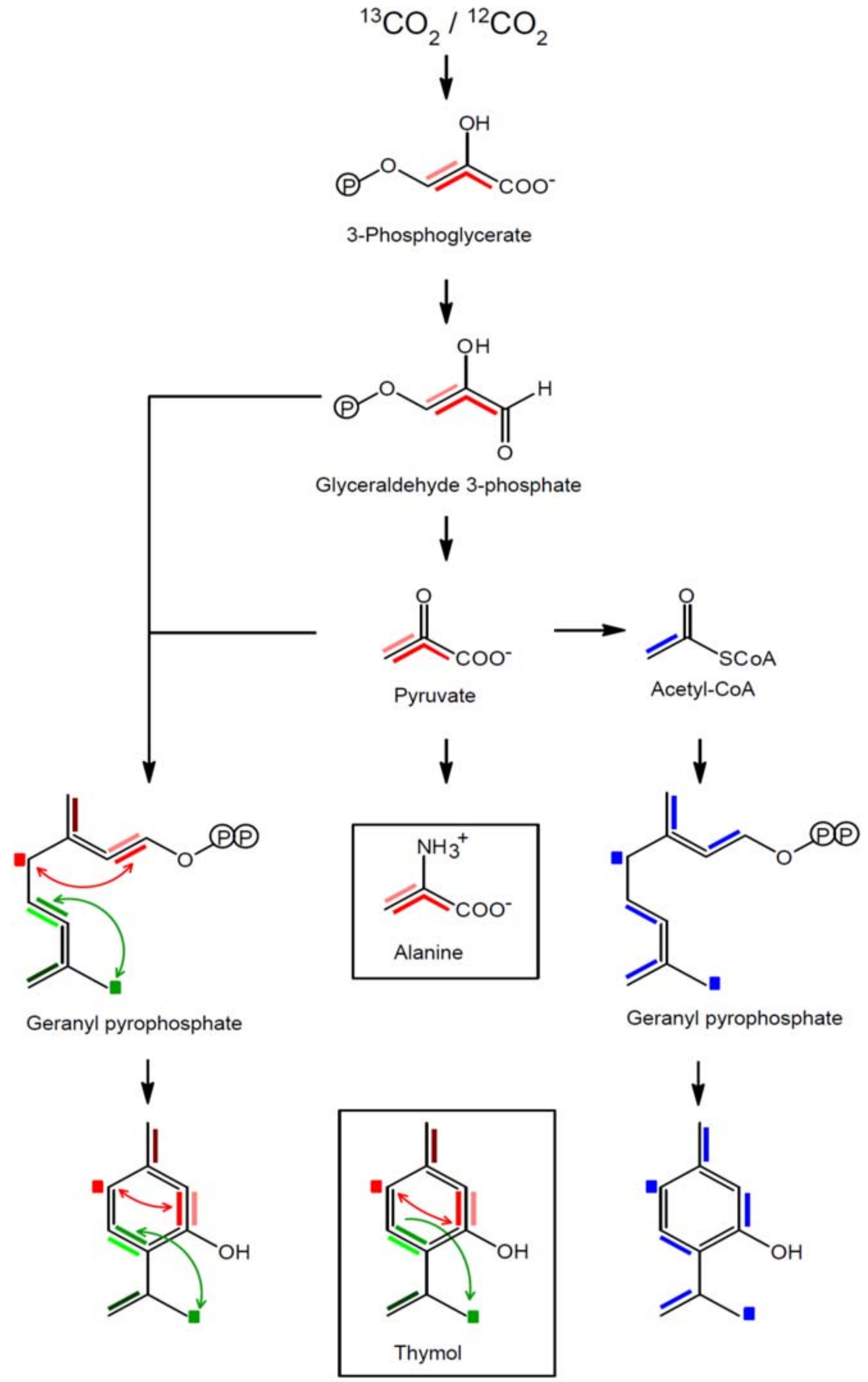

Figure 5- Incorporation of ^{13}C atoms derived from labelling experiments with $^{13}CO_2$ into various metabolites during the plant metabolism [70]

C2 block originating from pyruvate (dark red bar and dark green bar, respectively) and/or a C2 or C3 block originating from GAP carrying either two ^{13}C atoms (pink and light green bar, respectively) or three ^{13}C atoms (red/green bar and red/green box, respectively), respectively. Red color marks carbon atoms coming from IPP and green color marks carbon atoms coming from DMAPP. On the right side the predicted labelling pattern of thymol arising from the mevalonate pathway based on acetyl-CoA is shown. This path does not generate monoterpenes carrying three ^{13}C atoms. Boxed thymol is displayed with the labelling pattern observed by NMR spectroscopy [70].

Carvacrol

Carvacrol (2-methyl-5-isopropylphenol) is a phytochemical derived from aromatic plants of the genera oregano, thyme and satureja. Brownish liquid practically is insoluble in water and very soluble in ethanol (96%). Carvacrol is a monoterpene phenolic constituent of essential oils of various aromatic plants. The essential oils obtained from the genera *Origanum*, *Thymbra*, *Cordiothymus*, *Satureja*, and *Lippia* are rich sources of carvacrol (CRL). It is synthesized by the specialized secretory cells in the aerial parts of the plants and is stored in a subcuticular storage cavity. The essential oils containing the active substances are then extracted from the plant materials by mechanical pressing or steam distillation or by using organic solvents [71, 72].Carvacrol ($C_{10}H_{14}O$), is represented by the synonyms: isopropyl-o-cresol, p-cymen-2-ol, 2-hydroxy-p-cymene, 5-isopropyl-2-methylphenol and iso-thymol.

Carvacrol is an isomer and derivative of phenol, the chemical formula of carvacrol (cymophenol) is $C_6H_3CH_3(OH)(C_3H_7)$, a monoterpenoid phenol[73,74]. Carvacrol is a liquid and has the same taste of thymol.The density of carvacrol ranges from 0.976 g/cm^3 at 20 °C to 0.975 g/cm^3 at 25°C. The boiling point of carvacrol is 237~238 °C. It can bevolatile with steam. Carvacrol is highly lipophilic and insoluble in water [75]. Yadav and Kamble (2009) reported that formation of carvacrol could be resulted from alkylation of *o*-cresol with propylene or isopropyl alcohol (IPA) over solid acid catalysts [75]. Alkylation of *o*-cresol with propylene or IPA over solid acid catalysts results in the formation of carvacrol. Carvacrol does not have many long-term genotoxic risks. The cytotoxic effect of carvacrol can make it an effective antiseptic and antimicrobial agent. Carvacrol has been found to show antioxidant activity [76].

Biological activity of thymol and carvacrol

Effects of thymol on melanoma cells

Aromatic monoterpene, thymol, shows several beneficial activities, such as an anti-oxidative effect. However, the mechanism of their toxicity remains to be fully defined. Thymol was characterized as a melanin formation inhibitor in an enzymatic system. The antioxidant actions of thymol generate a stable phenoxy radical intermediate, which generates reactive oxygen species and quinone oxide derivatives. Thus, it is proposed that the primary mechanism of thymol toxicity at high doses is due to the formation of antioxidant-related radicals [77] (Figure 6).

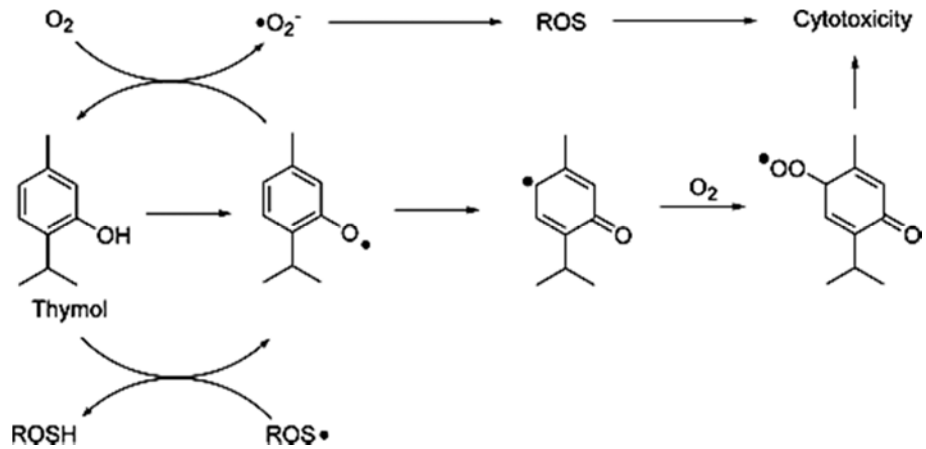

Figure 6-Effects of thymol on melanoma cells.

Biological activity of carvacrol

Several *in vitro* and *in vivo* studies described different bioactivity of carvacrol, including antibacterial, antioxidant, antiseptic, antispasmodic, growth promoter, antifungal, antiviral, anti-inflammatory, expectorant, antitussive, immunomodulatory and chemo preventive as well as modifier of rumen microbial fermentation and reduction of methane emission [9, 36, 78, 79]. Carvacrol plays a critical role as natural antioxidant in the reduction of lipid peroxidation which leading to oxidative destruction of cellular membranes [80]. Moreover, the deleterious effect of these compounds may lead to increase in the production of toxic metabolites (free radicals) and also to apoptosis (Figure 7) [79-81].

Improving nutrients bioavailability and growth/productive performance

Researcher reported that feed supplementation with thymol + carvacrol mixture by 60, 100, and 200 mg/kg of diet improved growth performance, digestive enzyme activities, and antioxidant enzyme activities besides inhibiting lipid peroxidation in broiler chicks [36].

Antimicrobial activity

The antimicrobial effects of essential oils have been due to the presence of phenolic compounds, such as carvacrol, thymol, eugenol, curcumin and cinnamaldehyde which are presented in essential oils of oregano, thyme, clove, turmeric and cinnamon, respectively [81-84]. Sikkema *et al*. (1995), Adam *et al*. (1998), Ben-Arfa *et al*. (2006) and Nostro and Papalia (2012) mentioned that the beneficial inhibitory effects of phenolic compounds could be attributed to the interactions between the effective compounds and cell membrane of microorganisms and is usually associated with the hydrophobicity of these compounds [84-87].

Arsi *et al*. (2014) showed that numbers of *Campylobacter* were reduced with 1% carvacrol supplementation, or a combination of both thymol and carvacrol at 0.5% [88]. Friedman *et al*. (2002), Nastro *et al*. (2004) and Baser (2008) found antimicrobial

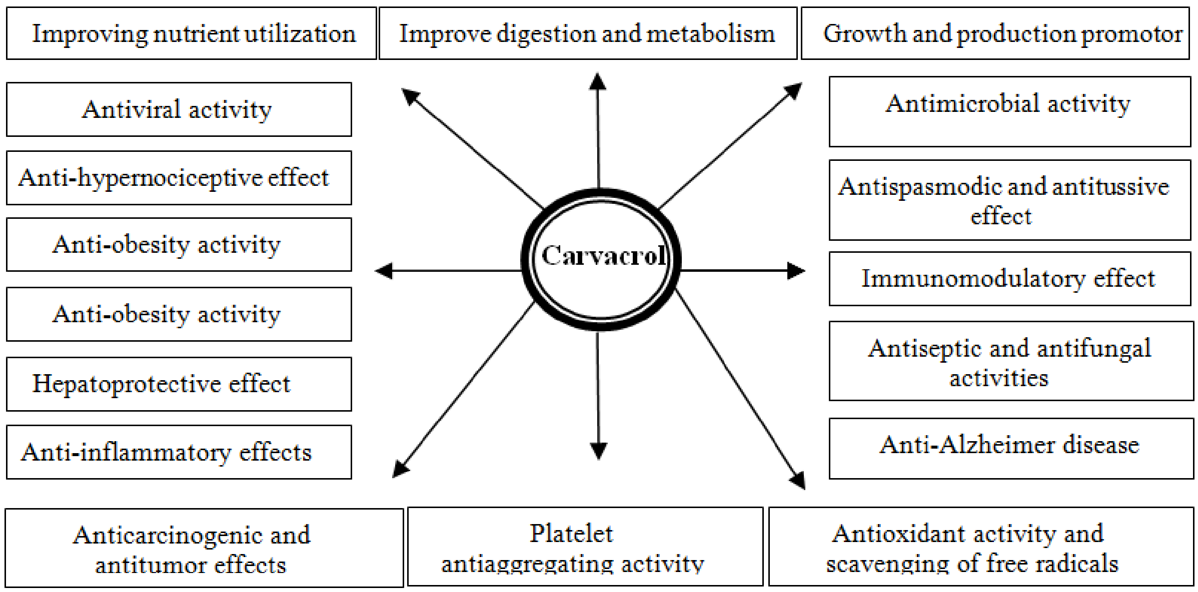

Figure 7-Modes of action and biological activities of carvacrol [79-81]

influences of carvacrol against many species of microbes such as *Pseudomonas, Aspergillus, Salmonella, Streptococci*, *Listeria*, *Bacillus* and *Fusarium* [89, 90, 71]. Also as carvacrol supplement as antimicrobial component has a significant impact on harmful bacteria including *Escherichia coli* and *Salmonella* numbers in chickens, this effect may be attributed to inhibit the growth of pathogenic bacteria by carvacrol vapor [89-91].

Antifungal activity of thymol and carvacrol

Thymol is normally the major phenolic component in common thyme [92]. *Zataria multiflora* effectively inhibited growth of *Aspergillus niger* [49]. Antifungal activity against *A. niger* were reported by Siddiqui *et al*. (1996) [92]. The high phenolic contents of *Z. multiflora* such as thymol, *p*-cymene and carvacrol may account for its strong antifungal and antimicrobial activity [93, 94, 95]. *Thymus daenensis* essential oils have significant antifungal properties against three pathogens: *Rhizoctonia solani, Fusarium solani* and *Alternaria solani*. In all samples, the maximum antifungal activity of the essential oil was observed in flowering stage but this different was not significant. The results showed that, the highest thymol content was obtained in flowering stage and the greatest antifungal activity was observed in this period. The amount of thymol contents was lower in pre flowering period than flowering and an antifungal activity was reduced in this period [93, 94, 95]. Ahmadi *et al.* (2015) reported that phonological and ontogenetically variation caused changed in the main components in *T. daenensis* essential oil and these components can be effect in antifungal properties in this plant [55]. According to Saez (1998) reported that essential oil of *T. hymalis* possessed a high level of *p*-cymene and γ-terpinene in flowering stage [63]. Also, Gergis *et al*. (1990) and Panizzi *et al*. (1993) were reported that essential oils rich in phenolic compounds (carvacrol and thymol) possessing high levels of antifungal activity [95, 96]. Karaman *et al.*, (2001) reported that *T. revolutus* Celak essential oil contains high level of carvacrol, *p*-cymene and γ-terpinene and has antibacterial and antifungal activity [97].

The marked difference between the metabolism of carvacrol and thymol by *Colletotrichum acutatum* may reflect the higher toxicity of thymol against this pathogenic fungus. In the same way, small differences between the metabolisms of both compounds by *Botryodiplodia theobromae* are consistent with the observed similarities in the antifungal activity against this fungus. Based on the structures of the metabolites, metabolic pathways for the biotransformation of thymol by *C. acutatum* and *B. theobromae* were proposed [31].

Antioxidant activity and scavenging of free radicals

Phenols are a class of compounds, which act as free radical scavengers and are responsible for the antioxidant activity in many medicinal plants [61, 98, 99]. Free radicals may cause many disease conditions such as heart diseases and cancer [46, 100].These compounds that can delay or prevent the oxidative damage of lipids or other molecules caused by free radicals, can probably be protective against the development of major diseases such as coronary heart disease and cancer in human and plant extracts containing antioxidants including phenolic compounds may prevent from free radical damage [101, 102, 103]. The antioxidant activity of plant extracts were assessed by the DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging method and ferric reducing antioxidant power (FRAP) assay [104]. The highest antioxidant activity of *T. daenensis* was observed in flowering stage, while the lowest value was detected in preflowering stage. Also the highest FRAP value was observed in flowering stage than preflowering stage. Also it was founded the coefficient correlation between total phenolic content and radical scavenging capacity. These results suggest that the major part of the antioxidant activity in *T. daenensis* results from the phenolic compounds. According to, their results showed that increasing of phenolic content, caused increase in antioxidant activity in different stages of harvesting times in *T. daenensis*. However, the antioxidant activity in plants is not caused only by phenols and may also come from the presence of other antioxidant secondary metabolites such as volatile oils, carotenoids and vitamins. Iranian *T. daenensis*are strong radical scavengers and can be considered as a good source of natural antioxidant for traditional and medicinal uses [104]. The antioxidant activity by radical scavenging capacity of Iranian and British *T. vulgaris* was shown in Table 4. The highest antioxidant activity was observed in Iranian *T. vulgaris* (7.76 µg/mL), while the lowest value was 8.05 µg/mL in British *T. vulgaris* [105]. This result suggests that the major part of the antioxidant activity in *T. vulgaris* results from the phenolic compounds. This is in line with the observation of other authors who found similar correlations between total phenolic content and antioxidant activity of various plants [106, 58].

Free radicals or reactive oxygen intermediates are generated by cells during the normal metabolism. When free radicals are accumulated excessively, this leads to damage in tissue and privation of many cellular functions. Carvacrol as an antioxidant protects the cells against free radicals. Moreover, antioxidants inhibit prostaglandin synthesis and induct drug-metabolizing enzymes in addition to many biological activities as reported by Azirak and Rencuzogullari (2008) [107]. Some studies assured the efficiency of carvacrol in scavenging free radicals i.e. nitric oxide, superoxide radicals, peroxyl radicals and hydrogen peroxide [79, 108-111]. The existence of hydroxyl group (OH) which linked to aromatic ring is suggested to be the reason for the highly antioxidant activity of carvacrol either *in vitro* or *in vivo* as explained by Aeschbach *et al.* (1994) and Guimaraes *et al*. (2010) [109, 110]. The reaction of carvacrol with a free radical is facilitated due to its weak acid character, so donating hydrogen atoms to an unpaired electron, producing another radical that is stabilized by electron scattering generated at a molecule resonance structure [111].

Anti-carcinogenic and antiplatelet effects

Several studies have been reported the addition of some phytogenic additives or their products such as cold pressed oil, essential oil or extracts to animal and poultry diets that improved live body weight, body weight gain, feed conversion ratio, immune response, antioxidant status, carcass traits and quality, and lowered morbidity and mortality rates [111-114, 82]. In fact, carvacrol component is added to various ingredients, such as nonalcoholic beverages (28.54 mg/kg), baked goods (15.75 mg/kg), chewing gum (8.42 mg/kg), *etc*. [115]. However, the mode of action of this compound was unknown by many researchers. A good knowledge of carvacrol mode of action is very required regarding application in nutritional systems. Formerly, Ultee *et al.* (1998) reported the antimicrobial activity of carvacrol on pathogen *Bacillus cereus* [115]. Carvacrol is a hydrophobic compound and has an effective impact on biological membranes. Carvacrol plays a key role as antiviral component against human rotavirus (RV). On the same context, Mexican oregano (*Lippia graveolens*) extract and oil as well as carvacrol component are able to reduce/inhibit the viral diseases in animal and human. Specifically, the antiviral activity of oregano and its phenolic components on acyclovir resistant herpes simplex virus type 1 (ACVRHHV-1) and human respiratory syncytial virus (HRSV) and of carvacrol on RV have been documented [116-118]. Acamovic and Brooker (2005) and Silveira et al. (2013) reported that herbs rich in flavonoids such as thyme and carvacrol could improve the immune functions through acting as antioxidants and extending the activity of vitamin C [118].

Anti-obesity effect of carvacrol

Obesity is a medical condition in which excess body fat accumulates to the extent that it may have a negative effect on health, leading to increased health problems. It is like other chronic diseases such as hyperlipidemia, cancers and diabetes. Umaya and Manpal (2013) stated that the main factor in enhancing obesity and attributed to the metabolic diseases in humans and animal models is the consumption of high levels of fat in the diet and they found that carvacrol caused an inhabiting of fat accumulation between cells and adipocyte differentiation in mouse embryo 3T3- L1 cells [119]. Also, results showed that diet high in fat and supplemented with carvacrol decreased total visceral fat, plasma and liver total cholesterol, HDL-cholesterol, triglyceride and free fatty acids of mice. Moreover, carvacrol decreased the expression of adipogenesis related genes- fibroblast growth factor receptor in visceral adipose tissues. Carvacrol decreased the expression of receptors which stimulates the intake of fat rich diet such as galanin receptor 1 and 2 [119]. Wieten *et al.* (2010) and Cho *et al.* (2012) found that free fatty acid levels and the mRNA and protein levels of toll-like receptors were reduced by carvacrol [120, 121]. Free fatty acids in high levels are reported in obese animals, because of their release either from high fat diet or from adipose tissues. Carvacrol as anti-obese drug needs for more detailed studies to be recommended for this purpose. Such properties could be due to its ability as antimicrobial, antioxidant, antifungal, immunomodulatory, anticancer and anti-inflammatory agents by preventing free radicals and hazardous compounds from interacting with cellular DNA and its ability to change the gut microflora, improving digestion coefficient and absorption of nutrient compounds [112, 122].

Application of thymol and carvacrolin postharvest technology

Food safety is one of the major issues related to fresh fruits and vegetables. More degradable natural compounds that can be regarded as safe to human health and environment are alternatives for synthetic chemicals used to control postharvest fruit rots. Results of recent studies on control of fungal rots using natural substances indicate that demands for research and development of natural fungicides are just gaining momentum globally [53]. Essential oil not only improved the storage life of lime fruits, but also caused no undesirable change in the appearance and quality of the fruits. Lime fruits treated with essential oil of *Z. multiflora* did not show any change in taste and odor even after 50 days of storage, a property that makes this essential oil an acceptable candidate for controlling postharvest lime fruit rot [50, 53].

Various factors affecting thymol and carvacrol contents in plants

In nature several factors affect production of secondary metabolites in commercially important plants [123] (Figure 8).

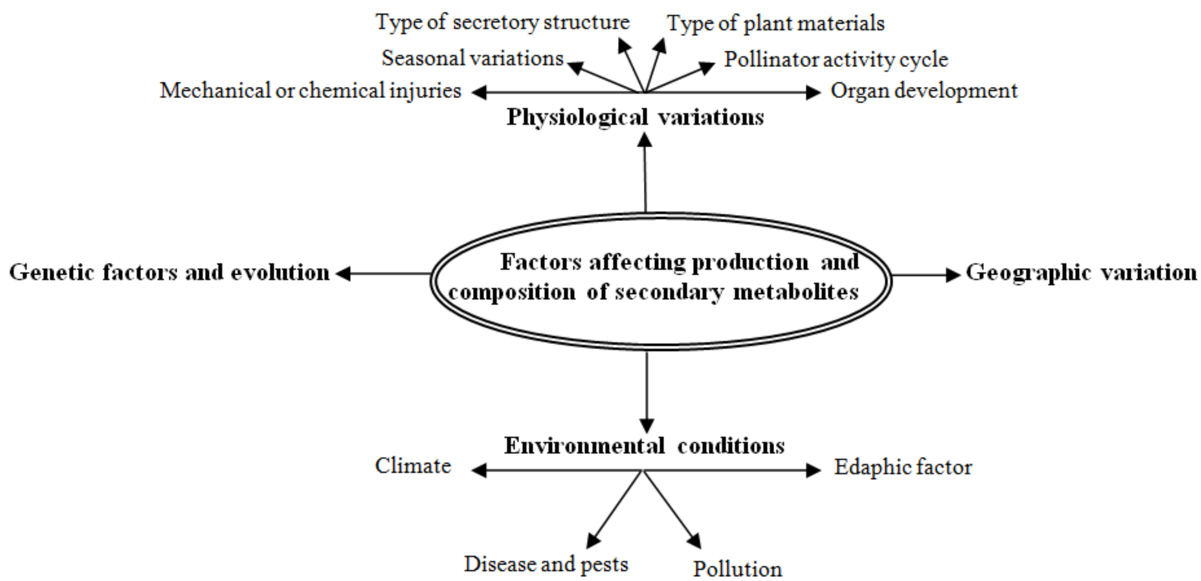

Figure 8- Factors affecting production of secondary metabolites in plant cells [81-123]

Origin of plant

According to Alizadeh *et al.* (2013), fresh and dry weight of British *Thymus vulgaris* was higher than those of Iranian *T. vulgaris*. Moreover, the essential oil yield and efficiency of the aerial parts in British *T. vulgaris* significantly increased compared with Iranian *T. vulgaris*. These variations may be attributed to the different origins (Esfahan and London) of cultivated seed [105]. Alizadeh *et al.* (2013) and Saez (1998) reported that, diversity of plant origins, seasonal variations or different ontogenesis and environmental conditions affected on essential oil yield and composition in *T. daenensis*, *T. vulgaris* and *T. hymalis* [105, 63].In a study by Santos *et al*., (2005), the essential oils of *Thymus caespititius* from populations collected in mainland Portugal and on Madeira were characterized to be *α*-terpineol, whereas those obtained from populations collected on nine Azorean islands showed a high variability, with carvacrol, thymol or α-terpineol as the major components [124].

Environmental conditions

The biosynthesis of secondary metabolites, although controlled genetically, is affected strongly by environmental factors. Agricultural practices have a critical effect on quantitative and qualitative characteristics of plant-derived metabolites, which finally result in plant growth and yield increment [125]. One way of increasing the consumption of health-promoting secondary plant metabolites in the diet would be by increasing metabolite levels in the fruit and vegetables themselves [57]. Upgrading vegetables and fruits in this respect could be done by breeding, genetic engineering, or modification of secondary metabolite biosynthesis by elicitor applications, which provides immediate response. In the latter case identification of the key enzymes or key genes targeted by these elicitor treatments is necessary. Several elicitors including chemical, physical, and biological elicitors for influencing glucosinolate biosynthesis has been applied. Piccaglia and Marotti (1993) reported that during their two-year study differences on relative amounts of thymol, carvacrol, γ-terpinene, and *p*-cymene in essential oils of *Satureja montana* which is grown in Italy, could be attributed to the effects of environmental conditions [125].

UV radiation

According to the international commission on illumination, ultraviolet (UV) wavelength (200-400 nm) is a small part of the solar radiation reaching the Earth's surface, which is divided into UV-A (315-400 nm), UV-B (280-315 nm) and UV-C (200-280 nm), and it affects negatively all living organisms. The harmful effect of UV radiation increases towards the shorter wavelengths. Solar radiation in the UV-B range corresponds to a minor percentage of the total solar energy but it is potentially harmful because these short wave lengths are capable of causing deleterious effect in cells. Plants are vulnerable to increased UV-B radiation because many cellular components such as nucleic acids, proteins, lipids and quinones can absorb UV-B radiation directly [126]. The treatment with UV light, particularly from the UV-B range, is an example for effective elicitor application. Secondary plant metabolites mediate many aspects of the interaction of plants with their environment by acting as feeding deterrents against herbivores, pollination attractants, protective compounds against pathogens or various abiotic stresses, antioxidants and signaling molecules. Low levels of UV-B radiation can cause distinct changes in the plant's secondary metabolism resulting in the accumulation of a broad range of secondary plant metabolites [127].

Light

In order to find the light effects on amount of essential oil, Thymus was put under several intensity of light (sunshade, cloudy, 15, 27, 45 and 100% light) and recognized that the maximum essential oil concentration and thymol found in 100% sun light, and length of leaf decreased with reduction of light levels. Amount of essential oil not only depended on grow stage of plant but also temperature, moisture, quality and quantity of light, rainfall are influenced on it [128].

Temperature

Omer (1998) on *Origanum syriacum* obligated that the phenolic compounds increase in hot seasons at the expense of their preceding precursors. In other words, the relative percentage of carvacrol was higher in the second cut than in the first cut. This may be attributed to the effect of the environmental factors especially non-edaphic factors, since these plants grew in summer months under high temperatures and received more solar energy than those grown in the spring summers. These conditions accelerate the transformation of terpinene and *p*-cymene to phenolic compounds [129].

Drought and salinity stress

By increasing drought stress, essential oil percentage and essential oil components had no significant difference, but there was a significant difference between essential oil yields (P≤0.01). Because essential oil yield is obtained from dry yield multiply to essential oil percentage [75]. Salinity stress had no effect on essential oil percentage and essential oil components but decreased essential oil yield [130, 131]. When irrigation volume decreased from 50 to 16 L/m^2 *Satureja khuzistanica* and *S. bachtiarica* dry matter were decreased up to 56 and 54%, respectively. Thus, essential oil yield decreased (> 50%). *S. bachtiarica* and *S. khuzistanica* essential oil had 14 and 11 components, respectively. Water stress caused an increase in oil production of thyme [57, 132]. It has been suggested that increased oil gland density, accompanied with higher absolute number of gland production during stress, could be the reasons behind accumulation of essential oil in some plant species. Other factors could be the net assimilation or the partitioning of assimilates among growth and differentiation processes. Sometimes, the decline in the primary metabolism of plants during stress could lead to the accumulation of certain intermediate products, which get channelized to form secondary metabolites like essential oil. In plants with decreased levels of essential oil under stress, the lowering could be due to the overall anabolism which gets inhibited on exposure to saline conditions [123, 130-132].

Plant growth

Both moisture and the plant growth phase had a significant effect on the plant material production. The crop yield increased significantly with increasing moisture and age of the Mexican oregano plants [133]. Although, older plants contained less oil than the younger plants, the differences were not statistically significant. Total thymol and carvacrol contents of oregano oils obtained from younger plants were higher than that of the mature plants. The amount of water received by the plant did not have a significant effect on the thymol and carvacrol content of the oil isolated from Mexican oregano [133].

Harvesting time (phenological stages)

Study about the effects of phenological stages such as harvesting times on herbage yield and quantity and quality of essential oil is very important goal to obtain maximum productivity of valuable medicinal plants [134, 135]. Harvest of Thyme is the critical point in agricultural management of this plant. Stahl-Biskup and Saez (2005) reported that the best time of harvest is early and middle of flowering period to get maximum effective material [65]. The yield of plant materials, essential oil composition and secondary metabolites in medicinal and aromatic plants are strongly influenced by harvest time, ecological and climatic conditions, ontogenetically variation and the others environmental factors such as soil type and nutrition [54, 136-138, 64, 45]. Knowledge of the factors that influence on yield and essential oil composition in medicinal plants is insufficient, and these factors seems to play an important role in drug yield and essential oil composition of medicinal and aromatic plants [138-140]. Sefidkon et al (2009) studied the effects of harvest stages and various methods of hydro-distillation on essential oil efficiency of garden thyme, phenological stages had significant effect on essential oil efficiency [138]. Mean comparison results showed that the highest efficiency (1.18%) belonged to beginning of blooming and vegetative phase had the lowest (0.86 %) value. Also, some studies showed that the highest herbage yield and essential oil of garden thyme were obtained in lower elevations and in full blooming stage [138]. Khorshidi et al (2010*b*) investigated four various stages in two regions to assess the effect of climate and phenological stages on essential oil percentage of Denaian thyme (*T. daenensis* Celak.) [54]. Results showed higher essential oil percentage in full blooming and fruit set stage in both regions. Golparvar (2013) recommended harvesting Kouhi thyme (*T. kotschyanus*) in fruit set stage to obtain the highest amount of essential oil and thymol yield as well as fresh and dry herbage [134].

Effects of different harvesting times on fresh and dry weights (g/plant) are presented in Table 2. *Thymus daenensis* fresh weight was increased at flowering stage but this is not significant. Dry weight of *Thymus daenensis* was significantly increased at flowering stage. It seems that increasing day-length, temperature and sunlight in flowering stage compared to pre-flowering stage causing an increase in fresh and dry weight of flowering stage [140]. Ozguven and Tansi (1998) reported that increasing day-length and sunlight significantly affected fresh and dry weight of *Thymus vulgaris* [141]. Essential oil yield and efficiency data are reported in Table 2. Essential oil yield and efficiency of *T. daenensis* increased at flowering stage, but not significant. This is due to high yields of fresh and dry biomass and content of oil and thymol in this stage.

Fertilizers

Physical, biological and chemical properties of soil have effect on grow and effective materials of medicinal plants [55]. With fertilizing of medicinal plants, initial and necessary elements of grow such as nitrogen, phosphorus and potassium are supplied. Foliar application of nitrogen significantly can be increased the plant height of thyme (*T. vulgaris*), percentage of thymol and essential oil [69-70, 128, 132].

Nitrogen

Nitrogen is essential and vital element for plant growth and development. Review of literature showed that the increasing amount of nitrogen fertilizer caused an increase in Thymus yield. Fertilizer treatments did not effect on amount of essential oil and thymol, but with attention to effect of it on plant yield, it can increase thymol and oil yield [38, 132]. Research showed that the application of nitrogen produced more thymol yield in Thyme oil [8]. Also, it has been reported that nitrogen increased *Thymus vulgaris* oil yield and thymol content [132, 142, 143].

Table 2- Means of *T. daenensis* oil content at different stages of plant growth by hydro-distillation [140]

Plant growth stage	Essential oil content (%)	Thymol (%)	Carvacrol (%)
Full flowering	2.28[a]	73.4	2.2
Beginning of flowering	2.04[b]	72.6	2.3
Before flowering	1.77[b]	70.1	3.1

Biological fertilizers

Now a day, use of beneficial soil microorganisms called biological fertilizers as the most natural and desirable solution for keeping the soil alive and active is widely considered. Biological fertilizers like *Azotobacter* absorb and increasing the concentration of essential elements such as nitrogen, phosphorus, potassium, zinc, magnesium, iron and protein in crops [57].

Effects of animal fertilizers

Akbarnia *et al*. (2004) have stated that chemical fertilizers have no effect on the oil content of the seeds of Bishop's weed (also known as Carom, Ajowan, Ajwain), however, application of animal fertilizers results in a dramatic increase in the seed yield and oil content of the plant [144]. Furthermore, application of animal fertilizers up to 20 tons per hectare results in a substantial increase in the percentage of thymol in the oil while no effect can be observed on the percentages of gamma-terpene and *p*-cymene. Mallanagoula (1995) has reported the positive effects of animal fertilizers on the improvement of quality in medicinal herbs [36]. Animal fertilizers due to having great advantages such as maintenance of water in the soil and inclusion of nutritive substances can help in the increase of the essential oil content of the plants by augmentation of vegetative growth, which comprises the most oil content. Through an experiment conducted on the medicinal plant Rosado Paraguayan garlic, Arguello et al (2006), have shown that application of vermi-compost may cause a considerable increase in the height of the shrub of the plant [145]. Similar results have also been obtained on fennel and Chamomile [146, 147].

Conclusion

Natural plant chemicals are the potential source of agrochemicals, flavors, and pharmaceuticals, known as secondary metabolites, which are synthesized by plants when exposed to different elicitors and/or inducer molecules. Thymol (2-isopropyl-5-methylphenol), a natural monoterpenephenol derivative of cymene, isomeric with carvacrol (5-isopropyl-2-methylphenol), are found as major constituents of essential oil in members of the Laminaceae, Verbenaceae and Ranunculaceae. Biosynthesis of thymol and carvacrol is thought to involve hydroxylation of γ-terpinene and *p*-cymene precursors. These components are known as biocides, with a wide spectrum of antimicrobial, anti-inflammatory, anti-leishmanial, antioxidant, hepato-protective and anti-tumoral activities, which have been the subject of several investigations *in vitro* and *in vivo*. The presence, content and constituents of these compounds in medicinal and aromatic plants may be affected in a number of ways, from their formation in the plant cell to their final isolation. These include: (a) plant origin (b) environmental conditions (c plant growth and physiological variations (d) phenological stages (e) and agronomical practices, *etc*. Exploration of thymol and carvacrol modes of action like pharmacological, nutritional, health benefits and biological properties may play critical role in their beneficial applications in different industries by providing further understanding of the health usages and improving performance parameters in agriculture species. Further experiments may be worthy of evaluation for future.

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