Chapter

Biological Application of Essential Oils and Essential Oils Components in Terms of Antioxidant Activity and Inhibition of Cholinesterase Enzymes

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Abstract

This chapter will be described oxidative stress related to modern age illness as well as biological activity of essential oils and essential oil components in terms of their antioxidant activity. The importance of essential oils and their constituents in terms of protecting lipids and proteins from oxidation will also be explained. Alzheimer's disease as a disease related to oxidative stress and strategies in their treatment by using essential oil components as cholinesterase inhibitors will also be described. As case studies will be pointed out medicinal plants, endemic *Saturejasubspicata* L., and widely used *Menthapulegium* L. growing in Bosnia and Herzegovina.

Keywords: oxidative stress, essential oils, biological activity, antioxidants, Alzheimer's disease, cholinesterase inhibitors, *Saturejasubspicata*, *Menthapulegium*

1. Introduction

Under normal physiological conditions, the production of harmful reactive species caused by oxidative processes and antioxidant defense are in balance. If the reactive oxygen species and other species production exceed the antioxidant capacity of a living system, reactive oxygen and nitrogen species (ROS and RNS) may react with macromolecules, causing structural and/or functional damage to cellular enzymes and genetic material. An excess of reactive species and damage caused by their action is called oxidative stress.

In a state of oxidative stress, an excess of ROS and RNS may damage lipids, proteins, carbohydrates, and nucleic acids. Free radicals attack unsaturated fatty acids in biological membranes causing lipid peroxidation. Lipid peroxidation is an enzymatic reaction catalyzed by the enzyme lipoxygenase [1]. This enzyme is found in the erythrocytes and leukocytes of animals, as well as in many plant organisms. Its substrate is linoleic and linolenic acid in plants, and arachidonic acid in animals, while oleic acid is not oxidized. Lipid peroxidation results in decreased membrane fluidity, loss of

enzymes and receptor activity, damage to membrane proteins and other macromolecules, which leads to apoptosis [2].

Oxidative modification of proteins, reversible and irreversible, occurs during redox signaling and other cellular processes. It also occurs as a result of oxidative stress. Exposure of proteins to hydroxyl $OH^{\bullet}$ and/or superoxide radicals $O_2^{\bullet -}$ leads to their structural modifications. Modified proteins may further undergo spontaneous fragmentation and cross-linking or show a significant increase in proteolysis. An oxidative attack of a polypeptide backbone is usually initiated by hydroxyl $OH^{\bullet}$. By an experimental generation of radicals, using water radiolysis or decomposing hydrogen peroxide H_2O_2 in a metal-catalyzed reaction - and in the interaction with lipids - alkyl, alkoxy, and alkylperoxyl radical intermediates can be formed, which affect peptide bond cleavage in several ways.

Tryptophan, histidine, and cysteine are the most sensitive to reactive oxygen species. In addition to fragmentation, oxidation of the amino acid residues of lysine, arginine, proline, and threonine increases carbonyl concentration, so the presence of carbonyl groups can be used as an indicator of protein oxidation.

Oxidative modification of proteins also occurs in reaction with aldehydes, which are formed during lipid peroxidation process. End products of lipid peroxidation, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), as well as oxidation products of polyunsaturated fatty acids cause oxidative damage to proteins [3].

Oxidative modification of proteins is present in diseases and changes associated with the aging process, such as atherosclerosis, tumors, neurodegenerative diseases, and aging. Protein carbonylation occurs with a large number of modifications and is a marker of oxidative stress. During the first two-thirds of life, the level of protein carbonylation slowly increases, while its level rises sharply in the last third. Protein carbonylation negatively affects the functions of proteins themselves, which suggests that this modification may be one of the causes of the aforementioned undesirable processes [4].

2. Oxidation and food

Apart from the living organisms, the oxidation process occupies an important place in the food, pharmaceutical, and cosmetic industries. It includes the oxidation of protein molecules, vitamins, but above all, the oxidation of lipid molecules [5].

Oxidation of lipid molecules is a major problem in the food industry, as it leads to changes in the organoleptic properties of food, a decrease in its nutritional value, as well as the formation of radical components that can endanger consumers' health.

Lipid oxidation in food implies a whole range of chemical changes that result from the reaction of lipids with oxygen. Triacylglycerols and phospholipids are hardly volatile molecules and do not directly affect the aroma of the product. During lipids oxidation from fatty acids, volatile compounds have formed that lead to an undesirable aroma of products known as rancidity [6].

Polyunsaturated fatty acids oxidize much faster than monounsaturated or saturated ones. The rate of lipid oxidation is influenced by the number and position of double bonds [1]. The methylene group ($-CH_2-$) located between the two double bonds is very susceptible to oxidation. Linoleic acid is subject to oxidation, as it has a methylene group between two double bonds, at position 11. Its oxidation produces two hydroperoxides. The main secondary product of linoleic acid autooxidation is hexanal. Lipid autooxidation is an autocatalytic reaction, which means that it progresses over time due to the formation of products that catalyze the reaction themselves.

Initiation: $LH \rightarrow L^{\bullet} + H^{\bullet}$

Propagation: $L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$
$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$
$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$

Termination: $2LOO^{\bullet}$
$LOO^{\bullet} + L^{\bullet}$
$L^{\bullet} + L^{\bullet}$

Figure 1.
The lipid oxidation phases [7].

Lipid peroxidation includes three phases: initiation, propagation, and termination (**Figure 1**). From the peroxides formed at the beginning, secondary oxidation products are formed: aldehydes, ketones, epoxides, and other compounds, which also have negative biological effects, such as loss of essential amino acids and lipid-soluble vitamins [7].

In the first phase, oxygen from the air attacks unsaturated fatty acids (LH), creating free radicals of fatty acids (peroxy $LO_2^{\bullet}$, alkoxyl $LO^{\bullet}$, or alkyl radicals $L^{\bullet}$). In the second phase of the reaction, hydroperoxides (LOOH) and free peroxide radicals ($LOO^{\bullet}$) are formed from free radicals by binding oxygen to free fatty acid radicals.

Hydroperoxides (primary oxidation products) are labile, so they are further decomposed into free radicals and decomposed oxidation products. These degradation products of oxidation (secondary oxidation products) are carbonyl compounds (aldehydes and ketones), fatty acids, alcohols, epoxides, etc., some of which give off an unpleasant, rancid odor characteristic of oxidized fat.

Lipid autooxidation is often initiated by free radicals from an unknown source. It is accelerated by rising temperatures, light and the presence of trace metals. Reductive forms of transition metals are more efficient in the hydrogen peroxide decomposition, so reductive components such as superoxide anion ($O_2^{\bullet -}$) and ascorbic acid further promote lipid oxidation. Redox cycling of iron in the presence of superoxide anions in lipid oxidation is known as the Haber-Weiss reaction, while the second step of this reaction is known as the Fenton reaction:

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2.$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}.$$

The resulting hydroxyl radicals ($OH^{\bullet}$) are the most reactive ROS species.

Ascorbic acid can also participate in the Haber-Weiss type reaction, but unlike superoxide anions, ascorbic acid may also act as an antioxidant at higher concentrations.

The control of the level of free radicals, prooxidants, and oxidation intermediates is used to protect the lipid components of food from oxidation. Free radical scavengers (FRS) inhibit lipid oxidation by reacting faster than unsaturated fatty acids with free radicals. They can react with peroxyl($LOO^{\bullet}$) or alkoxyl($LO^{\bullet}$) radicals in the following reaction:

$$LOO^{\bullet} \text{ or } LO^{\bullet} + FRS \rightarrow LOOH \text{ or } LOH + FRS^{\bullet}.$$

Phenolic components are known to be good free radicals scavengers, as they donate a hydrogen atom, and the resulting radical has low energy due to its delocalization in the structure of phenol ring (**Figure 2**) [6].

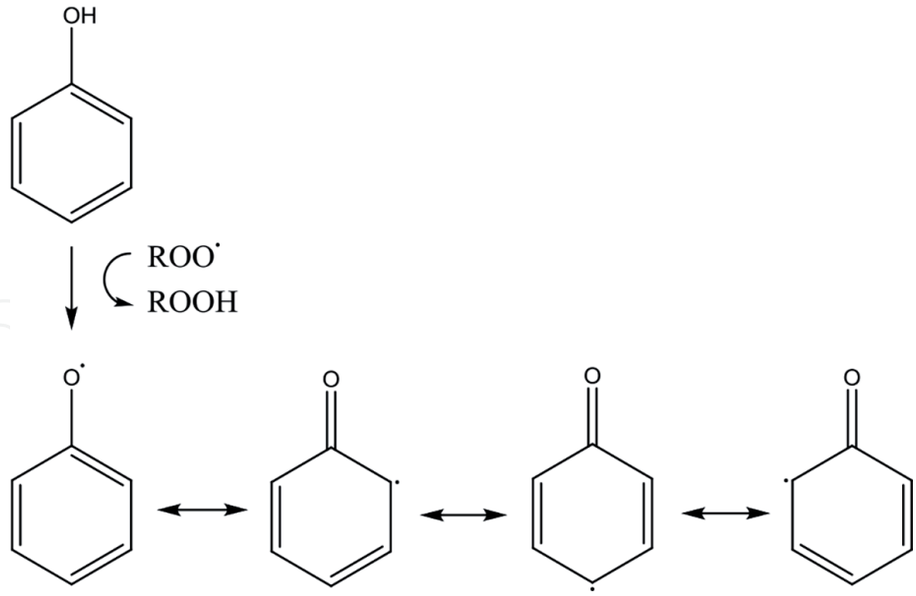

Figure 2.
Delocalization of phenol radical [6].

The most commonly used synthetic antioxidants are substituted monophenolic compounds, such as 2,6-di-*tert*-butyl-4-hydroxytoluene (BHT), 2-*tert*-butyl-4-hydroxyanisole and 3-*tert*-butyl-4-hydroxyanisole (2- and 3-BHA), propyl gallate(PG) and *tert*-butyl hydroquinone (TBHQ). The addition of the antioxidant BHA prolongs the stability time of lipid-based foods (e.g., butter, fat, meat, dairy, vegetable oils) by a few months to a few years. BHT is less effective than BHA because two tertiary butyl groups sterically affect the radical reaction. PG is poorly soluble in water. It is less commonly used in the food industry because it binds Fe^{3+} ions and reduces them to their Fe^{2+} form. When this antioxidant is used in food, it must be combined with chelating agents (such as citrate) to prevent this phenomenon. TBHQ is one of the best antioxidants added to oil intended for frying. Unlike PG, it does not complex with iron and copper ions. According to European rules, the permitted amount of BHT, BHA, and PG in food is 100 μg/g of lipids [8]. BHA and BHT are very effective in their role, but are easily volatile and thermolabile, which makes their use limited [9]. Some studies have shown that the use of some antioxidants of synthetic origin has negative effects on human health due to the promotion of carcinogenesis [10, 11].

For these reasons, there is a tendency to replace synthetic antioxidants, where possible, with non-toxic antioxidants of natural origin. More recently, essential oils have also been used as a substitute for synthetic antioxidants, in those food canning sectors where their use does not adversely affect product flavor [12].

3. Antioxidant activity of essential oil components

In addition to oxidative damage and death of cells, tissue damage and various pathological conditions may be the consequence of oxidative stress. Numerous forms of malignant disease are thought to be the result of oxidative DNA damage and the resulting mutations. The negative impact of free radicals is believed to lead to various autoimmune diseases, diabetes, rheumatic diseases, cardiovascular disease and heart attack, kidney disease, infectious diseases, neurodegenerative diseases (Alzheimer's

disease), etc. The aging process itself is described as the process of accumulation of numerous oxidative damage accumulated over time.

Given that the oxidative stress is associated with the etiology and pathogenesis of many diseases, it is believed that eliminating the causes of oxidative stress may prevent or delay the occurrence of pathological changes and reduce the occurrence of diseases. Numerous studies show that regular intake of fruits, vegetables, grains, and beverages have a positive effect on diseases that are mediated by the activity of free radicals. Therefore, natural antioxidants – alone or in the form of extracts – may be useful in the treatment of such diseases. Thus, the reason for the great interest in researching the antioxidant activity of aromatic, medicinal, and edible plants [13].

In situations of disturbed homeostasis, as well as in the prevention of disease development, the intake of antioxidants in food may be of great importance. In this regard, essential oils, plant extracts, or their individual components with good antioxidant activity may be used. From a chemical point of view, essential oils are complex mixtures of a large number of compounds, which makes their activity difficult to test.

With the exception of some phenolic components, whose antimicrobial and antioxidant activity is well known, such data are not available for most other components of essential oils. Numerous papers on essential oils mention synergism, antagonism, additivity, but such claims are rarely accompanied by experimental confirmation [12].

A study by Ruberto and Baratta [12] examined the antioxidant activity of 100 pure compounds, common constituents of essential oils, using two methods. Of the thirteen non-oxygenated monoterpenes, terpinolene, α-terpinene, γ-terpinene, and sabinen showed very high activity. The activity of α-terpinene and γ-terpinene was similar to that shown by α-tocopherol. An active methylene group is thought to contribute to this activity of the aforementioned compounds. Of the 34 oxygenated monoterpenes tested, thymol and carvacrol showed activity as did α-tocopherol. It is known that thymol and carvacrol contribute the most to the antioxidant activity of essential oils that contain them. Alcohols were the most active in this class of compounds, with the exception of linalool, which showed prooxidative activity. Ketones showed lower activity. Non-oxygenated sesquiterpenes were not active, while oxygenated sesquiterpenes showed activity similar to that of oxygenated monoterpenes. Germacron, a cyclic ketone, showed slightly more pronounced activity, while nerolidol showed prooxidative activity. Phenols, benzene derivatives, have shown the best results. They are more effective in preventing the formation of primary oxidation products, as opposed to preventing the formation of secondary oxidation products. Non-terpene compounds, which are present in essential compounds in a smaller amount, showed weak antioxidant activity – just like non-oxygenated sesquiterpenes [12].

More recently, essential oils have also been used as a substitute for synthetic antioxidants, in those food preservation sectors where their use does not adversely affect product flavor [12].

Due to their specific chemical structure, plant phenolic compounds may act as strong antioxidants, due to their ability to interrupt chain reactions by donating hydrogen atom or electron to a free radical, while taking on a stable non-reactive conformation. However, their activity depends on a number of factors: degree of hydroxylation, polarity, solubility, reducing potential, stability of the resulting radical, etc. Hydroxycinnamic acids, the components of essential oils, show stronger activity compared to hydroxybenzoic acids because they donate hydrogen atoms more

easily [14]. Polyphenols are proven to have a positive effect on cognitive abilities and neurodegenerative changes caused by aging [15].

Currently, there is a disparity in knowledge about the *in vivo* and *in vitro* effects of polyphenols as antioxidants [16]. Due to the lack of knowledge regarding the safety of higher doses intake, it is believed that the level of polyphenols, which are entered into the human body, should not exceed that in which they are otherwise found in food [17].

4. Neurotransmitter acetylcholine and cholinesterase inhibitors

The neurotransmitter acetylcholine (ACh) is present in the nervous system, where it enables cerebral-cortical activity and development, control of cerebral blood flow, control of sleep–wake cycles, as well as learning and memory processes (**Figure 3**). The enzyme cholineacetyltransferase (ChAT) catalyzes the production of acetylcholine (ACh) in cholinergic neurons, from choline and acetyl coenzyme A.

Releasing acetylcholine from the synaptic vesicle of the presynaptic membrane into the synaptic cleft, it binds to cholinergic receptors (nicotinic and muscarinic receptors) on the postsynaptic membrane of the cholinergic synapse or on muscle cells. This triggers a series of processes that result in membrane depolarization and further signal transmission [18].

ACh hydrolysis controls the transmission of nerve impulses at the cholinergic synapses of the central and peripheral nervous systems. The degradation of acetylcholine in the synaptic cleft by acetylcholinesterase (AChE) establishes the polarization of the postsynaptic membrane and impulse transmission ceases.

Two types of ChE are currently known: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). AChE is also called "true cholinesterase", while BChE is also known as "pseudocholinesterase" because it hydrolyzes many choline esters and other non-choline esters (butyrylcholine, succinylcholine, acetylcholine, acetylsalicylic acid, cocaine, and heroin).

Inhibition of AChE prevents the hydrolysis of ACh, thus prolonging its activity in the transmission of nerve impulses. This concept is applied in the treatment of diseases characterized by low ACh levels and is also being studied in toxicology because of health conditions and deaths caused by increased cholinergic stimulation [19].

Alzheimer's disease (AD) is the most common neurodegenerative disorder and the cause of dementia in the elderly population. It affects about 2% of the population in industrialized countries. AD is characterized by the formation of neuritic plaques; extracellular accumulations of fibrils and amyloid-β-peptides, as well as neurofibrillary tangles; intracellular accumulations of τ-protein, in regions of the brain responsible for learning, memory, and emotional behavior. These changes cause neuronal degeneration, loss of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), which is manifested in the loss of neurotransmitters and other neuromodulators, and

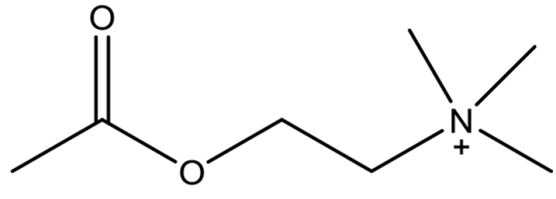

Figure 3.
Structural formula of neurotransmitter acetylcholine (ACh).

the disabling of synaptic transmission [18]. Currently, the treatment of this disease is limited to the treatment of symptoms of the disease, for which cholinesterase inhibitors (ChE) are used.

ChE inhibitors may be reversible, those which are bound by noncovalent interactions, or irreversible, which covalently bind to the serine of the catalytic triad. Reversible inhibitors bind to the active site, peripheral site or both, and the inhibition occurs as a result of conformational changes of the enzyme, electrostatic interactions of the inhibitor and the cationic part of the substrate, and steric and/orelectrostatic interferences with the substrate entry into the active enzyme center.

A feature of the structure of good cholinesterase inhibitors is the presence of a positive charge and/or aromatic or hydrophobic substituents that facilitate the entry and placement of inhibitors in the active site of the enzyme [18].

Synthetic AChE inhibitors such as physostigmine, tacrine, and donepezil cause side effects such as hepatotoxicity and gastrointestinal disorders. Irreversible inhibitors may cause serious consequences and even death, as is the case with sarin, a poison gas, so reversible inhibitors are preferred in this regard [20].

4.1 Essential oil components as cholinesterase inhibitors

Bioactive substances from fruits, vegetables, and medicinal plants play a major role in slowing down many pathogeneses and neurodegenerative disorders, such as Alzheimer's disease. In addition to alkaloids, food rich in phytochemicals contains terpenes and polyphenols, which can be good cholinesterase inhibitors, alone or in synergy with each other [20].

Donepezil, rivastigmine, and galantamine are currently used to treat AD symptoms, such as cognitive dysfunction and memory impairment [21]. The aforementioned galantamine is a reversible inhibitor of AChE, which has been used since 2007 in the treatment of mild to moderate AD. It shows good pharmacological and pharmacokinetic properties, as well as a small number of side effects [22]. The use of most of the ChE inhibitors tested so far has been accompanied by side effects such as fatigue, sleep disorders, cardiorespiratory, gastrointestinal disorders, and low bioavailability. This was an incentive for further research with the aim of finding new ChE inhibitors of natural origin, with greater efficiency and bioavailability, as well as with fewer side effects [23].

Essential oils contain a number of bioactive components; terpenes, terpenoids, phenylpropanoid and other compounds, so a large number of them have been tested in terms of their ability to inhibit ChE. The results showed that some of the tested oils have a good ability to inhibit ChE. Comparing the results of different studies, it was noticed that some essential oils of similar composition have different abilities to inhibit ChE. The differences in the mentioned results may be attributed to the synergistic or antagonistic effect between the individual components of the essential oil. To investigate these effects, a number of studies have been conducted to identify and isolate individual constituents of essential oils with a significant ability to inhibit ChE [24].

The majority of the data obtained thus far in the research pertains to the study of the ability of smaller individual components of essential oils to inhibit AChE, while a few pertain to the study of BChE inhibition. However, given the role of BChE inhibition in the treatment of AD in the later stages of the disease, the interest in testing BChE inhibition has increased [24]. In terms of ChE inhibition, IC_{50} values are impacted by the enzyme concentration, inhibitors, and substrates, as well as other

experimental conditions, making it difficult to compare the results obtained by different studies. It is important to standardize the protocols used in testing AChE and BChE inhibitors, so as to be able to detect them [25].

When it comes to the studies of the ability to inhibit ChE, most of these refer to the study of monoterpenes [24]. Of monoterpenes, 1,8-cineole and α-pinene are the most effective in inhibiting AChE. In addition to these two, the ability to inhibit AChE is shown by δ-2-carene (2-carene), δ-3-carene (3-carene), and mirtenal [18, 24], as well as geraniol, α-caryophyllene, and limonene [21]. Carvone also showed good AChE inhibitory activity [19].

Monoterpene carvacrol and its isomer thymol showed significant AChE inhibitory activity, with carvacrol activity being ten times higher, which indicates the importance of the hydroxyl group position for AChE inhibitory activity [26].

Among the monoterpenes with the *p*-menthane skeleton, pulegone was the most successful in terms of AChE inhibition [19]. Monoterpene camphor, bornyl acetate, carvone, β-pinene, fenchol, and fenchone show poorer ability to inhibit AChE [19].

Some studies show the existence of a synergistic effect of monoterpenes, especially between 1,8-cineole and α-pinene [19]. A synergistic effect is also present between the enantiomers of α-pinene and β-pinene (α-*S*-pinene: β-*S*-pinene, and α-*R*-pinene: β-*S*-pinene) in the mixture, at a ratio of 3:2 [27].

One of the ways in which terpenes inhibit AChE is through a hydrophobic ligand. The hydrophobic active site of AChE is the site where hydrophobic interactions take place, and terpene compounds, built from the skeletons of carbon and hydrogen atoms, thus contribute to the inhibition of cholinesterases [21].

Due to the differences in terpene compounds structure, it is difficult to determine the relationship between their structure and activity. When it comes to monoterpenes with a *p*-menthane skeleton, it has been established that ketone monoterpenes are better AChE inhibitors than the corresponding hydrocarbons and alcohols [19]. In the case of bicyclic monoterpenes, bicyclic hydrocarbons have a greater ability of inhibition than bicyclic alcohols and ketones. The position of the double bond increases the ability of inhibition, so 3-carene has a greater ability of inhibition than 2-carene [28].

Monoterpenes are much better inhibitors of AChE than BChE. Due to their low molecular weight, monoterpenes are more likely to inhibit ChE exerting steric or allosteric effects, whereby BChE does not affect the substrate's access to the enzyme site [18].

In a study examining 21 monoterpenes in terms of the ability to inhibit BChE, only 3carene showed BChE inhibiting ability (IC_{50} = 2000 μM) [29]. Monoterpenes α-pinene, 1,8-cineole, 1,8-cineole, linalool, terpinen-4-ol, linalyl acetate, thymol, γ-terpinene, and phenylpropanoid eugenol have shown good to moderate BChE inhibitory potential (IC_{50} = 0,1 to 1,0 mM) in various studies [24].

Of the flavonoids, flavones and isoflavones show the best activity, while xanthones and monoterpenes show weaker activity in the inhibition of cholinesterases (**Figure 4**) [18].

The most frequently studied sesquiterpenes for AChE inhibition are β-caryophyllene and α-humulene. In doing so, β-caryophyllene had a good ability to inhibit, in contrast to α-humulene (α-caryophyllene), which showed a weak ability to inhibit AChE [24]. In several studies, β-caryophyllene also showed good to moderate BChE inhibitory potential (IC_{50} = 0,1 to 1,0 mM) [24].

Diterpenes inhibit ChE at lower concentrations than monoterpenes, which indicates the importance of molecule size. Dihydrotanshinone and cryptotanshinone are non-competitive ChE inhibitors. Of triterpenes and steroids, ursolic acid, taraxerol, leucisterol, and oleanolic acid show ChE inhibitory activity [18].

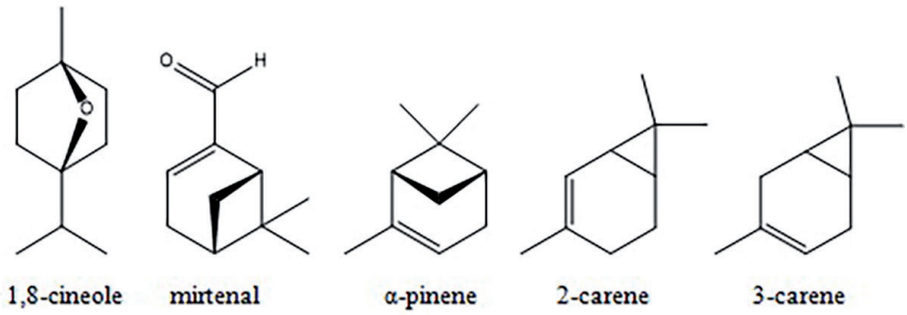

Figure 4.
Some monoterpenes with cholinesterase inhibition activity.

Given that BChE has a regulatory role in ACh hydrolysis, therapeutics that would inhibit both ChEs could exert additional positive effects in the treatment of AD, compared to inhibitors that inhibit only AChE. Thus, rivastigmine, which inhibits both ChEs, is very successful in the AD treatment. To date, there is no evidence that BChE inhibitors are more effective in reducing AD symptoms than AchE inhibitors [18].

In traditional medicine, many herbs are used in the treatment of cognitive disorders, including neurodegenerative diseases. The ethnopharmacological approach, testing of biological activity and isolation enabled the identification of potential AChE inhibitors of plant origin. Multifunctional compounds with several complementary biological functions are of particular interest. Plant extracts are the main sources of new compounds, AChE inhibitors [21]. In this regard, polyphenols are particularly interesting due to their positive effect on human health [20].

Many phytochemicals are bioactive compounds, some of which show ChE inhibitory activity and represent a model for the development of new drugs, ChE inhibitors. As terpenes and terpenoids have shown relatively weak inhibitory capacity in studies published so far, it is necessary to develop analogues with an improved efficiency [21].

Given the above, numerous plant extracts and essential oils, as well as their components, have been studied in terms of ChE inhibitory activity [18, 19, 20].

5. The case study of *Satureja subspicata* and *Mentha pulegium* growing in Bosnia and Herzegovina

Satureja subspicata (mountain savory) is a rare, endemic Dinaric species distributed in the eastern Mediterranean area [30]. *Satureja subspicata* (**Figure 5**) has long been utilized in Bosnia-Herzegovina's traditional medicine to treat leukemia and lymph node disorders. The herbal medicines of *Satureja subspicata* have been shown to be effective for a variety of cardiovascular diseases, especially arrhythmia, atrial fibrillation, and vascular diseases [31].

The essential oils obtained from various *Satureja* species have certain biological properties, such as antimicrobial [32, 33], antioxidant [33, 34], antiviral [35], antispasmodic and antidiarrheal [36, 37], anti-inflammatory and antinociceptive activities [38]. Carvacrol, thymol, β-caryophyllene, γ-terpinene, *p*-cymene, and linalool, all common compounds found in *Satureja* essential oil, have been shown to have strong antioxidant activity [39].

Figure 5.
Satureja subspicata *L. (mountain savory).*

Thirty-four (34) volatile compounds (98.0% of the total oil) in *Satureja subspicata* essential oil from Bosnia-Herzegovina were identified using GC/MS and GC/FID. The main classes of essential oil constituents were non-oxygenated monoterpenes (46.6%) and non-oxygenated sesquiterpenes (34.8%), followed by oxygenated monoterpenes (10.4%) and oxygenated sesquiterpenes (6.2%). The sesquiterpene ß-caryophyllene (14%) and non-oxygenated monoterpene *cis*-ß-ocimene (12.1%), as well as α-pinene (10.2%), were the main essential oil components. Other quantitatively important compounds were *trans*-ß-ocimene (8.8%), germacrene D (7.1%), caryophyllene oxide (6.2%), and myrtenol (6.1%) [40].

Mentha pulegium L. (pennyroyal) can be found in the area of Europe and Mediterranean [41]. In traditional medicine of Bosnia and Herzegovina, this plant is used for the treatment of nervous system disorders [31]. *Mentha pulegium* (**Figure 6**) stimulates digestive juices and helps with bloating and cramps. It is used for headaches and mild respiratory infections; it is a strong stimulant for the muscles of the uterus and can be used externally to relieve rheumatic problems, including gout [42].

Medicinal properties of *M. pulegium* are attributed to the monoterpenes present in the essential oil as well as polyphenol derivatives [43]. Essential oil of *M. pulegium* shows antifungal, insecticidal, antiparasitic, spasmolytic, and antioxidant properties [44]. Because of the mint-like odor, *M. pulegium* essential oil has a wide application; it is a constituent of foods and fragrances [45]. Pulegone, piperitone or piperitenone have been identified as dominant *M. pulegium* oil components [46]. Toxic effects of *M. pulegium* essential oil are mainly due to its main component pulegone. Reports suggest that the ingestion of up to 10 mL of *Mentha pulegium* oil causes gastritis and mild central nervous system toxicity without hepatorenal damage. The fatalities resulting from the ingestion of 15 to 30 mL of this oil [47]. Because of its potential toxicity pennyroyal is not recommended for children and other sensitive groups [45].

In *Mentha pulegium* essential oil growing in Bosnia and Herzegovina, 34 essential oil components (98% components of the oil), have been identified by GC/MS and

Figure 6.
Mentha pulegium *L. (pennyroyal).*

GC/FID. Monoterpenoids were the most represented group of compounds (90.4%), with pulegone (54.4%), *p*-menthone (14.0%), piperitenone (12.8%), piperitone (3.7%), and isopulegone (2.5%) being dominant. Monoterpenes accounted for a total of 3.3%. The most prevalent monoterpenes were: limonene (1.2%), α-pinene (0.9%), and ß-pinene (0.6%). Of sesquiterpenes (1.7%), germacrene D was the most prevalent (1.1%). The non-terpenic compounds accounted for 2.5%, with 3-octanol being the most prevalent (2.3%) [48].

The antioxidant capacity of the essential oils of *Satureja subspicata* and *Mentha pulegium* were evaluated by the commonly used DPPH and FRAP assay. In DPPH test, in comparison to reference antioxidants ascorbic acid (IC_{50} = 0.35 g/L) and hydroxyanisole (BHA) (IC_{50} = 0.37 g/L), essential oil of tested plants showed lower antioxidant potential for *Satureja subspicata* essential oil (IC_{50} = 3.3 g/L) [40] and good antioxidant potential for *Mentha pulegium* essential oil (IC_{50} = 94.3 μg/mL) [48].

The antioxidant potential of *S. subspicata* and *M. pulegium* essential oils, in concentration of 1 g/L tested by FRAP assay were for *S. subspicata* essential oil 73.89 (Eq Fe^{2+}μM), [40] and for *M. pulegium* essential oil 6.71 (Eq Fe^{2+}μM) [48]. Ascorbic acid and BHA had antioxidant potentials of 5568.43 and 5586.27 (Eq Fe^{2+}μM) respectively, for the same tested concentration [40, 48].

Low quantities of phenol compounds or monoterpenoids (such as carvacrol and thymol), which are good antioxidant compounds, may explain low antioxidant activity of *S. subspicata* essential oil [49]. As pulegone and menthone are known for their antioxidant properties, [50] the obtained results for *M. pulegium* essential oil can be explained by the high content of pulegone (54.4%) and the significant content of *p*-menthone (14.0%). Good antioxidant activity is also shown by 1,8-cineole, [51] which was also identified as one of the components of the tested *M. pulegium* oil (0.2%) [48].

The ability of *Satureja subspicata* and *Mentha pulegium* essential oils to inhibit enzymes acetylcholinesterase and butyrylcholinesterase (AChE and BChE) was tested by Ellman's method. The essential oils were tested at initial concentrations of

1 and 2 mg/mL. At an initial concentration of 0.1 mg/mL, eserine - the well-known cholinesterase (ChE) inhibitor - inhibited AChE with 95.9%, while BChE inhibited with 79.1%. In comparison to eserine, *S. subspicata* essential oil demonstrated good inhibitory activity of AChE at starting concentrations of 1 and 2 mg/mL (72.82% and 76.89%, respectively), and moderate inhibition of BChE (51,51% i 27,15%, respectively) [52]. *M. pulegium* essential oil at the same starting concentrations showed moderate inhibitory activity with inhibition of AChE (28.8 and 50.6%, respectively) and BChE (63.7 and 71.1%, respectively) [48].

These good results for *S. subspicata* essential oil may be due to presence of the well-known cholinesterase inhibitors α-pinene (10.2%) and β-caryophyllene (14%), among the main components. The moderate inhibitory activity of *M. pulegium* essential oil could be explained by the presence of moderate ChE inhibitor pulegone, as its primary component (54%). 1,8-cineole, a highly strong ACh inhibitor, is also present in *M. pulegium* oil in a smaller amount (0.2%), as well as α-pinene and ß-caryophyllene [51]. It is worth noting that our results reveal better inhibition of less specific BChE than AChE, which has to be further examined.

6. Conclusions

An excess of reactive species and damage caused by their action is called oxidative stress. In a state of oxidative stress, an excess of ROS and RNS may damage lipids, proteins, carbohydrates, and nucleic acids. Free radicals attack unsaturated fatty acids in biological membranes causing lipid peroxidation. Oxidative modification of proteins is present in diseases and changes associated with the aging process, such as atherosclerosis, tumors, neurodegenerative diseases, and the aging. In addition to fragmentation, oxidation of the amino acid residues increases carbonyl concentration, so the presence of carbonyl groups can be used as an indicator of protein oxidation.

Oxidation of lipid molecules is a major problem in the food industry, as it leads to changes in the organoleptic properties of food, a decrease in its nutritional value, as well as the formation of radical components that can endanger consumers' health. Polyunsaturated fatty acids oxidize much faster than monounsaturated or saturated ones. Lipid autooxidation is an autocatalytic reaction, which means that it progresses over time due to the formation of products that catalyze the reaction themselves.

The use of some synthetic antioxidants has negative effects on human health due to the promotion of carcinogenesis [10, 11], and there is a tendency to replace synthetic antioxidants, where possible, with non-toxic antioxidants of natural origin. More recently, essential oils have also been used as a substitute for synthetic antioxidants, in those food canning sectors where their use does not adversely affect product flavor [12].

Given that the oxidative stress is associated with the etiology and pathogenesis of many diseases, it is believed that eliminating the causes of oxidative stress may prevent or delay the occurrence of pathological changes and reduce the occurrence of diseases. Therefore, natural antioxidants – alone or in the form of extracts – may be useful in the treatment of such diseases. Thus the reason for the great interest in researching the antioxidant activity of aromatic, medicinal, and edible plants [13]. With the exception of some phenolic components, whose antimicrobial and antioxidant activity is well known, such data are not available for most other components of essential oils.

Inhibition of acetylcholinesterase prevents the hydrolysis of acetylcholine, thus prolonging its activity in the transmission of nerve impulses. This concept is applied in the treatment of diseases characterized by low ACh levels, such as Alzheimer's disease. Synthetic AChE inhibitors such as physostigmine, tacrine, and donepezil cause side effects such as hepatotoxicity and gastrointestinal disorders. This was an incentive for further research with the aim of finding new ChE inhibitors of natural origin, with greater efficiency and bioavailability, as well as with fewer side effects. Many phytochemicals are bioactive compounds, some of which show ChE inhibitory activity and represent a model for the development of new drugs, ChE inhibitors. As terpenes and terpenoids have shown relatively weak inhibitory capacity in studies published so far, it is necessary to develop analogues with an improved efficiency [21].

The obtained results show that the tested essential oils of *Satureja subspicata* and *Mentha pulegium* growing in Bosnia and Herzegovina contain compounds that, in addition to antioxidant activity, also show activity in terms of cholinesterase inhibition. Therefore, they may be important in the prevention and treatment of Alzheimer's disease and other neurodegenerative disorders, as well as in conditions of impaired homeostasis caused by oxidative stress, and also in food as protecting antioxidants [40, 48].

Conflict of interest

The authors declare no conflict of interest.

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