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Antimicrobial Activity and Antioxidant Capacity of Thyme, Rosemary and Clove Essential Oils and Their Mixtures

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Abstract

In this study, antimicrobial and antioxidant properties of thyme (*Thymus vulgaris* L., TEO), rosemary (*Rosmarinus officinalis* L., REO) and clove essential oils (*Syzygium aromaticum* L., CEO) and their mixtures (TEO/REO, TEO/CEO, REO/CEO and TEO/REO/CEO) at 1/1 ratio have been evaluated. The agar well diffusion method has been used for screening the antimicrobial activity against *Bacillus subtilis*, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Staphylococcus aureus*. For the antioxidant capacity of essential oils and their mixtures, FRAP and DPPH scavenging activity methods have been applied. All of the essential oils and their mixtures have shown an antimicrobial activity against the test microorganisms and an increased antioxidant capacity. TEO has displayed the highest inhibition zones against *B. subtilis*, *E. coli* O157:H7, *L. monocytogenes* and *S. aureus*. In general, the mixing of TEO with other essential oils has caused a decrease of its antimicrobial activity when compared with TEO alone. The lowest antimicrobial activity has been observed from REO alone and mixing REO with TEO and/or CEO has led to an increase of the antimicrobial activity of REO. The FRAP value of essential oils and their mixtures have ranged from 254.83 to 721.16 mM Fe (II)/mL, while the DPPH scavenging activity values have ranged from 0.155 to 4.121 µL oil. All the essential oils and their mixtures have displayed an antioxidant capacity, however the highest antioxidant capacity have been determined by using CEO in both methods, followed by TEO, and REO has showed the lowest antioxidant capacity. These results support the utilization of essential oils extracted from thyme, rosemary and clove and their mixtures at 1/1 ratio as a natural antimicrobial and antioxidant agent in the food industry.

Keywords: Antimicrobial activity, Antioxidant capacity, Thyme, Rosemary, Clove essential oils

1. Introduction

Concerns over the safety of synthetic food additives and possible toxicity of synthetic chemicals used as antimicrobials or antioxidants increased in recent years. In the food industry, there is considerable demand for using natural antimicrobial and antioxidant compounds rather than synthetic formulas, in order to extend the shelf life of foods [1]. Recently, plant essential oils are considered as natural antimicrobial and antioxidant additives and they are becoming more acceptable than synthetics for consumers. Another advantage is, that natural compounds overall are considered to be safe and a safety test is not required by legislation. These substances are identical to the food consumed over hundreds of years and they not only extend the shelf life of foods but also add nutraceutical value to the foods [2].

Essential oils are oil-like mixtures obtained by using different purification methods from plants or various parts of plants, which are liquid at room temperature, can be dragged by water vapor, easily crystallize, have a volatile characteristic and sharp odor. These essential oils, which are secondary metabolite products of plants, are responsible for the unique smell of plants [3]. Essential oils are important sources of natural antimicrobial and antioxidants. They are used in pharmacology, medical, food and the cosmetic industry [1, 4]. Essential oils contain active compounds called as terpenoids (carvacrol, carvone, thymol) and phenyl propanoids (cinnamaldehyde, eugenol, anthol, etc.) and the vast majority of these active compounds are composed of terpenoids, which are terpenes and isoprene derivatives, mono and diterpenes and their oxygenated derivatives [5]. The antioxidant and antimicrobial effects of essential oils vary depending on the chemical composition, amount of the active compounds they contain, the geographical region and climate in which they grow, harvesting time and method of production. The mechanism of antioxidant action of essential oils is explained by the reduction of the formation of peroxide radicals

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developed during the first step of oxidation of phenol hydroxyl groups [6]. The antimicrobial activity of essential oils against Gram (-) and Gram (+) bacteria can be explained by certain active compounds, such as terpenoids and phenyl propanoids. The mode of action of terpenoid-derived compounds, such as carvacrol and thymol, is to break down the bacterial cell membrane, while the phenyl propanoid derivatives such as cinnamaldehyde and eugenol, affect the mitochondrial lipid membrane by penetrating the bacterial wall and reaching the inner parts of the cell because of its lipophilic properties [7]. Bacteria cells use their energy to repair damaged parts of the cell and wall rather than for growth hence, cell growth slows down and death occurs [8, 9].

Thyme (*Thymus vulgaris* L.), rosemary (*Rosmarinus officinalis* L.) and clove (*Syzgyium aromaticum* L.) grow naturally in Mediterranean regions of Turkey [10]. Thyme is a member of Labiatea family and has many beneficial effects such as carminative, antiseptic, antioxidant and antimicrobial [11]. Thyme essential oil (TEO) consists of various terpenoids, such as α-pinene, myrcene, p-cymene, γ-terpinene, linalool, thymol and carvacrol. Quantitatively, the last two terpenoids are major components of thyme extracts [12, 13]. Thymol and carvacrol disturb the cell membrane resulting in an inhibitory effect on microorganisms [8]. These compounds are also responsible for the antioxidant capacity of the plant [14]. Rosemary is a member of Lamiaceae family and grows naturally on dry rocky slopes and hillsides. It is used fresh, dried or in essential oil extracts [15]. It is widely known that rosemary essential oil (REO) has antimicrobial and antioxidant properties [4, 16]. The major components of REO are 1,8 cineol (20%), α-pinene (20%), camphor (18%), camphen (7%), borneol (5%), myrcene (5%), bornylacetat (3%) and α-terpineol (2%), whereas linalool, limonene and caryophyllene are minor components. These minor and major components of REO are highly responsible for the antimicrobial and antioxidant properties [17, 18]. Clove is the aromatic dried flower buds of an evergreen tree in the family of Myrtaceae. It is used as a spice in the world's cuisine [19]. Clove essential oil (CEO) has two different fractions; light and heavy from water and its main component is eugenol. Essential oil yield of clove is close to 15-16% and 70-85% of the CEO is eugenol [20]. CEO, has anesthetic and antimicrobial properties and it is used to eliminate bad breath or ameliorate the ache of a bad tooth [19]. It was shown in several studies, that CEO has a lethal or anti-inflammatory effect against various bactericides [21-25]. Moreover, it has been reported, that the CEO has an increased antioxidant capacity when compared with commercial antioxidant substances [20, 22].

There are many studies on the antimicrobial and antioxidant properties of thyme, rosemary, clove plants and their essential oils. Nevertheless, there is limited research focusing on the antimicrobial and antioxidant properties of TEO, REO and CEO mixtures. In a previous study, Tural and Turhan [15] reported antimicrobial activity of thyme, rosemary and laurel essential oils and their mixtures against *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Staphylococcus aureus*, as well as its antioxidant capacity. Therefore, the objectives of present study is to determine the antimicrobial activity of TEO, REO and CEO and their mixtures against pathogenic food bacteria such as *Bacillus subtilis*, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Staphylococcus aureus.* In addition, the antioxidant capacity of essential oils and their mixtures with two different test methods have been evaluated.

2. Material and Methods

2.1. Materials

2.1.1. Plants

Dried thyme (*Thymus vulgaris* L.), rosemary (*Rosmarinus officinalis* L.) and clove (*Syzygium aromaticum* L.) plants were purchased from local markets in Samsun, Turkey in May, 2016. Plants were stored in plastic bags at dark and room temperature (25 °C) until use.

2.1.2. Bacterial strains

Food borne pathogenic bacteria such as *Bacillus subtilis* (NRRL-B209), *Escherichia coli* O157:H7 (ATCC 25922), *Listeria monocytogenes* (ATCC 7644) and *Staphylococcus aureus* (ATCC 33862) were used as test organisms. Microorganisms were kindly provided from Biotechnology laboratory of Ondokuz Mayis University, Department of Food Engineering, Samsun, Turkey.

2.2. Methods

2.2.1. Extraction of essential oils

Hydro-distillation technique was used for essential oil extraction, and for this purpose two different (lighter and heavier than water) Clevenger apparatus (Sesim Chemical Laboratory, Ankara, Turkey) were used (Fig. 1). Thyme (TEO) and rosemary (REO) essential oils were extracted using lighter than water type apparatus (Figure 1-a). Heavier than water type Clevenger apparatus (Figure 1-b) was used for extraction of clove essential oil, since it was heavier than water.

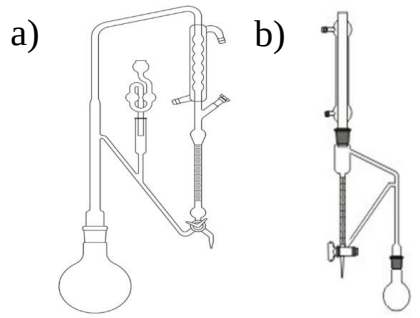

Figure 1. Clevenger type hydro-distillation apparatus used for essential oil extraction, (a) is lighter and (b) is heavier than water

Thyme and rosemary plants (50 g) were mixed with 500 mL of distilled water and then, placed in the apparatus for 4 h. The extracted essential oils were dehydrated with anhydrous sodium sulphate (Na_2SO_4) and stored in amber vials at +4 °C. However, 100 g of clove buds were mixed with 1000 mL of distilled water and solution was extracted for 6 h, then the mixture of water-oil was collected and separated with a funnel. Clove essential oil (CEO) was stored in amber vials at +4 °C until use. The essential oil yield of dried thyme, rosemary and clove plants were 1.45, 1.06 and 12.5 %, respectively.

2.2.2. Antimicrobial activity of essential oils

The antimicrobial activity of TEO, REO and CEO and their mixtures at a ratio of 1/1 (TEO/REO, TEO/CEO, REO/CEO and TEO/REO/CEO) were analyzed by the agar well diffusion method as described by [26]. Firstly, *B. subtilis*, *E. coli* O157:H7, *L. monocytogenes* and *S. aureus* were grown in Tyriptic Soy Broth (TSB) at 37 °C for 24 h, and after incubation, each of the bacterial suspensions was adjusted to 0.5 Mc Farland unit turbidity in TSB. Each 100 mL of the Nutrient Agar was inoculated with 0.1 mL of bacterial solution prior to casting into the petri dish. After the petri dishes were dried, 5 mm diameter wells were opened with cork borer to agar plates under aseptic conditions and 50 µL of each essential oil and their mixtures were placed in the well. Plates were incubated at 37 °C for 24 h and inhibition zones (free from bacterial growth) were measured with a digital caliper in mm. Antimicrobial activity tests of each bacteria were performed in triplicate and results are given as average values of zone diameter.

2.2.3. Antioxidant capacity of essential oils

Ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity methods as described by Gao, Björk [27] and Odabaş and Koca [28], were used with slight modifications for determination of antioxidant activity of essential oils. For FRAP analysis, essential oils and their mixtures were diluted with methanol for a suitable concentration and 50 µL of diluted samples were mixed with 0.95 mL of ferric-2,4,6-tripyridyl-s-triazine (TPTZ) reagent, which prepared with mixing 300 mM acetate buffer, 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$ at the ratio of 10/1/1. Samples were held in a dark environment for 5 min and then, the absorbance of the samples was measured with a spectrophotometer (Helios Gamma, Thermo Scientific, USA) at 593 nm wavenumber. The antioxidant capacity was calculated as mmol Fe (II)/mL from the calibration curve prepared with iron (II) sulfate hepahydrate ($FeSO_4.7H_2O$).

For the DPPH analysis, essential oils and their mixtures were dissolved in methanol and diluted samples were vigorously mixed with 1 mL of 100 μM DPPH solution. Samples were kept in a dark chamber for 30 min and then, the absorbance of samples were determined at 515 nm with a spectrophotometer. DPPH scavenging activity was calculated by:

$$\text{DPPH scavenging activity (\%)} = \frac{A_{Blank} - A_{Sample}}{A_{Blank}} \times 100 \quad (1)$$

where A_{Blank} is the absorbance of the control, and A_{Sample} is the absorbance of the sample. The amount of sample required to reduce the initial DPPH concentration by 50% (EC_{50}) was calculated as μL oil, and results are given as EC_{50}.

2.2.4. Statistical analysis

Statistical analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA) and the results were expressed as means and standard deviation calculated from three measurements. Data were analyzed as a completely randomized design procedure using one-way analysis of variance (ANOVA). The differences among means were determined by Duncan's multiple range tests at a significance level of 0.05.

3. Results and Discussion

3.1. Antimicrobial activity

The antimicrobial activity of essential oils and their mixtures against foodborne pathogenic bacteria are given in Figure 2. As seen, all of the essential oils used in this research displayed an inhibitory effect against all tested microorganisms and inhibition zones ranged from 10.67 to 35.00 mm. TEO displayed the highest inhibition zones against *B. subtilis*, *E. coli* O157:H7, *L. monocytogenes* and *S. aureus*, which were 27, 28.67, 30 and 35 mm respectively, and it was also significantly different from other essential oils ($P<0.05$). REO had the lowest antimicrobial activity against *B. subtilis*, *E. coli* O157:H7 and *L. monocytogenes*, however antimicrobial activity against *S. aureus* was higher than CEO ($P<0.05$).

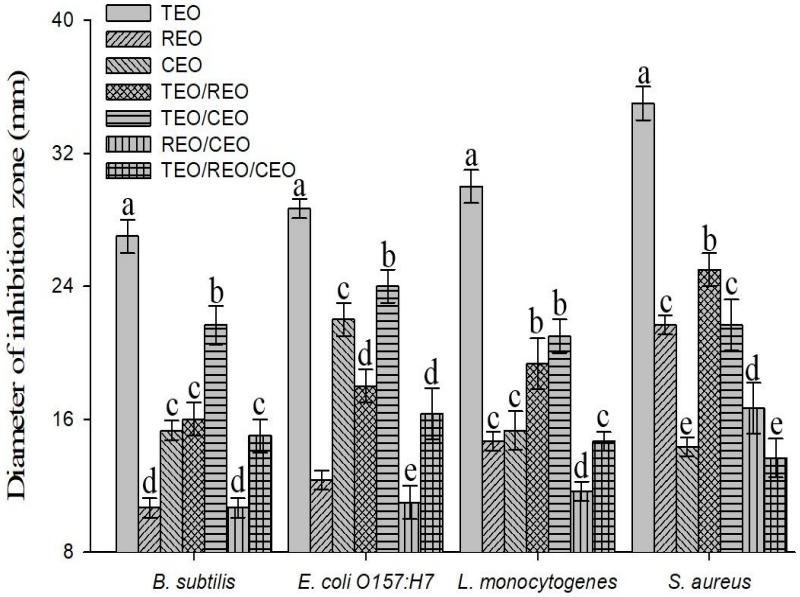

Figure 2. Antimicrobial activity of thyme (TEO), rosemary (REO) and clove essential oils (CEO) and their mixtures against *B. subtilis*, *E. coli* O157:H7, *L. monocytogenes* and *S. aureus*. Bars represent means ± Standard deviation of three replicates. Different letters (a-e) on the bars in each group refer to significant differences ($P<0.05$).

Among the essential oil mixtures, TEO/CEO had the highest antimicrobial activity against all tested microorganisms, except for *S. aureus*, in which TEO/REO had higher inhibition zone than other mixtures due to higher antimicrobial activity of TEO and REO alone against *S. aureus*. In general, mixing of essential oils having high inhibition diameter with other essential oils, which have low inhibition diameter led to an increase in antimicrobial activity, but essential oil mixtures were observed to have no synergistic or antagonistic effect on microorganisms.

Phenolic compounds in plant essential oils are responsible for the antimicrobial properties of essential oils. In general, essential oils, which contain phenolic compounds such as carvacrol, eugenol and thymol at high levels, exhibit antimicrobial activity against pathogenic microorganisms [8, 29]. Essential oils exhibit antimicrobial activity by coagulating the cytoplasm content, causing structural damage to the cell membrane, destroying the cell structure and causing loss of cytoplasmic material [30]. Moreover, phenolic compounds in the essential oils inactivate enzymes, which are vital for microorganisms, disturb the genetic material functionality, disturb energy production and structural material synthesis [31]. The effectiveness of essential oils against microorganisms may vary depending on the cellular structures of microorganisms. For instance, Gram-positive bacteria are more susceptible to the essential oils or antimicrobial compounds than that of Gram-negative bacteria. This is mostly because of that Gram-negative bacteria contain cellular walls which consist of lipoproteins and lipopolysaccharides, and hence these compounds form a barrier to restrict the entry of hydrophobic compounds such as essential oils [32]. Essential oils are natural and therefore their antimicrobial activities have widely been reported by various researchers in order to substitute with chemical antimicrobial agents. Tural and Turhan [15] investigated the antimicrobial activities of thyme, rosemary and laurel essential oils and their mixtures against *S. aureus*, *E. coli* O157:H7 and *L. monocytogenes*, and they reported that thyme essential oil had the highest antimicrobial activity against all test microorganisms. CEO displayed 22, 25 and 20 mm inhibition zones against *B. subtilis*, *E. coli* and *S. aureus*, respectively [33]. Rao, Jesmi [34] reported that TEO, REO and CEO had antimicrobial activity against *E. coli*, *S. aureus* and *L. monocytogenes*, and inhibition diameters for TEO were 22.8, 28.3 and 33.3 mm, for REO 20, 40 and 40 mm, and for CEO 20, 17.7 and 30.3 mm, respectively. As seen, the results of the present work are similar to the above results with slight differences, which could be attributed to environmental and ecological characteristics of plants, extraction method of essential oils and method of antimicrobial activity analysis.

3.2. Antioxidant capacity

Antioxidant capacity of plant extracts is generally analyzed by at least two different methods due to complicated composition of extracts [11]. Therefore, in this study FRAP and DPPH methods were used for determination of antioxidant properties of essential oils and their mixtures. The DPPH method is based on the ability of antioxidants to act as radical scavengers while the FRAP method measures the ability of antioxidants to perform as reducing agents [35]. In the FRAP method, in which the antioxidant capacity is determined, the higher the analysis result, the higher the antioxidant capacity, while the higher the EC_{50} value calculated as the result of the DPPH analysis, the lower the antioxidant capacities. The antioxidant capacity results of essential oils and their mixtures are given in Table 1.

Table 1. Antioxidant capacity of essential oils and their mixtures

Essential oils	FRAP (mmol Fe (II)/mL)	DPPH (EC_{50}, µL yağ)
TEO	310.42±13.99^{c}	0.814±0.02^{c}
REO	278.54±11.03^{cd}	4.121±0.32^{a}
CEO	721.16±30.01^{a}	0.155±0.03^{d}
TEO/REO	254.83±9.50^{d}	3.238±0.26^{b}
TEO/CEO	556.83±9.83^{b}	0.442±0.03^{cd}
REO/CEO	564.17±15.85^{b}	0.594±0.02^{c}
TEO/REO/CEO	532.59±11.01^{b}	0.527±0.02^{cd}

Values are means ± Standard Deviation. $^{a-d}$ Means within the same column with different letters are different ($P<0.05$). TEO: thyme essential oil; REO: rosemary essential oil; CEO: clove essential oil.

As can be seen, all the essential oils and their mixtures displayed antioxidant capacity, however the highest antioxidant capacity among essential oils alone was determined in CEO for both methods and followed by TEO and REO had the lowest antioxidant capacity ($P<0.05$). When the antioxidant capacities of the oil mixtures were examined, the TEO/REO mixture showed the lowest activity in both methods ($P<0.05$) and no significant difference was determined between the other oil mixtures ($P>0.05$). Since REO had the lowest antioxidant capacity, low antioxidant capacity was also observed in mixtures containing REO, and the antioxidant capacity results determined by both methods were generally similar to each other.

The antioxidant capacities of plant essential oils are derived from phenolic compounds, which are present in large quantities in their compositions. Phenolic compounds, due to phenol structures or phenolic sequences in molecular structures, give rise to hydrogen from phenolic hydroxyl groups and prevent oxidation by inhibiting the formation of free fatty acid radicals at the beginning [36]. It is considered that the antioxidant capacity of CEO is highly related to eugenol content, whereas thymol and carvacrol are responsible for the antioxidant capacity of TEO [14, 37]. Yanishlieva-Maslarova and Heinonen [38] reported that REO has antioxidant capacity due to epirosmanol, carnosol, rosmanol, carnosic acid, rosmaridiphenol, rosmadial, rosmarinic acid, isorosmanol and rosmariquinone content. It has been reported by several researchers that essential oils and extracts of thyme, rosemary and clove have strong antioxidant capacity [15, 39-41].

Viuda-Martos, Ruiz-Navajas [37] studied the essential oils of various plants such as thyme, rosemary and clove in terms of antioxidant capacity and compared their results with commercial antioxidant compounds such as ascorbic acid and BHT. Researchers have reported that clove essential oil has higher antioxidant capacity than commercial antioxidants and other plant oils and similar to our findings, they have also found that rosemary essential oil has the highest EC_{50} value, which means the lowest antioxidant capacity. EC_{50} values of thyme, rosemary and clove essential oils were reported as 83.21, 204.27 and 5.20 μg oil/mL and FRAP results of the thyme and clove essential oils were determined as 67.99 and 34.64 μM Fe (II)/g oil, respectively [40]. The antioxidant capacities of essential oils, obtained from thyme plants collected from different regions were determined as EC_{50} value of 273.36 to 693.75 μg/mL oil, and it was reported that antioxidant capacities of plant essential oils change significantly depending on the collecting region [42]. In another study in which antioxidant capacities of essential oils of different rosemary species were identified, EC_{50} values ranged from 6 to 28.5 μL/mL oil and FRAP values ranged from 16.53 to 21.77 mmol Fe (II)/L [4]. As seen, the antioxidant capacity results of the present study are in accordance with the literature mentioned above with slight differences, causing from the differences of plant variety, harvesting time, environmental and regional conditions, amounts of active substances, extraction methods and solvent type.

4. Conclusion

In recent years, researchers have been focused on natural antimicrobial and/or antioxidant additives such as essential oils due to toxicity and carcinogenicity of synthetic additives. This study reveals that all the essential oils and their mixtures exhibit remarkable antimicrobial activity and antioxidant capacity. TEO shows the highest antimicrobial activity among essential oils and mixtures, however CEO has the highest antioxidant capacity in both analysis methods. The mixing of essential oils with each other, have not exhibited a synergistic or antagonistic effect on microorganisms. Moreover, the mixing of essential oils with high antioxidant capacity and oils with low antioxidant capacity, reduce the results of oils with high antioxidant capacity. According to these results, it can be suggested that TEO, REO and CEO, both alone and in mixture forms can be used as natural antimicrobial and antioxidant agents in food processing.

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