INHIBITORY ACTIVITY OF ESSENTIAL OILS ON *ESCHERICHIA COLI*

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Abstract. *Bacteria are prokaryotic, single-celled microorganisms, among the first life forms to appear on Earth, and are present in most existing habitats. Escherichia coli is a Gram-negative, short bacillus that does not form spores; it is mobile, usually with peritric flagella, forming individual colonies or pairs. The genus Escherichia is part of the large Enterobacteriaceae family, having as a habitat, largely, the intestines of humans and animals. Because some strains cause serious infections and are resistant to conventional treatments, herbal extracts are studied and used, at least as an adjuvant, in the treatment of these conditions in humans and domestic animals. Herbal essential oils are increasingly popular, being used both in perfumery, cosmetics, and natural medicine industries. Currently, there are numerous scientific studies that have experienced the properties of these oils. In this research, the inhibitory effect of 19 essential oils on Escherichia coli was studied. The method used was the Kirby-Bauer diffusion method, often used in microbiology laboratories to test the antibiotic sensitivity of various bacteria. The reading of the results was achieved by measuring the inhibition zone in mm using a ruler, including the disc diameter (6 mm). The essential oils used were purchased commercially and the bacteria tested was Escherichia coli, a resource obtained from the "Horia Cernescu" Research Laboratory Complex of the "King Michael I of Romania" Banat University of Agricultural Sciences and Veterinary Medicine in Timisoara, Romania. The results obtained show that Escherichia coli is highly sensitive to oregano (34 mm), wild thyme (32 mm), garden thyme (27 mm), rosemary (25mm), tea tree (22 mm), and clove (21 mm) oils. It is very sensitive when treated with cinnamon oil (17 mm), peppermint (15 mm), and basil (15 mm), and sensitive when treated with lavander (10 mm), fennel (9 mm), dill (9 mm), garden sage (9 mm), and cumin (9 mm) oils. It is non-sensitive when treated with patchouli (0 mm), grapefruit (8 mm), vervain (8 mm), black cumin (7 mm), and rose geranium (7 mm) oils. The main objective of this study is to test some commercially obtained essential oils on E. coli bacteria to verify the veracity of these products and their effect on in vitro bacterial cultures.*

Keywords: *Escherichia coli, essential oils, disk diffusion method, inhibition zone.*

INTRODUCTION

German paediatrician Theodore Escherich managed to describe, in 1885, for the first time, the microorganism that bears his name. Since then, *Escherichia coli* has become one of the best known and understood organisms on Earth. Escherich discovered the microorganism while studying the faecal bacterial flora of new-borns. He noted that *E. coli* was universally present in healthy individuals, being the most abundant anaerobic optional in the intestines of humans, but also in other warm-blooded species (DONNENBERG, 2012).

E. coli is a Gram-negative, short bacillus that does not form spores; it is mobile, usually with peritric flagella, forming colonies individually or in pairs, capable of fermentative and respiratory metabolism. The optimum development temperature is 37°C, increasing easily over a wide range of culture media. On solid media, colonies are unpigmented and can be smooth or rough, usually circular in shape (SUSSMAN, 1997).

E. coli remains one of the most common causes of many bacterial infections, such as enteritis, urinary tract infection, septicaemia or clinical infections, as well as neonatal meningitis. It is commonly associated with diarrhoea in pets and farm animals. Therapeutic treatment of these infections is threatened by the appearance of resistance to antimicrobial substances. The multi-resistance of *E. coli* strains and, in general, microbial resistance occurs worldwide, becoming a continuing concern for public health (World Health Organization, 2012). Since the introduction of penicillin, a large number of bacteria have responded to the use of antibiotics with their ability to evolve and transmit microbial resistance to other species (Von Baum & Marre, 2005). Increased consumption of antimicrobial agents and their misuse are among the factors that have further accelerated this phenomenon. In addition, continuous migration of people, international tourism, and business travel play an important role in the acquisition and spread of multi-resistant strains (Van Der Bij & Pitout, 2012).

E. coli is resistant to therapeutic levels of penicillin, the first β-lactam introduced into clinical practice, but is resistant to several classes of antibiotics, with distinct mechanisms of action (Johnson *et al.*, 2011; Erb *et al.*, 2007). Because of this, solutions have been sought to increase conventional treatments with phyto-therapeutic products.

Essential oils are natural products derived from medicinal, aromatic plants, traditionally used worldwide for disinfection, as anti-inflammatory, antibacterial and antifungal substances, relaxing and stimulating, with potential for use in clinical medicine. Starting from their traditional use, for the treatment of problems of the respiratory tract, digestive system, gynaecological, endocrine, cardiovascular, nervous system disorders or skin infections, essential oils are increasingly used as natural alternatives to synthetic preparations for the prevention and treatment of infectious diseases, although often not prescribed by specialists, are not properly dosed and do not take into account the cumulative effect of mixed oils, the patient's possible sensitivity, the amount of pesticides or toxic substances extracted from improperly grown, harvested or preserved plants.

Ancient Egyptians have used aromatic oils since 4500 B.C. in cosmetics and ointments. They used to make a mixture of different sources of herbal preparations such as anise, cedar, onions, myrrh and grapes in perfumes or medicines (Hüsnü Can Baser & Buchbauer, 2010). On the other hand, the use of aromatic oils was recorded for the first time in traditional Chinese and Indian medicine between 3000 and 2000 B.C. For China and India, historical sources recorded more than 700 substances or plants used in this way, including cinnamon, ginger, myrrh, and sandalwood. Greek historiography has documented the use of various essential oils between 500 and 400 B.C., including thyme, saffron, marjoram, cumin, and peppermint (Pauli & Schilcher, 2010).

In the 18th and 19th centuries, chemical researchers documented the active components of medicinal plants and identified many substances such as caffeine, quinine, morphine, and atropine, which were considered to play an important role through their biological effects (Tisserand & Balacs, 1995). Numerous essential oils, such as lavender, peppermint, and myrrh are commonly used pharmaceutically as alternatives for synthetically produced medicines (Ali *et al.*, 2015).

The main objective of this study is to test some commercially obtained essential oils on *E. coli* bacteria to verify the veracity of these products and their effect on *in vitro* bacterial cultures.

MATERIAL AND METHODS

The bacterium used to test sensitivity to essential oils is *E. coli* (Migula 1895) Castellani and Chalmers 1919 (syn. *Bacillus coli communis* Escherich 1885), obtained from the "Horia Cernescu Research Laboratory Complex" at the University of Agricultural Sciences and Veterinary Medicine of Banat "King Michael I of Romania" in Timisoara, Romania. The essential oils used were bought from naturist pharmacies, from different manufacturers. The list of essential oils and plant species is given in Table 1.

Table 1

Herbal essential oils used to test the sensitivity of *E.coli*

Essential oil		**Plant**	
Romanian name	**English name**	**Botanical name**	**Botanical family**
Oregano	Oregano	*Origanum vulgare* L.	*Lamiaceae*
Cimbru	Garden thyme	*Thymus vulgaris* L.	*Lamiaceae*
Cimbrişor	Wild thyme	*Thymus serpyllum* L.	*Lamiaceae*
Lavandă	Lavender	*Lavandula angustifolia* Mill.	*Lamiaceae*
Mentă	Peppermint	*Mentha x piperita* L.	*Lamiaceae*
Busuioc	Basil	*Ocimum basilicum* L.	*Lamiaceae*
Rozmarin	Rosemary	*Rosmarinus officinalis* L.	*Lamiaceae*
Salvie	Garden sage	*Salvia officinalis* L.	*Lamiaceae*
Patchouli	Patchouli	*Pogostemon cablin* (Blanco) Benth.	*Lamiaceae*
Fenicul	Fennel	*Foeniculum vulgare* Mill.	*Apiaceae*
Chimion	Cumin	*Cuminum cyminum* L.	*Apiaceae*
Mărar	Dill	*Anethum graveolens* L.	*Apiaceae*
Chimen negru	Black cumin	*Nigella sativa* L.	*Ranunculaceae*
Grapefruit	Grapefruit	*Citrus x paradisi* Macfad.	*Rutaceae*
Verbină	Vervain	*Verbena officinalis* L.	*Verbenaceae*
Geranium	Rose geranium	*Pelargonium graveolens* L'Hér.	*Geraniaceae*
Cuişoare	Clove	*Syzygium aromaticum* (L.) Merrill & Perry	*Myrtaceae*
Melaleuca	Tea tree	*Melaleuca alternifolia* (Maiden & Betche) Cheel	*Myrtaceae*
Scorţişoară	Cinnamon	*Cinnamomum* spp. Schaeff.	*Lauraceae*

The working method is the Kirby-Bauer diffusion method, commonly used in microbiology laboratories to test the antibiotic sensitivity of bacteria species. The culture medium used was agar supplemented with blood (BAB 5%) in Petri boxes.

A tube containing Mueller Hinton Broth (MHB) inoculated with the microorganism was incubated during 18 h, at 37°C. Decimal dilutions up to 10^{-6} cfu/ml were made. 200 µl of inoculum were spread over Petri plates. Subsequently, the discs 6 mm in diameter were added at a distance of at least 25 mm and at least 15 mm from the edge of the plate. After fixing the discs, the essential oils were added, 10 microliters on each disc. The plates were left for 15 minutes at room temperature to allow the oil diffusion; they were kept in the incubator for 24 hours at 37ºC. After this time, the antibacterial activity was evaluated by measuring the diameter of inhibitory zones around the discs using a calliper.

All tests were carried out three times and the entire experiment was carried out within the “Horia Cernescu” Research Laboratory Complex of the B.U.A.S.V.M. Timisoara.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC). The essential oils, which exhibited antimicrobial activity in the paper disk diffusion assay, were selected for determining the minimum inhibitory concentration and the minimum bactericidal concentration using broth dilution method (WIEGAND *et al.*, 2008; DE AGUIAR *et al.*, 2017). The bacterium was grown in Mueller Hinton Broth at 37ºC for 18-24 h. In sterile tubes with MHB, decimal dilutions were achieved with each essential oil (1/4-1/16) after which the bacterial suspension was inserted into each tube to result in a final volume of 4 ml. Final solutions were incubated at 37°C overnight. The MIC was defined as the lowest concentration that inhibited visible growth. The MBC was determined by subculturing 100 μL from the negative test tube onto plates (BAB 5%). MBC was defined as the lowest concentration resulting in a negative subculture. The experiments were carried out in three replicates.

Interaction between Components of Essential Oils. Based on the information in literature on the biochemical composition of essential oils and the existence of synergistic relationships between them, we tested, by the same Kirby-Bauer diffusion method, the antibacterial effect produced by the combinations between 2 essential oils, selected after the results obtained in the first stage of the study.

RESULTS AND DISCUSSION

Antimicrobial activity of essential oils

Currently, there are numerous scientific studies that have tested the properties of essential oils, proving their antioxidant, antibacterial, antifungal, and antiviral effects, which are a recognized source of bioactive compounds.

In this study, we chose to test the quality of commercially available essential oils, i.e. antimicrobial plant species, by checking the inhibitory capacity of 19 such products compared to *E. coli*.

To process the measurements, we opted for a classification of essential oils based on a scale used in similar studies (MELO *et al*., 2015; BABU *et al.*, 2011) starting from the diameter of the inhibition zone, as can be seen in Figure 1. For oils that produced an inhibition area diameter of more than 20 mm, *E. coli* was considered to be extremely sensitive; for values between 15 and 19 mm, we characterized it as very sensitive; and, for diameters between 9 and 14 mm, it was called sensitive; for all values below 8 mm, *E. coli* was considered not sensitive.

Extremely sensitive: Inhibition zone >20 mm	Very sensitive: Inhibition zone 15-19 mm	Sensitive: Inhibition zone 9-14 mm	Non-sensitive: Inhibition zone <8 mm

Figure 1. Classification of essential oils by inhibition zone diameter

Inhibition zones obtained by the average of the 2 measurements are shown in Table 2.

Table 2

Average diameter of inhibition zones (mean values)

Essential oils	**Inhibition zone (mm)**	**Essential oils**	**Inhibition zone (mm)**
Oregano	34	Fennel	9
Wild thyme	32	Dill	9
Thyme	27	Garden sage	9
Rosemary	25	Cumin	9
Melaleuca	22	Grapefruit	8
Cloves	21	Vervain	8
Cinnamon	17	Geranium	7
Peppermint	15	Black cumin	7
Basil	15	Patchouli	0
Lavander	10		

The inhibitory effect of some essential oils can be seen in Figures 2-6.

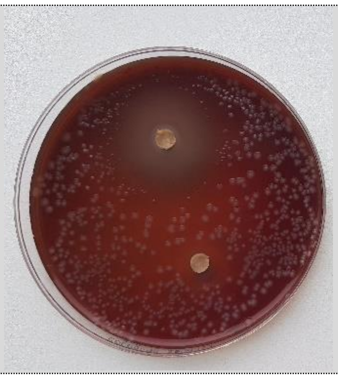


Figure 2. Inhibition zone for oregano and basil oils

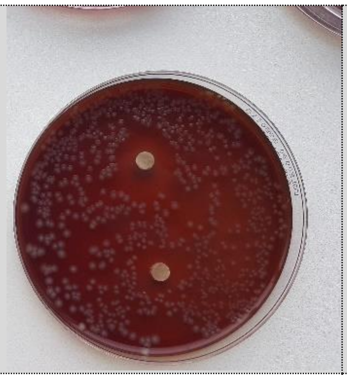


Figure 3. Inhibition zone for melaleuca and peppermint oils

Figure 4. Inhibition zone for rosemary and lavender oils

Figure 5. Inhibition zone for wild thyme and cinnamon oils

Figure 6. Inhibition zone for thyme and clove oils

After reading the results, it was noted that the diameter of the inhibition zone is 34 mm in the case of oregano oil, 32 mm in wild thyme oil, 27 mm in thyme oil, 25 mm in rosemary oil, 22 mm in melaleuca (tea tree) oil, and 21 mm in cloves oil (Figure 7). Compared to cinnamon oil, *E. coli* is very sensitive (17 mm), a category that includes peppermint and basil oils (15 mm).

Extremely sensitive: Inhibition zone >20 mm	Very sensitive: Inhibition zone 15-19 mm	Sensitive: Inhibition zone 9-14 mm	Non-sensitive: Inhibition zone <8 mm
Oregano	Cinnamon	Lavender	Grapefruit
Wild thyme	Peppermint	Garden sage	Vervain
Thyme	Basil	Cumin	Black cumin
Rosemary		Fennel	Geranium
Melaleuca		Dill	Patchouli
Clove			

Figure 7. Classification of oils by their inhibitory effect on *E. coli* species

Lavender (10 mm), dill (9 mm), cumin (9 mm), fennel (9 mm), and garden sage (9 mm) had less inhibitory activity. The least effective ones were the oils of grapefruit (8 mm), vervain (8 mm), black cumin (7 mm), and geranium (7 mm), while patchouli oil was the only one that did not show inhibitory effect.

An analysis of the results of the study by Melo *et al*. (2015) indicates certain differences in the diameter of the inhibition zone compared to our results: oregano essential oil had an inhibition zone of 38.5 mm ±0.99 mm, compared to 34 mm in our case; tea tree essential oil had a diameter of 35 mm ±1.87 mm compared to 22 mm; in thyme essential oil, the diameter was 64 mm±1.44 mm, compared to 27 mm. These differences can be explained by the concentration of the chemical compounds of the essential oil, related to the different origin of these products, i.e. plants. The chemical composition of plants or essential oils may vary greatly, depending on the geographical location of the species used, the quality of the plant material (harvesting at the optimal time phenological and climatic, the time between harvesting and processing), cultivation technologies in the case of plants not derived from spontaneous flora, bacterial endophytes, extraction techniques (Imbrea *et al*., 2016; Castronovo *et al*., 2020). Oregano essential oil is often cited, in literature, as one of those with antibacterial effect, as it has been shown in our tests.

For other essential oils, such as patchouli, our results indicate the absence of any antibacterial effect, although there are studies showing it with good results against bacteria such as *Staphylococcus aureus*, *Streptococcus pneumonia* and *Pseudomonas aeruginosa* (Vieira-Brock *et al*., 2017). In some studies, patchouli oil completely inhibits the growth of *E. coli* at 0.05% (Lin *et al*., 2016). Such differences show that some commercially available essential oils are not true, a common situation for those obtained from exotic species.

Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC)

MIC represents the lowest concentration that can inhibit the growth of the bacteria. For this, essential oils were selected that had a higher antibacterial effect in the first stage of the study. We considered ½ to be the beginning of dilutions in which essential oil is present in the greatest amount.

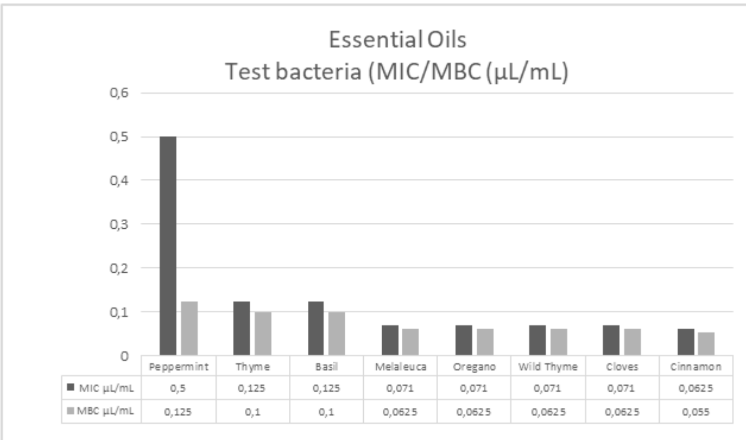


Figure 8. MIC and MBC concentrations of selected essential oils against *E. coli*

Transformed into µL/mL, dilution ½ was transposed as 0.5µL/mL, showing that peppermint inhibited the bacteria of interest only at a very high concentration of oil compared to the other essential oils used, e.g. oregano, for the latter MIC being visible at a dilution of

1/14 (0.071µL/mL). MIC was determined by the clarity of the macrodilutions, visually interpreted (Figure 8).

The MBC can be explained as the µL quantity of the essential oil that we need for the inhibition of *E. coli*. For most essential oils used, which inhibit the development of *E. coli*, MBC values are in the range of 0.125µL/mL and 0.055µL/mL.

Four combinations made from 2 essential oils were also tested.

The combination between essential oils oregano / basil and oregano / thyme inhibits the bacteria at a lower concentration than the combination between cinnamon / thyme and thyme / basil. That can be attributed to oregano essential oil and its high concentration of carvacrol (Figure 9), which confirms the results obtained in the first part of the study.

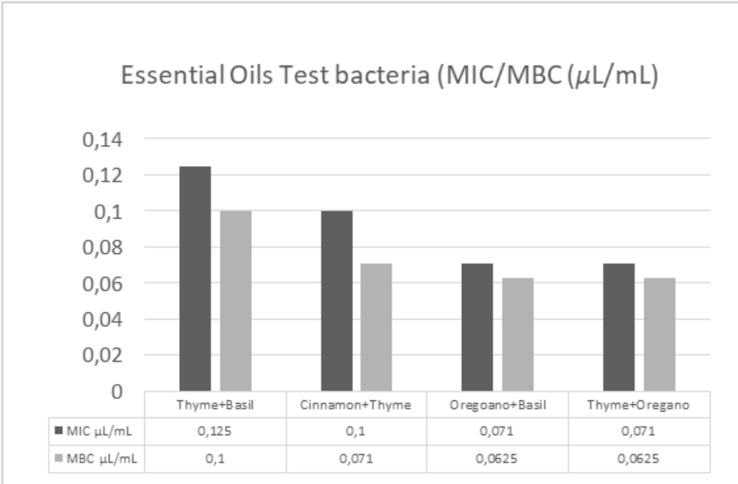


Figure 9. MIC and MBC concentrations of combined essential oils against *E. coli*

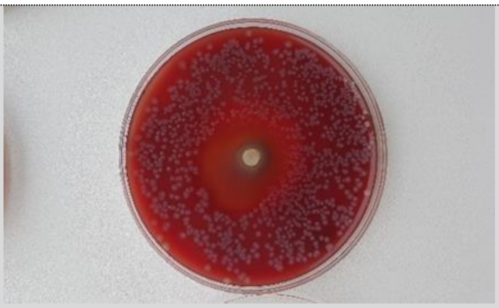
Although sometimes the disk method "may present a weak correlation with the quantitative microdilution technique because of heterogeneity of some oils when diffusing through the agar or their different volatility, depending on the chemical composition or external temperature" (DE AGUIAR *et al*., 2017), in our case the best results were obtained with oregano oil, both methods.

Interaction between components of essential oils

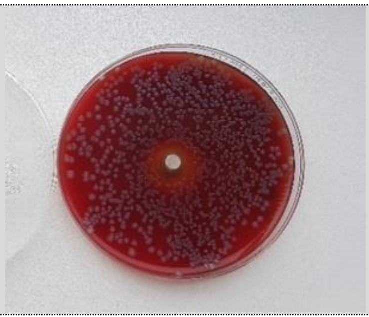
Classes of components are known, in essential oils, such as phenols, ketones, aldehydes, alcohols, esters, ethers and hydrocarbons (Burt, 2004), some having significant antibacterial effect. The bioactivities of essential oils are related with the activity of the main components of the oils (Juliani *et al*., 2002). Studies have found that some of these compounds exhibited antimicrobial properties when tested together (Harris, 2003).

It has been reported that essential oils contained phenols, such as carvacrol, eugenol, thymol, cinnamaldehyde or citral as major components showed the highest antibacterial activity, followed by essential oils containing terpene alcohols (*Chrysanthemum* essential oils). High antimicrobial activity of *Thymus* and *Origanum* species has been attributed to their phenol components such as thymol and carvacrol (Bassole & Juliani, 2012; Karatzas, 2001) and *Ocimum basilicum* (Santiesteban-Lopez *et al*., 2007) to eugenol. Terpinen-4-ol is considered to be the main active component of *Melaleuca alternifolia* (tea tree) oil (Gutierrez *et al*., 2008).

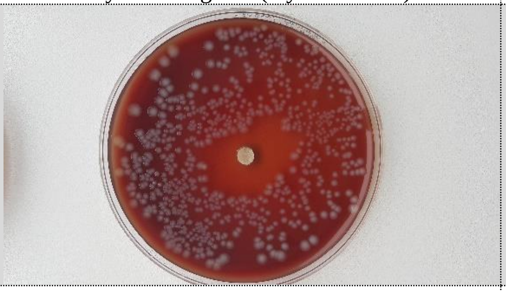
In the case of oils tested by us also (Figure 10, Table 3), the combination of phenols (thymol with carvacrol, and both components with eugenol) were synergistically active against *E. coli* strains (Bassole & Juliani, 2012).



Thymol / Eugenol (thyme & basil)



Cinnamaldehyde / Thymol (cinnamon & thyme)



Carvacrol / Eugenol (oregano & basil)

Thymol / Carvacrol (thyme and oregano)

Figure 10. The effect of interaction between the components of essential oils

Table 3

Mean inhibition zone diameter on combinations of two essential oils (mean values)

Essential oils	**Inhibition zone (mm)**
Carvacrol / Eugenol	30
Thymol / Carvacrol	25
Thymol / Eugenol	23
Cinnamaldehyde / Thymol	15

CONCLUSIONS

Our study verified the inhibitory effect of commercially available essential oils against *E. coli*. Numerous essential oils showed medium to high antibacterial activity, with very good results in oregano, thyme, wild thyme, rosemary, melaleuca, and cloves. Patchouli essential oil, a species proven to have antibacterial action for *E. coli*, was the only one that had no inhibitory effect at all, which means that some commercially available essential oils are not genuine, especially common in those obtained from exotic species.

Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC), together with tests performed with combinations of two oils, have confirmed oregano essential oil as the most effective of all those tested by us.

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