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THE EFFECT OF PEPPERMINT, CLOVE, CINNAMON, SAGE AND ORANGE ESSENTIAL OILS ON *LACTOBACILLUS BULGARICUS* BACTERIA

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ABSTRACT

Essential oils have been mainly used in aromatherapy, but they have gained the attention of the food industry in the last decade due to their ability to control the growth of pathogenic microorganisms. The essential oil content of plant tissue varies according to the stage of development of the plant, the parts of the plant (leaves, flowers, roots, etc.) as well as the method of extraction. Commonly used techniques for extracting essential oils include hydrodistillation, steam distillation (Chialva et al.,1982), solvent extraction, (Chialva et al.,1982) and CO2 extraction (Takeoka et al.,1985). The composition of the extracted oil may vary from one extraction method to another. The essential oils used in this study were: clove, peppermint, cinnamon, sage, orange. Observations were made on extraction yield, MIC for Lactobacillus bulgaricus, and comparative antimicrobial activity with two commonly used antibiotics.

INTRODUCTION

Essential oils mainly comprise aromatic and volatile compounds naturally present in all parts of plants, and are known to be highly effective antimicrobial agents. The antimicrobial (prevention of spoilage by inhibiting the growth of microorganisms) and antioxidant (prevention of oxidation and peroxidation of lipids) activities of essential oils are attributed to their volatile compounds. Thus, they have been widely used for medicinal, bactericidal, virucidal, fungicidal, antiparasitic, insecticidal and antioxidant purposes. Essential oils, which are synthesised by the secondary metabolism of plants, extracted by hydro-distillation can be used to extend the shelf life of some foods with their antioxidant and antimicrobial qualities (Ionica et al. 2022). Their composition may vary, due to intrinsic factors: depending on the part of the plant used (roots, stems, leaves, seeds, flowers and others), and extrinsic factors: extraction method - water or steam distillation, solvent extraction, microwave-assisted extraction, ultrasound-assisted extraction, supercritical fluid extraction and subcritical water extraction (Cassel et al., Essential oils have antimicrobial activity against a broad spectrum of Gram-positive and Gram-negative bacteria. One of the first in vitro studies on the antimicrobial activity of essential oils was conducted by Buchholtz in 1875. This author investigated the inhibitory activity of caraway, thyme, phenol and thymol oil on bacteria grown in a tobacco decoction and found that thymol had stronger antibacterial activity than phenol (Husnu et al. 2009). Subba et al. (1967) reported the inhibitory activity of orange and lemon oil on a wide range of spoilage bacteria (Subba et al.1967). Thyme, cinnamon, bay, clove, almond, nutmeg, angelica and nutmeg oils were reported to be the most inhibitory essential oils (Deans et al. 1987).

Clove*, Eugenia caryophyllata* (*Syzigium aromaticum L. Myrtaceae*) is a spice obtained from the flowering buds of an exotic tree in the myrtle family, which also includes myrtle and eucalyptus. The essential oil isolated from the buds of *Eugenia caryophyllata L. (Myrtaceae*) is widely used and is well known for its medicinal properties. Traditional uses of clove oil include use in dental care as an antiseptic and analgesic, where undiluted oil can be rubbed on the gums to treat toothache. It is active against oral bacteria associated with tooth decay and periodontal disease and effective against a large number of other bacteria: *Escherichia coli, Listeria monocytogenes, Salmonella enterica, Campylobacter jejuni, Salmonella enteritidis, Staphylococcus aureus* (Hayden et al., 2003, Kalemba et al., Previous studies have reported antifungal (Chami et al. 2005), anticarcinogenic (Zheng et al. 1992), antiallergenic (Kim et al. 1998) and antimutagenic (Mitsu et al. 2001) activity. Eugenol, the main component of clove oil, exhibits antioxidant properties (Ogata et al. 2000). Antibacterial activity of various *E. caryophyllata* extracts has been demonstrated against pathogenic bacteria including *Campylobacter jejuni, Salmonella enteritidis, Escherichia coli* and *Staphylococcus aureus* (Feres et al. 2005). A recent study reported that growth rates of *Listeria monocytogenes* strains observed at 15°C and 5°C were significantly reduced by treatment with 1% and 2% clove oil (Mytle et al.2006) and, in addition, Ogunwande A. et al. (2005) found that fruit essential oil exhibited strong antibacterial activity against *Staphylococcus aureus,* while leaf oil strongly inhibited the growth of *Bacillus cereus*, with an MIC of 39 µg/mL.

Mentha x piperita L. (*M. aquatica x spicata*) - Good izma, Mint. It is a drab perennial plant, approx. 100 cm., with reddish branches. The leaves are petiolate, lanceolate, with serrate margins. The flowers are pink, with a weakly bilabiate corolla, grouped in terminal, dense, globose spikes. It is cultivated as a medicinal and aromatic plant because of the menthol-rich essential oil it secretes. Peppermint oil and its by-products are generally used for candy, teas, mouthwash, toothpaste, drinks, alcoholic liqueurs, jellies, syrups, ice creams, cough drops, chewing gum, confectionery, soaps, detergents and mosquito repellents. Peppermint essential oil and its ethanol extract showed antifungal activity against *Candida albicans, C. tropicalis, C. glabrata* and *C. parasilosis*, but its infusion had no antifungal activity (Carretto et al. 2010). Peppermint oil showed good antimicrobial activity against *Aspergilus niger, Rhizopus solani* and *Alternaria alternata* (Hussain et al. 2010), *Pseudomonas, Xanthamonas campestris, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhimurium* (Iscan et al. 2010). The antibacterial activity of mint leaf juice against Gram-negative bacilli was higher than that of its stem juice (Saeed et al. 2007). This oil has good antioxidant activity in two free radical scavenging systems DPPH and -carotene/linoleic acid systems (Yadegarinia et al. 2006). The main active compounds of mint (*Mentha spp*.) are limonene, cineole, menthone, mentofuran, isomenthone, mentil acetate, isopulegol, menthol, pulegone and carvone (Shrivastava et al. 2009). Other components include glycosides, flavonoids (e.g. Narirutin, Luteolin-7-o-rutinoside, Isorhoifolin and Hesperidin, etc.), polyphenols (e.g. rosmaric acid, eriocitrin, cinnamic acid, caffeic acid and narigenin-7-oglucoside). The luteolin diglucoronide and the glucopyranosyl-ramnopyranoside eriodictyol have also been purified from aerial parts of mint (Areias et al., 2001).

Peppermint oil and its extracts have been tested on various microorganisms such as: *Escherichia coli, Salmonella pullorum, Streptococcus faecalis, Acinetobacter sp, Streptococcus thermophilus, Lactobacillus bulgaricus, Staphylococcus, Streptococcus, Serratia marcescens, Mycobacterium avium, Salmonella typhi, Salmonella paratyphi A/B, Proteus vulgaris, Enterobacter aerogenes, Yersinia enterocolitica* and *Shigella dysenteriae* (Shaikh et al. 2014; Bohnert et al. 2016).

Cinnamon (*Cinnamomum zeylanicum)* contains antioxidants and other active ingredients that are found in the water-soluble parts of cinnamon, not in the cinnamon oil. To date, more than 300 volatile substances have been found to be constituents of cinnamon essential oils. It has been established that cinnamon oils and extracts possess a distinct antioxidant activity, which is mainly attributed to the presence of phenolic and polyphenolic substances.

Sage (*Salvia officinalis* L.) is one of the largest and most important aromatic and medicinal genera in the *Lamiaceae* family and comprises about 900 species, spread throughout the world (Ogunwande et al. 2005). Several species of sage are used in folk medicine around the world to treat microbial infections, cancer, malaria, inflammation and to disinfect homes after illness (Kamatou et al. 2008). Essential oils produced from leaves are recognised worldwide for their beneficial uses: there are a large number of studies on the analysis of essential oils from plants of this genus and morphological and genetic variations are also observed depending on their geographical origin. Salvia is one of the most appreciated medicinal plants for its rich essential oil and for the multitude of biologically active compounds widely used in folk medicine (Penso 1983).

Orange (*Citrus sinensis*) is a source of essential oil concentrated in the exocarp of the fruit, which is composed of the epidermis and a layer of glandular cells. According to Mahato et all. (2018), large amounts of by-products are generated during orange processing and can be used in the food industry for essential oil extraction. In a study of essential oils from plants belonging to the genus *Citrus*, including orange essential oil, against different foodborne pathogens, antibacterial activity against both Gram-positive and Gram-negative bacteria was shown (Frassinetti et al. 2011). Torrez-Alvarez et al. (2017) reported the antibacterial and antioxidant potential of orange essential oil, highlighting it as an alternative for the development of safer products accepted by consumers who prefer natural ingredients. Probiotics are microorganisms that alter the host's intestinal microbial balance (Song et al., 2013). They can maintain and repair the balance of intestinal flora, and improve the immune system (Zhao et al. 2020). In addition, probiotics inhibit the growth of harmful bacteria.

Lactobacilli have been used in food since ancient times in food fermentation processes. They are part of probiotic products, i.e. bifidobacteria, strains of *L. acidophilus* or related species (so-called *L. acidophilus*). Lactic acid bacteria (LAB) have been used in the prevention of antibiotic-associated diseases such as acute infantile and recurrent diarrhea (Turgay & Erbilir 2006). *Lactobacillus bulgaricus* is considered to be a probiotic, a friendly bacterium found in both the enteric system and the oral cavity (Naseer et al. 2021). In addition, in food, *L. bulgaricus* increases flavour, reduces blood glucose levels and promotes glycopyranoside conversion (Si et al. 2019). Oral administration of *L. bulgaricus* also attenuates Aflatoxin B-induced liver damage which may become a new therapeutic approach for treating Aflatoxin B poisoning (Chen et al. 2019). In the food industry *L. bulgaricus* is traditionally used along with other species (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* *ssp*.) to make yoghurt, one of the most popular fermented dairy products (Zhang et al. 2020).

The present study aimed to test the effect of 5 essential oils extracted by hydrodistillation on a strain of lactic acid bacteria (*Lactobacillus bulgaricus*) used in yoghurt production technology.

MATERIAL AND METHODS

Plant material

The strains used for essential oil extraction were purchased from the market from organic culture in Pakistan (Organic Kitchen by Pansari). For obtaining mint and sage oil, naturally dried leaves, harvested at harvest maturity, were used from plants cultivated in Cârcea, Dolj County (44°16′32″N 23°53′46″E). The cultivation technology applied did not include treatments to control diseases and pests, only irrigation with moderate doses of water was performed on the plants. Ceylon cinnamon (*Cinnamomum verum*) used for oil extraction was in powdered form (grounded) from organic crops purchased from the market (Z Natural Foods, 5407 N Haverhill Rd #336,West Palm Beach, FL 33407). Essential oil was extracted from commercially purchased organically grown orange peels (Bioagros S.A., Vasileos Pavlou 2, Kria Vrisi 583 00).

Obtaining essential oils by hydrodistillation

The plant material was placed in a 2 litre round-bottomed flask with distilled water, (1000 ml for 75 g dry material) connected to a Machita-type heating nest. The essential oil was extracted by water distillation using a Clevenger-type equipment. The distillation time varied between 2 and 3 hours. The amount of oil extracted was related to the amount of plant material used.

Testing the antimicrobial activity of the oils obtained

Lactobacillus bulgaricus strain was isolated from yoghurt by seeding on MRS agar medium. For the microbial suspension 24-hour cultures were used. Inoculum harvested from plates was suspended in sterile saline (0.85 g/l). The resulting suspensions were adjusted to correspond to a turbidity of 0.5 McFarland units (1.5 x10^8UFC/ml). The MIC was determined by the 96-well microplate method (Preuss et al., 2005). 100µl sterile nutrient broth supplemented with 0.5% v/v Tween 80 was added to each well and then 100µl undiluted oil was added. Dilutions were made by transferring 100µl medium-Tween 80-oil mixture to the next well. From the last well 100µl were removed. The microbial suspension was added to each well (10µl). The plates were covered with a lid and thermostated at 37ºC. After thermostatting, 20 µl resazurin aqueous solution (0.2 mg/ml) was added to each plate well. The plates were covered with the lid and thermostatted for another 2h at the same temperatures. The added resazurin stained the medium-oil-microbial suspension mixture blue.

The growth of the microorganisms resulted in oxidation of resazurin to resorufin which has a fluorescent pink colour. The goblets where the mixture stained pink demonstrate cell viability, the blue ones are considered as negative, i.e. they inhibited microorganism growth (MIC).

Antimicrobial activity was also followed by the standard disc diffusion method (Saeed et al. 2007). Sterilized filter paper discs (6 mm diameter) were soaked in10 µL. The medium used was nutrient agar. The bacterial suspension was obtained in nutrient broth. Seeding was done using a sterile swab over the entire surface of the medium. The oil disc was placed on the surface of the inoculated plates using sterile tweezers. After incubation, the diameters of the inhibition zones for each oil were measured to the nearest millimetre (mm).

Statistical Analysis

All tests and analyses were performed in triplicate, with the mean values for each sample used for statistical analysis. To summarize the variability in the datasets, standard deviation was used using Microsoft 365 Excel and the data are presented as means ± SD.

RESULTS AND DISCUSSIONS

Obtaining essential oils

All tested oils were extracted by hydrodistillation. The time interval was between 2.5-3h. The amount of essential oil obtained ranged from 0.9-2.4%. The highest amount of oil was obtained from orange peels (Table 1), while cinnamon had the lowest oil extraction yield. A good yield was also found for cloves, while plant material represented by dried mint and sage leaves had average extraction yields.

Table 1.

Essential oil extracted (%)

Plant	Essential oil (%)
Cloves (powder)	2,2± 0.10
Mint (dried leaves)	1,6±0.08
Cinnamon (powder)	0,9±0.04
Sage (dried leaves)	1,40±0.06
Orange (fresh peel)	2,4±0.12

The results obtained are consistent with those obtained by other authors for different herbs (Tavakolpour et al. 2016, Durling el al. 2007, Denys el al. 1990, Jamshidi et al. 2009).

Effect of tested oils on Lactobacillus bulgaricus

It was found that essential oils of clove, peppermint, cinnamon, sage, orange did not show significant antimicrobial activity on *Lactobacillus bulgaricus* species. The MIC (minimum inhibitory concentration) is given in Table 2.

Table 2.

Minimum inhibitory concentration of the *Lactobacillus bulgaricus*

Essential oil	CMI (%)
Cloves	1,56±0.07
Mint	6,25±0.28
Cinnamon	12,5±0.64
Sage	3,12±0.14
Orange	12,5±0.60

From the data in the table it can be seen that the oils tested show antimicrobial activity on *Lactobacillus bulgaricus* only at very high concentrations. Antimicrobial activity of the oils was also followed by comparison with two antibiotics - penicillin and doxycycline. Sage and clove oil were found to show antimicrobial activity, but the antibiotics used had a much greater effect compared to the oils (photos 1-5).

Table 3 shows the diameter of the inhibition zones of *Lactobacillus bulgaricus* bacteria for penicillin, doxycycline and the oils tested.

Table 3.

Inhibition zone`s diameter for *Lactobacillus bulgaricus* (mm)

Essential oil	Diameter (mm) of the inhibition zone		
	Doxiciclin	Penicilin	Oil
Cloves	25	25	9
Mint	25	25	NI
Cinnamon	25	25	NI
Sage	28	28	12
Orange	28	15	NI

NI = no inhibition (<9 mm diameter)

Mint, cinnamon and orange showed no inhibitory activity on the *Lactobacillus bulgaricus* bacteria tested (Fig. 2,3,5). Clove oil (Fig.1) showed very weak inhibitory activity. Sage oil (Fig.4) was found to have inhibitory activity on *Lactobacillus bulgaricus* bacteria, but far below the activity of doxycycline and penicillin. The data obtained are in agreement with those published by Rania Ahmed (2018) who found that useful bacteria of *Lactobacillus* and *Streptococcus* genus are not inhibited by essential oils of peppermint, clove, cinnamon, black thyme and black cumin.

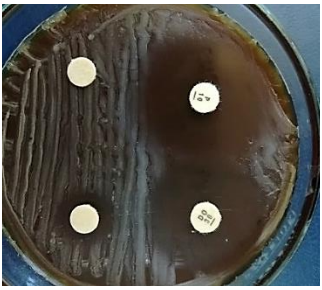

Photo 1. Effect of clove oil *on Lactobacillus bulgaricus*

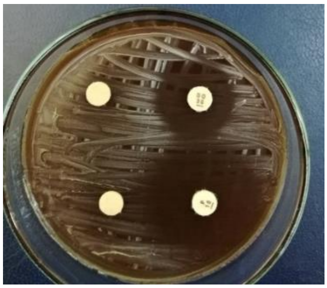

Photo 2. Effect of mint oil on *Lactobacillus bulgaricus*

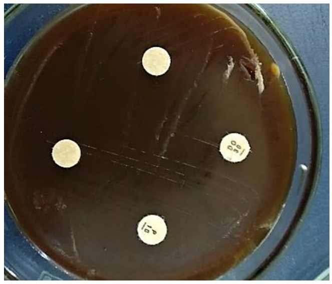

Photo 3. Effect of cinamon oil on *Lactobacillus bulgaricus*

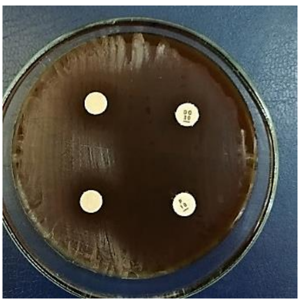

Photo 4. Effect of sage oil on *Lactobacillus bulgaricus*

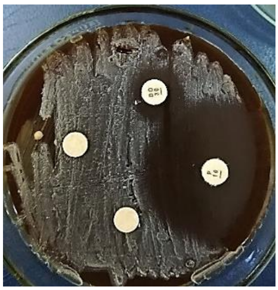

Photo 5. Effect of orange oil on *Lactobacillus bulgaricus*

CONCLUSIONS

Herbs are a very rich source of volatile compounds that have been shown to have very good antimicrobial and antioxidant activity. Cloves, mint, cinnamon, sage and orange peel are important sources of essential oil, the richest being orange peel.

The antimicrobial activity of these oils was found to be weak for useful lactic acid bacteria of the *Lactobacillus* genus, which may open new perspectives for their use in the food industry without inhibiting useful bacteria in fermentative processes.

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