

**Investigation of antimicrobial activities of some herbs containing essential oils
and their mouthwash formulations**

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INTRODUCTION: The aim of this study was to prepare pharmaceutical formulations of mouthwashes and examine the antimicrobial activities of essential oils obtained from plants used traditionally in Turkey for oral infections.

METHODS: Essential oils were obtained from herbal drugs with water distillation method using of Clevenger apparatus. Antimicrobial capacities of mouthwash formulations containing mixture of essential oils with proportions of 4.5 % and 9.0 % were examined by disc diffusion method and microbroth dilution method.

RESULTS: In study, antimicrobial activity was evaluated by disc diffusion method and microbroth dilution method. The inhibition zone diameters were determined to vary between 7-59 mm. It has been observed that the static and cidal activity was generally 50% and greater than 50%, when pure essential oil samples were applied

on microorganism specimens. Formulation F2 containing mixture of essential oils with proportions of 4.5%, showed 6.25 % minimum bactericidal effect on *Staphylococcus aureus* ATCC 25923 and 3.125 % the minimum inhibitor concentration and minimum bactericidal concentration on all other microorganisms. It is found that the antimicrobial effect of pure essential oil samples applied on microorganisms, was lower than mouthwashes formulations; the antimicrobial effect of F2 formulation containing mixture of essential oils with proportions of 4.5% was higher than formulation F1 containing mixture of essential oils with proportions of 9%.

DISCUSSION AND CONCLUSION: The results obtained by these methods allow us to conclude that the essential oils and prepared F1 and F2 mouthwash formulations exert activity against microorganisms affecting the oral cavity. It has also been reported that the F2 formulation has significantly antimicrobial activity on the tested microorganisms.

Keywords: Antimicrobial activity, *Laurus nobilis* L., *Origanum vulgare* L. ssp. *hirtum*, *Rosmarinus officinalis* L., *Salvia fruticosa* Mill.

Uçucu yağ içeren bazı bitkilerin ve gargara formülasyonlarının antimikrobiyal aktivitelerinin araştırılması

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GİRİŞ ve AMAÇ: Bu çalışmanın amacı; Türkiye'de halk arasında yaygın olarak kullanılan bitkilerden elde edilen uçucu yağların antimikrobiyal etkilerini araştırmak ve bu etkilerden yola çıkarak ağız enfeksiyonlarında kullanılmak üzere gargara formülasyonu hazırlamaktır.

YÖNTEM ve GEREÇLER: Uçucu yağlar, bitkilerden (*Origanum vulgare* L. ssp. *hirtum*, *Rosmarinus officinalis* L., *Laurus nobilis* L., *Salvia fruticosa* Mill.) su distilasyonu metodu ile Clevenger aparatı kullanılarak elde edilmiştir. Elde edilen uçucu yağlar ve % 4.5 ve % 9.0 oranlarında uçucu yağların karışımını içeren gargara formülasyonlarının antimikrobiyal aktiviteleri disk difüzyon metodu ve mikrobroth dilüsyon metodu ile araştırılmıştır.

BULGULAR: Bu çalışmada antimikrobiyal aktivite disk difüzyon metodu ve mikrobroth dilüsyon metoduyla değerlendirilmiştir. İnhibisyon zon çaplarının 7-59 mm arasında değişkenlik gösterdiği belirlenmiştir. Uçucu yağlar, mikroorganizmalar üzerine saf olarak uygulandığında statik ve sidal etkinliğin genellikle % 50 ve % 50' nin üzerinde olduğu gözlenmiştir. % 4.5 oranında uçucu yağ karışımı içeren F2 formülasyonunun *Staphylococcus aureus* ATCC 25923 üzerindeki minimum bakterisidal etkisinin % 6.25 olduğu ve diğer tüm mikroorganizmalar üzerinde

minimum inhibitör konsantrasyon ve minimum bakterisidal konsantrasyonunun % 3.125 olduđu bulunmuştur. Uçucu yağ örneklerinin mikroorganizmalar üzerine saf olarak uygulandığında gösterdikleri antimikrobiyal etkinin, gargara formülasyonlarına göre daha düşük olduđu; ayrıca % 4.5 oranında uçucu yağ karışımı içeren F2 formülasyonunun, % 9.0 oranında uçucu yağ karışımı içeren F1 formülasyonuna göre test edilen tüm mikroorganizmalar üzerine daha yüksek antimikrobiyal etkiye sahip olduđu bulunmuştur.

TARTIŞMA ve SONUÇ: Elde edilen sonuçlar uçucu yağların karışımı ile hazırlanan F1 ve F2 formülasyonlarının, test edilen mikroorganizmalara karşı antimikrobiyal etkiye sahip olduğunu ortaya koymuştur. Ayrıca çalışma sonucunda F2 formülasyonunun test edilen mikroorganizmalar üzerinde önemli oranda antimikrobiyal aktiviteye sahip olduđu rapor edilmiştir.

Anahtar Kelimeler: Antimikrobiyal aktivite, *Laurus nobilis* L., *Origanum vulgare* L. ssp. *hirtum*, *Rosmarinus officinalis* L., *Salvia fruticosa* Mill.

INTRODUCTION

Diseases that affect the buccal cavity and teeth are a public health concern nowadays. Mouth bacteria have been linked to plaque, tooth decay and toothache. Plaque which is a layer that forms on the surface of a tooth, principally at its neck; composed of bacteria in an organic matrix has been linked to gingivitis, periodontal disease, or dental carries (1). Patient compliance and acceptance is extremely important for oral topical products. Ointments, creams and some emulsions are rarely used for oral topical treatment while the patients have lower acceptance for application of ointments in mouth (2). Nowadays mouthwash is one of the oral formulations that are available in the market. A mouthwash is identified as a non-sterile liquid solution used mostly for its deodorant, refreshing or antiseptic action and also these rinses are planned to decrease oral bacteria, eliminate food particles, temporarily decrease bad breath and offer a pleasant taste (3). Mouthwashes are very useful in reduction of microbial plaques (4). It is important to make sure the aqueous mouthwashes formulated provide a comfortable feeling in the mouth during using, and it must have a pleasant flavor to obtain the consumer acceptance.

Plants have been used for centuries in herbal tea preparation, as spices and for therapeutic purposes in the world (5). The number of plants used for therapeutic purposes and spices is reported to be around 20 000. It is estimated that the number of plant species used for medical purposes in the world is 350 000 and 5% of these species are formed by aromatic plants (6, 7). Herbal products have recently experienced more thorough investigation for their potential in preventing oral illnesses, particularly plaque-related diseases, such as dental caries (8). Natural substances obtained from medicinal plants and used in the alternative medicine were reported to possess antibacterial activity. The development of antibiotic resistant

strains in recent years has become a serious health problem. In this direction, the antimicrobial effects of plant extracts and essential oils obtained from various parts of the plant against bacteria and fungus became important (5, 9). Researchers are trying to pay more attention to these natural products aiming to find an effective antimicrobial mouthwash having the advantage of decreasing the side effects of synthetic one. The use of natural antimicrobials may conduce to control the disordered growth of oral microbiota, thus overwhelming problems caused by species resistant to conventional antimicrobials (10, 11). Natural materials have proved antibacterial action mainly because most plants used in alternative medicine are composed of flavonoids, which act on bacterial cells disrupting the cytoplasmic membrane and inhibiting the enzymatic activity (12). **Hata! Başvuru kaynağı bulunamadı.**

Turkey is a Mediterranean country rich in medicinal and aromatic plants. Most of these are used in the local folk tradition for many purposes (13). *Laurus nobilis* L. is a plant belonging to the Lauraceae family, which comprises approximately 2500 species. The genus *Laurus* is found in Europe and consists of the two species *Laurus azorica* and *Laurus nobilis*. Leaves of the plant which are not shed during winter, are 5-10 cm long, 2-5 cm wide, and green in color. The fruits are small and olive-like (14,15). The antimicrobial, analgesic, anti-inflammatory, acetylcholine esterase inhibiting properties of the essential oil of *Laurus nobilis* L. have been reported (15,16).

There are four reports on the essential oil content and composition of *Origanum vulgare* L, ssp. *hirtum* of Turkish origin. This plant is known in Turkey as “Istanbul kekiği” and is widely used as thyme in Marmara and Thrace regions (17,18).

Rosemary (*Rosmarinus officinalis* L.), belonging to the Lamiaceae family, is a pleasant smelling perennial shrub that grows in several regions all over the world. It is known as 'Biberiye, Kuşdili' in Turkey (13,19). Sage is one of the most valued herb known for its essential oil richness and its plethora of biologically active compounds extensively used in folk medicine. Sages are cultivated in many countries. The garden sage (*Salvia officinalis* L.) is grown in Canada, USA, Spain, Italy, Greece, Albania, Germany, France, Turkey and England (20).

Essential oils are volatile and oily mixtures with a strong odor, consisting of a large number of chemical compounds which gives off the characteristic odor and color of the plant, which can be obtained from aromatic plants or from various herbal sources by methods such as water or hydro distillation. Essential oils can be found in different parts such as root, stem, leaf, fruit, seed, wood, crust, bud, rhizome and flower. Also they are found in particular secretory canals such as secretory trichome, secreting vesicle, or parenchyma and epidermal cells, depending on the family in plants (5, 21, 22).

Essential oils are called 'essential oils' because they are fragrant and 'volatile oil' and 'etheric oil' because they are volatile and easily evaporate (6, 21). The term 'essential oil' was derived from effective ingredient of the drug 'Quinta essentia', named by Paracelsus von Hohenheim in the 16th century (23). Aromatic materials have been used scientifically and commercially for many purposes such as especially cosmetics, medicine, food industry, perfumery, aromatherapy and phytotherapy for many years (21). Essential oils contain terpenic hydrocarbons and their oxygenated derivatives, as well as a little amount volatile aliphatic hydrocarbons and mixtures of aromatic substances derived from phenylpropene. They are consisting of a lot of compound, which have different structures contained different functional groups.

These functional groups also determine the characteristic chemical properties of volatile oil. Essential oils contain phenylpropene as well as terpenes. Terpenes are present in much greater amounts than the phenylpropenes, while phenylpropenes are responsible for odor and taste of essential oil (6, 7).

Hence, the purpose of the present study was to prepare and evaluate antimicrobial activities of mouthwashes.

EXPERIMENTAL

The plant materials were provided from the market and identified in Pharmaceutical Botanical Department, Istanbul University Faculty of Pharmacy. Plant materials were determined as *Origanum vulgare* L. ssp. *hirtum*, *Laurus nobilis* L., *Rosmarinus officinalis* L., *Salvia fruticosa* Mill. Voucher specimens were kept in Medipol University.

In this study sodium chloride, sodium bicarbonate, sodium saccharin, ethanol was purchased from Sigma Aldrich Company (Germany).

Obtaining essential oil

Plant materials were hydro distilled for 4 hours using a Clevenger apparatus. The temperature of the heater was set at 100 ± 2 °C. The obtained essential oils were dried over anhydrous sodium sulfate and stored at 4 °C (24). Plant materials and plant registration numbers were shown at Table 1.

Preparation of mouthwashes

The mouthwash was prepared according to Table 2. Mouthwash solutions were formulated 4.5-9 % of essential oil, 1-2 % of *Origanum vulgare* L. ssp. *hirtum* and *Salvia fruticosa* Mill, 2-4 % of *Rosmarinus officinalis* L. and 0.5-1 % of *Laurus nobilis* L. Ethanol, sodium chloride and sodium bicarbonate were also added to formulations. Saccharine sodium was used as sweetener. Essential oils were weighed and dissolved in a part of the ethanol and the other ingredients were added gradually with the aid of a mechanical stirrer 500 rpm for 30 minutes. The mixture was filtered and the filtrate volume was made up to 10 mL by distilled water. No preservative was necessary to be added due to the high content of ethanol (> 15 %) in the formulations (25).

Determination of pH

The pH of the mouthwashes was determined using calibrated pH meter (Mettler Toledo, Switzerland). Determinations were carried out three times and an average of these determinations was taken as the pH of the prepared mouthwashes.

Determination of antimicrobial activity of essential oils and mouthwash formulations

Kirby-Bauer disc diffusion method

Kirby - Bauer disc diffusion method was used to determine the antimicrobial susceptibilities of microorganisms to essential oils. Antimicrobial activities of sage, rosemary, bay and thyme essential oils were determined against various microorganisms (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis*

ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* DSM 4312, *Escherichia coli* ATCC 25922, and *Candida albicans* ATCC 10231). For this purpose, the bacteria were incubated in Brain Heart Infusion Agar (BHI) medium at 37°C, for 24 hours. *Candida albicans* was incubated in Sabouraud Dextrose Agar (SDA) medium at 30°C, for 48 hours. After incubation, the microorganisms were adjusted to 0.5 McFarland turbidity standard (10^8 CFU / mL) in 0.85 % physiological saline. The prepared microbial suspension was seeded on the Mueller Hinton Agar (MHA) medium with a swab. Petri dishes were allowed to stand for 15 minutes to dry. At the end of the period, aseptically, taking discs prepared from Whatman 42 number filter paper with the help of a pen, 10 μ L of essential oil was dropped onto the disks and the disks were placed on the petri dishes. After placement of the disks, the petri dishes were allowed to stand for 15 minutes, the bacterial specimens were incubated for 24 hours, and the yeast specimens were incubated for 48 hours. After the incubation, the zone diameters around the discs were recorded with the scale and the results were evaluated. The experiment was carried out in double parallel (11).

Microbroth dilution method

Microbroth dilution method was applied to determine minimum inhibitor concentrations (MIC) and minimum bactericidal concentrations (MBC) of F1 and F2 mouthwashes and essential oil samples. For this purpose, 100 μ L double-strength Mueller Hinton Broth (MHB) medium for antibacterial activity and SDA medium for antifungal activity were added 100 μ L to each well of a 96-well plate. 100 μ L of the essential oil samples and formulation samples were added and 1/2 dilutions were

made. Subsequently, bacterial specimens (*Staphylococcus aureus* ATCC 25923, *Salmonella typhi* ATCC 14028, *Escherichia coli* ATCC 25922) incubated in BHI medium at 37°C, for 24 hours and yeast specimen (*Candida albicans* ATCC 10231) incubated in Sabouraud Dextrose Broth (SDB) medium at 30°C, for 48 hours, were adjusted to a 0.5 McFarland turbidity standard (10^8 CFU / mL) in the 0.85% physiological saline. Microorganism samples adjusted the McFarland turbidity was added to the 100 μ L. The wells only contain the medium were used as a negative control, while the wells contain the microorganisms and the medium were used as positive controls. After incubation, minimum inhibition concentrations and minimum cidal concentrations were determined and were recorded. The experiment was carried out in double parallel (12).

RESULTS AND DISCUSSION

In current study the *Origanum vulgare* L. ssp. *hirtum*, *Laurus nobilis* L., *Rosmarinus officinalis* L. and *Salvia fruticosa* Mill. essential oils were collected (Table 1) and the mouthwash formulations were prepared according to Table 2. There are studies showing antimicrobial activities of volatile oil obtained from various plants and spices (26). Since essential oils contain different components, the antibacterial effect ratings vary depending on the variety and amount of the compounds. Essential oils have antimicrobial effects on various Gram (+) and Gram (-) bacteria and many other microorganisms. Carvacrol and thymol provide to move out of the cell the membrane-related substances by breaking down the bacterial membrane; terpenoids and phenylpropanoids have been reported to reach more internal parts of the cell by penetrating the bacterial wall due to their lipophilic nature. It is known that plant extracts and essential oils have antimicrobial effects on Gram (+) and Gram (-) bacteria, as well as against various fungi (27).

In a study, Al-Howiriny has extracted the essential oil of the *Salvia lanigera* and has reported that it has a good inhibitory effect against *Mycobacterium smegmatis*, *Candida albicans*, and *Candida vaginalis* (27). Holley and Patel showed that essential oils obtained from *Coriandrum sativum*, *Cinnamomum zeylanicum*, *Cymbopogon citratus*, *Satureja montana* (Coriander, cinnamon, lemon grass, geysey) are effective against *Aspegillus niger*, *Candida albicans*, *Rhizopus oligosporus* and showed that essential oils obtained from *Thymus vulgaris*, *Pimpinella anisum*, *Cinnamomum zeylanicum* plants (thyme, anise and cinnamon) have fungicidal activity against *Aspergillus flavus*, *Aspargillus parasiticus*, *Aspergillus ochraceus*, and *Fusarium moniliforme*. Also, they showed that thyme essential oil is fungi toxic. This effect is thought to be due to the hydrogen bonds formed between

the hydroxyl groups of phenolic compounds in the volatile oil composition and the active part of the target enzymes (28). Another study shown that the essential oil obtained from *Rosmarinus officinalis* are effective against *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, and *Pseudomonas aeruginosa* (29).

For many centuries, plants have been used to provide food flavor and aroma to extend the shelf life of foods and to treat especially diseases. The antimicrobial effects of plants used for many years as traditional have been investigated from the beginning of the 20th century. Due to the increase in antibiotic resistant infections in recent years, there is a growing interest in natural compounds and essential oils obtained from plants in particular (7, 30). In this study the *Origanum vulgare* L. ssp. *hirtum*, *Laurus nobilis* L., *Rosmarinus officinalis* L. and *Salvia fruticosa* Mill. essential oils were collected and used for preparing mouthwashes formulations. Flavors are added to the formulas to improve the consumer acceptability of the mouthwash ingredients. In this study saccharine sodium was used as sweetener. Sodium bicarbonate was used at F1 and F2 formulations. Several studies have shown that bicarbonate is one of the salivary components that potentially modify the formation of caries. It increases the pH in saliva, and in this way, creates a hostile environment for the growth of aciduric bacteria. Sodium bicarbonate can also change the virulence of the bacteria that cause tooth decay. Animal studies have shown that dentifrices containing sodium bicarbonate reduce the amounts of both *Streptococcus sobrinus* and *Streptococcus mutans* and this may reduce caries. Studies on human show statistically reduction in number of mutants streptococci. Sodium bicarbonate can also prevent caries by reducing enamel solubility and increase remineralization of enamel (31).

Antimicrobial activities of essential oil samples were determined by disc diffusion method. Furthermore antimicrobial activities of F1 and F2 mouthwashes and essential oil samples were determined by microbroth dilution method. By disc diffusion method, antimicrobial activities of tested samples against various microorganisms (*Staphylococcus aureus* ATCC 25923, *Streptococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* DSM 4312, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231) are given at Table 3. According to these results, it has been determined that the diameters of the zones vary between 7-59 mm (Table 3).

Bacillus sp., *Enterococcus* sp., *Salmonella* sp., *Staphylococcus* sp., *Streptococcus* sp., and *Candida* sp. are found in the oral microbiome. Therefore, we worked with these bacteria in our study (32, 33).

It has been shown that *Origanum vulgare* L. ssp. *hirtum* essential oil has the highest inhibition zone (59 mm) on *Candida albicans* ATCC 10231 and *Salvia fruticosa* Miller essential oil has the lowest inhibition zone (7 mm) on *Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* DSM 4312; *Rosmarinus officinalis* essential oil has the lowest inhibition zone (7 mm) on *Staphylococcus aureus* ATCC 25923. As a result of the study, it has determined that essential oil obtained from *Origanum vulgare* subsp. *hirtum* shown highest zone diameter on the tested microorganisms (Table 3).

Antimicrobial activity results determined by microbroth dilution method against various microorganisms (*Staphylococcus aureus* ATCC 25923, *Salmonella typhi* ATCC 14028, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231) are given at Table 4. According to these results; it has been observed that the static and cidal activity is generally 50 % and greater than 50 %, when pure essential oil

samples are applied on microorganism specimens. Minimum inhibitor concentration of essential oil *Salvia fruticosa* Mill. was registered as 6.25% on *Escherichia coli* ATCC 25922 and *Salmonella typhi* ATCC 14028.

Formulation F1 contained 9.0 % of essential oil was observed that the minimum inhibitor concentration and bactericidal concentration were 25% on *Escherichia coli* ATCC 25922, and *Salmonella typhi* ATCC 14028; was 50 % on *Staphylococcus aureus* ATCC 25923 and was over 50 % on *Candida albicans* ATCC 10231. Formulation F2 contained 4.5 % of essential oil in its composition, has been found that was 6.25 % the minimum bactericidal effect on *Staphylococcus aureus* ATCC 25923 and were 3.125 % the minimum inhibitor concentration and minimum bactericidal concentration on all other microorganisms. It has been determined that the solvent formulation does not exhibit antimicrobial activity (Table 4).

In this study, it has investigated antimicrobial effects by disc diffusion method on various microorganisms (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC DSM 4312, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231) of essential oils obtained from *Origanum vulgare* L. ssp. *hirtum*, *Salvia fruticosa*, *Rosmarinus officinalis*, *Laurus nobilis* plants by water distillation method. According to these results, most of the tested plant materials were observed to have antimicrobial activity against microorganism. It has been reported that the highest antimicrobial activity is against *Candida albicans* ATCC 10231 strains of *Origanum vulgare* subsp. *hirtum* essential oil. In addition, it has also been reported that the lowest antimicrobial activity of *Salvia fruticosa* essential oil is against *Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* DSM 4312 bacteria; that the lowest antimicrobial activity of *Rosmarinus officinalis* essential oil is against *Staphylococcus aureus* ATCC 25923 bacteria. As a result of this study, *Origanum vulgare* subsp. *hirtum* essential oil has been detected the strongest antimicrobial activity against the tested microorganisms.

According to the results of the microbroth dilution test, it has been observed that essential oil samples showed antimicrobial activity at a certain rate against the tested microorganisms (*Staphylococcus aureus* ATCC 25923, *Salmonella typhi* ATCC 14028, *Escherichia coli* ATCC 25922, and *Candida albicans* ATCC 10231). The antimicrobial effect that essential oils show separately on microorganisms is less effective than mouthwash formulation. The antimicrobial effect of pure essential oil samples applied on microorganisms, was lower than mouthwashes formulations; the antimicrobial effect of F2 formulation containing mixture of essential oils with

proportions of 4.5% was higher than formulation F1 containing mixture of essential oils with proportions of 9%.

Conclusion

In this study, essential oils and essential oil containing mouthwashes have been successfully prepared. The results obtained by these methods allow us to conclude that the essential oils and prepared F1 and F2 mouthwash formulations exert activity against tested microorganisms affecting the oral cavity. It was also concluded that static and cidal activity on the microorganisms of the F2 formulation had markedly higher than the F1 formulation. It has been observed that the static and cidal activity is generally 50% and greater than 50%, when pure essential oil samples are applied on microorganism specimens. Formulation F2 contained 4.5% of essential oil has been found 6.25% the minimum bactericidal effect on *Staphylococcus aureus* ATCC 25923 and 3.125% the minimum inhibitor concentration and minimum bactericidal concentration on all other microorganisms.

F2 formulation contains lower essential oil than F1 formulation, antibacterial and antifungal effect on the microorganisms of the F2 formulation is markedly higher than F1 formulation.

The pH of a formulation is important for patient compliance. The pH of the prepared mouthwashes ranged between 7.37 and 7.63. The pH of the formulations was appropriate for mucosal delivery since they were iso-hydric. This indicated the nonirritancy of the formulation in oral mucosa.

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Declaration of interest

The authors declare no conflict of interest.

Uncorrected proof

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