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Antibacterial Activity of *Melissa officinalis*, *Hypericum perforatum*, *Sideritis amasiaca and Echinacea sp.* Extracts against Some Nosocomial Pathogens

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Abstract: ***New plant-derived antibacterial agent researches have increased in recent years, because of increased resistance to antimicrobial agents that used against to pathogen microorganisms, and unwanted side effects of food additives. In this study we aimed to determine in vitro antibacterial activity of the extracts of M. officinalis L., S. amasiaca, H. perforatum and Echinacea sp. on some nosocomial bacterial agents. Disc diffusion and broth microdilution tests were used to determine the antibacterial activity of plant extracts on E. coli, K.pneumoniae, P. aeruginosa, methicillin resistance S.aureus (MRSA) and E. faecalis. While, in the disc diffusion test; M. officinalis L. and H. perforatum (8mg/disc) had showed antibacterial activity to all bacteria, S. amasiaca (8mg/disc) had showed antibacterial activity all bacteria exception of E.faecalis, Echinacea sp. (8mg/disc) had showed antibacterial activity only P.aureginosa. MRSA showed the highest sensitivity to all plant extracts with the exception of Echinacea. While, in the broth microdilution test; the values of the minimum inhibitory concentration were between 100 to 1,56 mg/ml for the four plant extracts (200mg/ml). It was observed that all plant extracts caused an inhibitory effect on all the bacteria in different levels (1,56-25mg/ml). In addition, extract of Sideritis amasiaca L. whose antibacterial activity was for the first time investigated and observed against some nosocomial infections agents.***

Keywords*:* Antibacterial resistance, Broth microdilution, Disc diffusion, *E.coli, S.aureus.*

1. Introduction

The natural plants and their volatile oils have been studied. Particularly since 1940, significant results were obtained in many studies in terms of their antimicrobial activity, as they are the raw materials used in many branches of industry such as pharmaceutical, food, perfumes and cosmetics. Plants having antimicrobial activity are used to control weeds and plant pests. They are also used for medical purposes such as anti-bacterial, anti-fungal, anti-helminthic and as preservatives in foods [1].

Turkey is rich in flora structure and has nine thousand species of plants that grow naturally. However, it is estimated that only a thousand plants are used in folk medicine. In our country as well as worldwide, there is an increasing and intense interest regarding the acquisition of antimicrobial and antioxidant agents from plant materials rather than synthetic antioxidants and protective agents. Antimicrobial compounds are generally found in the essential fatty part of the plants [1].

Antimicrobial activity depends on the plant type, composition and concentration of the target microorganism species, the food composition and storage conditions. Proteins, lipids, salts, pH, and temperature are factors that affect the antimicrobial activity of phenolic compounds [2]. Studies conducted on this subject revealed that the plant materials have numerous phytochemical compounds with the capability for powerful antioxidant and antimicrobial activity [3]. Until recently, many studies have been conducted on the antimicrobial and antioxidant activities of plant essential oils [4, 5, and 6].

The bee balm, namely sweet balsam or melissa (*Melissa officinalis* L.), which originates from the *Lamiaceae* family and includes 45 kinds and 546 species in Turkey, is one of the most important medicinal plant species. *Melissa* which is one of the most important medicinal plants, grows wildly particularly in Turkey in the Mediterranean region. This plant also grows in Southern Europe, North Africa, in the eastern Caucasus and northern Iran [7, 8].

Melissa, as a traditional medicinal plant has been used since ancient times, and continues to be used globally in pharmaceutical, cosmetic and food industries [9]. Another medicinal plant "*Hypericum perforatum* L. type" known in Turkey as Tipton's weed belongs to the *Clusiacea* (*Guttifera A=Hypericaceae*) family and generally grows in Europe, Asia, North Africa and America. Currently, extracts of this plant species are mostly used in antidepressants. In addition, it is known that the extract of this plant is useful against liver hypertrophy, lung infection and scarring. The various extracts of this plant have been proven to possess antimicrobial activity. In recent literature, it was reported that the alcoholic extracts (methanolic/ethanol) of this plant have higher antibacterial activity in gram-positive bacteria compared to gram-negative bacteria [4, 10].

The plants in the *Lamiaceae* genus- which also includes the *Sideritis* species- have flavones, essential oil and various chemicals in their contents. The essential oils and certain ferments found in their structure, prevents the development of bacteria causing them to be natural food-preservers [1].

Sideritis L. (*Lamiaceae*) mainly grows in the Mediterranean basin and includes more than 150 species. There are 25 species belonging to 31 *Sideritis* taxa that are endemic to Turkey and some species are exported. *Sideritis* genus is

important, because its endemism rate is higher compared to other aromatic plants and all types are consumed as tea by the public [11].

The plant commonly known as purple coneflowers are classified in *Asteraceae* genus and include the species belonging to the genus *Echinacea* [12]. The *Echinacea* plant is known to increases the number of our white blood cells that fight against infection, thus strengthening our defense system, and has been used for medical purposes both in the past and in the present day [13].

Echinacea, known by its powerful antibacterial and antiviral properties, originates in North America. Today, it has spread to South America, Canada, Europe, Russia, Africa and the Pacific. It has been reported that said plant constitutes 10% of Medicinal plants in the United States [14].

In this study, the antibacterial activity and minimum inhibitory concentrations of extracts from *Melissa officinalis*, *Hypericum perforatum*, *Sideritis amasiaca and Echinacea sp.* on the *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and MRSA were investigated in vitro using both the disc diffusion and broth microdilution tests.

2. Materials and Methods

Plant material

The *Melissa officinalis*, *Hypericum perforatum*, *Sideritis amasiaca and Echinacea sp.* medicinal plants used in this study were either collected from the wild or were cultivated on herb farms in Turkey.

Extraction

Plants were properly dried and pulverized into a coarse powder. All dry samples were extracted using methanol [15]. After being concentrated, stock solutions of extracts (200 mg/ml) were obtained by dissolving a certain amount of crude extract in the solvent dimethyl sulfoxide (DMSO-Sigma). The liquid plant extracts diluted with 5% DMSO were sterilized with 0.45 µM filters.

Bacterial strain

Gram negative *Escherichia coli* (ATCC 35218), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853) and gram positive *Enterococcus faecalis* (ATCC 29212), methicillin - resistant *Staphylococcus aureus* (MRSA) (clinical isolate) which cause nosocomial infection were used to investigate the antibacterial activity of plant extracts. All bacteria were incubated for 24 h at 37 °C in nutrient broth plates prior to testing for antibacterial activity, then bacteria were inoculated on nutrient agar plates and incubated at 24 h at 37 °C.

Bacterial susceptibility tests

The antibacterial activity of the sterilized plant extracts on the bacteria involved in nosocomial infections such as *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *E. faecalis* and MRSA were investigated using the disc diffusion and broth microdilution method [16, 17].

In the disc diffusion method, the bacterial suspensions whose turbidity was adjusted to McFarland 0.5 (~10^8 kob/ml) in Mueller-Hinton Broth, were spread over a Mueller-Hinton agar surface. In the following phase, the discs containing 40 µl of plant extracts were dried at 30 °C and placed on an agar surface by sterile forceps. Gentamicin (10 µg/disc, Bioanalyse) discs were used as reference antibiotics and 5% DMSO were used negative control. The medium was incubated at 37 °C for 24 hours and in the end, the zones of inhibition greater than 7 mm was recorded. The experiments were repeated three times, and the mean and standard deviation were calculated.

In the broth microdilution method, 100 µl cation adjusted Mueller-Hinton Broth (BBL) was placed into all the wells of 96-well microplates. Then, 100 µl of the plant extract in the concentration of 200 mg/ml was added to the first row of wells. Each sample was studied three times. Two-fold serial dilutions were made and the concentrations were obtained between 100 mg/ml to 1,6 mg/ml. 10 µl of bacterial suspensions of $5x10^6$ kob/ml was then added to all wells. A well without a plant extract was reserved for growth control of each bacteria and another well without containing bacteria or a plant extract was reserved for sterility control, and gentamicin (40 µg/ml) (Sigma) was used as reference antibiotic. Microplates were incubated for 20-24 hours at 37 °C. At the end of incubation, the last dilution wells with an absence of visible turbidity were determined as the MIC value. In addition, 50 µl of %1 2,3,5-triphenyl-tetrazolium chloride (TTC, Sigma) was added to each well, the plates were incubated for 30 min at 37 °C and the MIC values were verified depending on the occurrence of color change [18,19].

Statistical Analysis

Disc diffusion results were analyzed by one-way analysis of variance (ANOVA) to determine differences at the $p < 0.05$ significance level. Statistical analysis was performed using Minitab 16 Statistical Software package (Minitab Inc. State College, PA).

3. Results and Discussion

The level of antibacterial activity exhibited by any plant material depends on many factors, including the plant part, geographical source, soil conditions, harvest time, moisture content, drying method, storage conditions, and post-harvest processing. The relatively high temperatures that can be generated during tissue grinding can denature chemical constituents and the extraction solvent, time period, and temperature can affect the level and composition of secondary metabolites extracted from plant tissues [20].

The four plants chosen for this study are commonly used for the treatment of infectious diseases in traditional herbal therapy and are reported to produce a wide range of antimicrobials compounds ([1, 20, 21]. In this study, it can be said that disc diffusion and MIC tests showed compatible results on antibacterial activity. In the present study, when disc diffusion (8mg/disc) and MIC (200 mg/ml) tests are evaluated together, MRSA which is a gram positive bacteria had highest antibacterial susceptibility for the *Melissa officinalis* L., *Sideritis amasiaca* L., *Hypericum perforatum*

L. plants extracts used against five different bacteria. *P.aeuroginosa* had highest antibacterial susceptibility for *Echinacea sp.* plant extract.

For the disc diffusion method, the inhibition zone diameter of five bacteria species exposed to four different plant extracts are indicated in table 1. It was determined that the *Echinacea sp.* plant extract (8mg/disc) did not display antibacterial activity exception *P.aeruginosa and S. amasiaca* (8mg/disc) did not display antibacterial activity against *E.faecalis*. However, the other plant extracts showed activity in different values against five bacterial strains. In addition, the highest inhibition zone diameters were observed against MRSA.

Table 1: Antibacterial activity of plants extracts according to the inhibition zones

Plants Extracts (8 mg/disc)	Antibacterial activity inhibition zone diameters (mm)				
	E. coli	***P. aeruginosa***	***K. pneumoniae***	***MRSA***	***E. faecalis***
Melissa officinalis	12 ± 1^{Aab}	$11\pm0,577^{Aab}$	$10\pm2,51^{ABb}$	$14\pm1,00^{Ba}$	$12\pm0,577^{Bab}$
Hypericum perforatum	$11\pm2,51^{Ab}$	$10\pm0,577^{ABb}$	$9\pm0,00^{Bb}$	$15\pm0,577^{Ba}$	$14\pm,00^{Aa}$
Sideritis amasiaca	$14\pm0,00^{Ab}$	$12\pm1,00^{Ac}$	$14\pm0,577^{Abc}$	$16\pm0,577^{Aa}$	R
Echinacea sp.	R	$9\pm0,577^{B}$	R	R	R
Gentamicin (10 µg/disc)	20±0,577	19±2,00	19±0,577	18±0,577	16±0,00

R: Resistant Values; mean ± standard deviation, A, AB, B: Differences between the groups in the same column, a, b, c: Differences between the groups on the same line, $p<0.05$ accepted as significant.

When the effect of plant extracts (8 mg/disc) against each bacteria compared, we found significant differences ($p <0.05$) between the groups. According to the results obtained from the study, *H. perforatum, M. officinalis and S. amasiaca* showed the highest antibacterial activity against *E.coli* (*f 3,45/p 0,1*). Moreover *M. officinalis and S. amasiaca* showed the highest antibacterial activity against *K.pneumoniae (f 12,61/p 0,002)* in the study. Also *S.amasiaca* showed the highest antibacterial activity against MRSA *(F 45,80/P 0,000)* and *H. perforatum* showed the highest antibacterial activity against *E.faecalis* (*F 25/P 0,007)* in this study.

When the effect of each chemical on bacteria is compared; differences between groups were significant at $p <0.05$ level. According to the results obtained from this study, we determined the statistically highest activity of *M. officinalis* and *S. amasiaca* against *MRSA (*respectively f 4,70/p 0,022-f 87,67/p 0,000*), H.perforatum* against *MRSA* and *E.faecalis* (f 16,83/p 0,000), *Echinacea sp*. against *P. aeruginosa*.

For the broth microdilution method, the MIC values of four plants extracts (200mg/ml) against five different bacteria are indicated in Table 2. It was determined that the MIC value of *Echinacea sp.* plant extract was 6,25-25 mg/ml against five different bacteria; other plants extracts were lower than 25 mg/ml especially against MRSA. The different results obtained in the two tests are related to the applied extraction doses. When the doses were equal in the two test, antibacterial activity was determined to be the same. Thus, it was seen that the two methods support each other.

Table 2: Antibacterial activity of plants extracts according to the minimum inhibitor concentrations

	Minimum inhibitory concentration (MIC) values (mg/ml)				
Plants Extracts (200mg/ml)	*E. coli*	*P.aeruginosa*	*K.pneumoniae*	*MRSA*	*E.faecalis*
Melissa officinalis	6,25	6,25	6,25	3,12	6,25
Hypericum perforatum	6,25	6,25	6,25	3,12	1,56
Sideritis amasiaca	6,25	6,25	6,25	3,12	12,5
Echinacea sp.	12,5	6,25	12,5	12,5	25
Gentamicin (40 µg/ml)	0,0025	0,005	0,0025	0,005	0,005

According to the data obtained from this study, the extracts of four plants showed activity at various levels against five different pathogenic bacteria which are the important factors of nosocomial infection.

Melissa officinalis L. extract was showed antibacterial activity against all bacteria in the disc diffusion test in different inhibition zones diameter. The highest inhibition zone diameter of 14 mm was recorded for MRSA. The MIC of this plant extract was 6,25 mg/ml against *E.coli, P. aeruginosa, K. pneumonia, E. faecalis* and 3,12 mg/ml against MRSA. Stefanović and Comic [22] has been reported that MIC values of different *M. officinalis* extracts were in the range of 20 to 40 mg/ml against *P. aeruginosa* and *E.coli*, 5 to 10 mg/ml against *K. pneumoniae* and *S. aureus*, respectively. The disc diffusion and MIC tests findings obtained for other bacteria with the exception *of K. pneumoniae* has been found in almost the same values for both the specified study and present study. In accordance with the present study's data, Abu-Shanab *et al.* (23) reported that ethanol extract of *M. officinalis* was effective on MRSA and MIC values were in the range of 3,125 to 12,50 mg/ml.

Hypericum perforatum L. extract was showed antibacterial activity against all bacteria in the disc diffusion test in different inhibition zones diameter. The highest inhibition zone diameter of 15 mm was recorded for MRSA in the disc diffusion test. The MIC of this plant extract was 6,25 mg/ml against *E.coli, P. aeruginosa* and *K. pneumonia*, 3,12 mg/ml against MRSA and 1,6 mg/ml against *E. faecalis*. In accordance with the present study's data, Duman and Sevimli [21] reported that *H. perforatum* extract did not show activity against *E.coli, P. aeruginosa* and *K. pneumoniae* which are gram negative bacteria but an inhibition zone diameter of 9-10 mm was recorded for *S. aureus* in the disc diffusion test. Meral and Karabay [24] reported that methanol extract of *H. perforatum* L. had broad

spectrum antibacterial activity against *S. aureus* and *E. faecalis* which are gram positive bacteria; against *E.coli, P. aeruginosa* and *K. pneumoniae* which are gram negative bacteria. As determined in the present study, Conforti *et al.* [25] reported that methanol extract of *H. perforatum* L. had antibacterial activity against generally gram positive bacteria.

Sideritis amasiaca L. extract whose antibacterial activity was for the first time investigated with this study, was showed activity against all bacteria except *E. faecalis* in different inhibition zone diameter. Highest was recorded to have an inhibition zone diameter of 16 mm for MRSA in disc diffusion test. The MIC of this plant extract was 6,25 mg/ml against *E. coli, P. aeruginosa* and *K. pneumonia*, 3,12 mg/ml against MRSA and 12.5 mg/ml against *E. faecalis*. Thus, it can be said that *Sideritis amasiaca* L. extract showed antibacterial activity in different variations among bacteria treated for both test types. This plant extract very effective for MRSA. Temel *et al.* [26] reported that *Sideritis akmanii* L. extract (4 mg/disc) did not show activity against *K. pneumoniae* in the disc diffusion test. These results appear to be consistent with the results obtained in our study. But in contrast to the our study, they determined that the inhibition zone was not observed for *S. aureus* and inhibition zone diameters in the range of 7,75 to 17 mm were recorded for *P. aeruginosa and E.coli*. It is believed that this contrasting situation may depend on different species.

Echinacea sp. extract did not show activity against all studied bacteria except *P.aeuroginosa* in the disc diffusion test. The inhibition zone diameter of 9 mm was recorded for *P.aeuroginosa*. The minimum inhibitory concentration (MIC) of this plant extract was recorded in the range of 6,25- 25 mg/ml against all studied bacteria. In accordance with the present study's data, Wendakoon *et al.* [2] reported that *Echinacea angustifolia* L. extract did not form either an inhibition zone against MRSA or any antibacterial activity. But Barnes *et al.* [27] suggested that *Echinacea* might have significant immunomodulatory activity and could be used for the prevention and treatment of upper respiratory tract infections.

4. Conclusions

It is believed that all plant extracts in this study whose antibacterial activity were identified, upon consideration of their organic property, appropriate dose and content, will be effective against bacteria in many fields such as pharmaceutical (in animal and human health), cosmetics and the food industry. Thus, it was aimed to contribute to the efforts of developing alternative antibacterial agents for the struggle against infectious diseases (esp. nosocomial infections) by using certain plants with medicinal properties.

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