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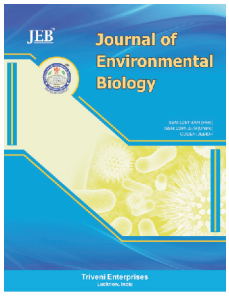

Comparative analyses of phytochemical composition and antimicrobial properties of different solvent extracts of *Melissa officinalis* leaves

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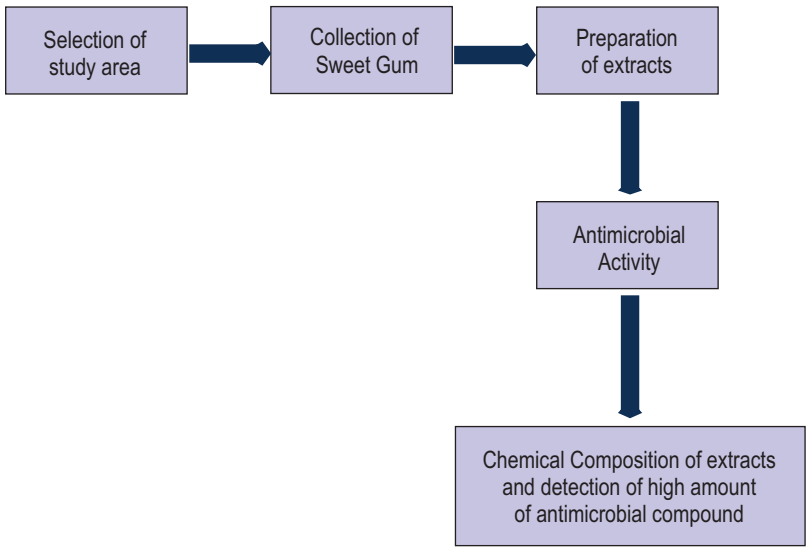
Abstract

Aim : The ethanol, methanol, hexane, chloroform, isopropanol and water extract of *Melissa officinalis* leaves were tested for antimicrobial activity against eleven bacteria and one yeast by disc diffusion method.

Methodology : The antimicrobial activity was assessed and the volatile components of chloroform and dimethyl sulfoxide extracts of *Melissa officinalis* leaves were analysed by GC/MS.

Results : In the present study, chloroform extracts of *Melissa officinalis* leaves showed the best inhibition zone (27 mm) against *Candida albicans*. Inhibition zones of the all the extracts varied from 8 to 27 mm against tested microorganisms. The minimum inhibitory concentration (MIC) of *M. officinalis* subsp. *officinalis* extracts obtained by broth serial dilution method, chloroform extracts were among the most active with the MIC values ranging from 0.016-128 mg ml^{-1}. *C. albicans* showed lowest sensitivity to 0.016 mg ml^{-1} concentration of chloroform extracts. GC/MS analyses of dimethyl sulfoxide extracts identified three compounds; the primary content of lemon balm leaves extract was dimethyl sulfoxide (98.53%). Similarly, GC/MS analyses of chloroform extract identified eight compounds; the primary contents of the of lemon balm leaves extract were chloroform (96.6%).

Interpretation : Different solvent extracts of *Melissa officinalis* leaves showed a broad-spectrum activity against both Gram-positive and Gram-negative bacteria and fungi. Chloroform and dimethyl sulfoxide extracts, especially showed better antimicrobial activity due to high content of chloroform and dimethyl sulfoxide.



Introduction

Climate plants have been classified as an essential source of medicinal agents for centuries and a large number of novel drug components have been isolated from natural plant sources. Medicinal plants play a key role in health care with about 80% of the world's populations relying on the use of plant based traditional medicine (Owolabi *et al.,* 2007; Keskin and Toroglu., 2011). Plant derived medicines have made large contributions to human health (El-Astal *et al.,* 2005). Plant-derived compounds of therapeutic value are mostly secondary plant metabolites traditionally used for medicinal purposes. They have a wide activity range, according to the species, the topography and climate of the country of origin, and may contain different categories of active principles (Arruda *et al.,* 2011; Assob *et al.,* 2011). Variations in the chemical composition modifies their antimicrobial activity. The phytochemical composition of medicinal plants, their antioxidant and antimicrobial activities are centre of attraction for researchers (Barros *et al.,* 2007; Keskin *et al.,* 2010; Ceyhan *et al.,* 2012). Medicinal plants have been used since time immemorial to treat and prevent human ailments because of therapeutic value (Hassan *et al.,* 2006; Gulluce *et al.,* 2006; Parekh and Chanda, 2007; Moghadamtousi *et al.,* 2014).

Melissa officinalis belongs to family Lamiaceae and is commonly known as lemon balm. It is used to treat a number of health concerns, including insomnia, anxiety, migraines, hypertension, diabetes, herpes and dementia. Three subspecies, namely *M. officinalis* subsp. *officinalis, M. officinalis* subsp. *altissima* and *M. officinalis* subsp. *inodora* are well known and native to the Mediterranean region. The dry leaves of *M. officinalis* are used as a herbal tea (Meftahizade *et al.,* 2010), whereas dried or fresh leaves and top aerial section of the plant parts are used as medicine, food and cosmetics (Janina, 2003; Tullio *et al.,* 2007; Mencheriniet *et al.,* 2007; Romeo *et al.,* 2008; Hussain *et al.,* 2011; Vitullo *et al.,* 2011). Lamiaceae consist of about 236 genera with approximately 6900-7200 species. Several other herbal species of Lamiaceae like thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), oregano (*Origanum vulgare*), peppermint (*Menthapiperita*) and sage (*Salvia officinalis*) also possess antimicrobial activity (Kozlowska *et al.,* 2015; Nair *et al.,* 2016). In light of the above, the objective of the present study was to determine the antimicrobial activities of different solvent extracts of *Melissa officinalis.* Further, the antibacterial effect *M. officinalis* was compared with antibiotics.

Materials and Methods

Plant material and storage: Samples of *Melissa officinalis* was procured from market stall in Mugla, Turkey. The plant sample was sent for taxonomic identification to the Department of Biology, Ege University, Turkey. The leaves of the plants were cut into small pieces and stored at 4°C until further analysis.

Solvent extraction of *Melissa officinalis*: The ethanol, methanol, hexane, chloroform, isopropanol and water extracts were prepared by boiling 50 g of dry leaves in a water bath at 80°C. Following filtration, the extract was evaporated in a water bath, while the extracts were evaporated under vacuum at 40°C. The solutions of different concentrations of all dried extracts were resuspended in 5% DMSO.

Microorganisms and inoculum preparation: The microorganisms used for antimicrobial tests were: Gram-positive bacteria (*S. epidermidis* ATCC 12228, *B. subtilis* ATCC 6633, *B. cereus* CCM 99, *S. aureus* ATCC 6538/P, *S. faecalis* ATCC 8043.), Gram negative bacteria (*E.coli* ATCC 35218; *P. aeruginosa* ATCC 27853, S. typhimurium CCM 583, *A. hydrophila* ATCC 19570; *K. pneumoniae* CCM 2318) and *C. albicans* ATCC 10239. Nutrient agar medium was used for bacteria, while Potato Dextrose Agar was used for *C. albicans.* The colonies were taken directly from the plate and suspended into 5 ml of sterile saline solution. The turbidity of initial suspension was adjusted comparing with 0.5 McFarland standard.

Antimicrobial activity with paper disc diffusion assay: The paper disc diffusion assay was used to test the antimicrobial activity, following the method of Ali *et al.* (2001). Sterile paper discs (6 mm; Oxoid) were loaded with different amounts (0.25, 0.5 and 1 mg) of the extracts dissolved in 10% dimethyl sulphoxide (Lab-Scan) and left to dry for 12 hrs at 37°C in a sterile room. The microbial suspensions were diluted to match 0.5 Farland standard scale (approximately 1.5×108 CFU ml^{-1}). The antibiotic disc were obtained from oxoid. Ampicilin (10 µg ml^{-1}), Penicillin G (10 µg $disc^{-1}$), Chloramphenicol (30 µg), Nystatin (30 µg $disc^{-1}$) and erytromycin (15 µg $disc^{-1}$) were used as positive controls and paper discs treated with ethanol, hexane, isopropanol chloroform, methanol and DMSO were used as negative control. The plates were then incubated at 35°C for 24 hrs in an incubator. After incubation time, inhibition zone diameter around each disc was measured.

MIC determination: The minimum inhibitory concentration (MIC) was measured following the method of Magiatis *et al.* (1999). Initial emulsion of extracts were prepared at 0.008 mg ml^{-1} in sterile distilled water. Serial dilution of stock solutions in broth medium (100 µl of Müller-Hinton broth or on Potato Dextrose Broth) were prepared in a microtiter plate (96 wells). Then, 1 µl of microbial suspension (in sterile distilled water) was added to each well. For each strain, the growth conditions and sterility of the medium were checked and the plates were incubated. The incubation condition for bacteria was 24 hrs at 37°C and for *C. albican* sit was 48 hrs at 28°C. MICs were determined as the lowest concentrations preventing visible growth. Standard antibiotics (ampicilin, penicillin and chloramphenicol) were used to control the sensitivity of the tested bacteria, whereas nystatin was used as control against *C.albicans.*

GC/MS analysis of the extracts: The steam-distilled components were analysed by GC/MS. A HP 6890 gas chromatograph equipped with a HP-PTV and a 0.32mX0.60m HP-Innowax capillary column (0.5 µm coating) was employed for

Table 1 : Antimicrobial activity of lemon balm extracts against test microorganisms by disc diffusion method

Microorganisms	Methanol	Ethanol	Hexane	Chloroform	Isopropanol	Water
S. faecalis	23	-	9	23	10	-
S. typhimurium	12	13	8	18	13	17
E. coli	15	12	12	16	14	9
P. aeruginosa	14	10	8	11	10	9
A. hydrophyla	8	11	9	14	11	10
S. epidermidis	9	10	10	14	13	10
S. aureus	11	8	8	19	16	16
K. pneumoniae	15	21	9	12	15	-
B. cereus	16	10	9	16	11	15
B. subtilis	14	22	14	15	12	-
C. albicans	23	22	14	27	17	15

*Each inhibition zone value is a mean of three tests ($p<0.05$ for antimicrobial activity), (-): No inhibition

GC analysis (Adams,1995). A computerized search was carried out using Wiley 7n.l GC/MS library and ARGEFAR GC/MS library created with authentic samples.

Statistical analyses: All the studies were carried out three times. A mean of three tests and standard deviation from average value were calculated (data not given). Statistical analyses with p value was found out for the percentage calculation of inhibition zone diameters. The results were analyzed by t-test seeking at least 95 % confidence.

Results and Discussion

The extract of methanol, ethanol, hexane, chloroform, isopropanol and water of *Melissa officinalis* leaves were tested for antimicrobial activity against ten bacteria and one yeast by disc diffusion method. In this study, chloroform extracts of *Melissa officinalis* leaves showed the best inhibition zones (27 mm) against *C. albicans.* Inhibition zones of all the extracts varied from 8 to 27 mm against tested microorganisms (Table 1). On comparing the antimicrobial activity of lemon balm extracts, chloroform and methanol extarcts showed the best antimicrobial activity against *S. faecalis* with 23 mm inhibition zone. Also, ethanolic extracts of lemon balm showed the best antimicrobial activity against *B. subtilis* and *C. albicans* with 22 mm inhibition zone (Table 1). The antimicrobial activity of methanol, ethanol, hexane, chloroform, ısopropanol and DMSO was tested as a negative control. *S. typhimurium* and *S. epidermidis* showed no inhibition zone in all the extracts (Table 2) however lemon balm extract showed antimicrobial activity with 8-17 inhibition zones against the tested extracts.

Abdellatif *et al.* (2014) reported that the *in vitro* antimicrobial activity was determined by paper disc agar diffusion testing and minimum inhibitory concentration (MIC) using 7 bacteria (3 Gram-positive and 4 Gram-negative), 2 yeasts and 3 fungi. The target microorganisms studied were human pathogenic strains (*Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Salmonella enterica*) and common foodborne pathogen (*Listeria*

Table 2 : Antimicrobial activity of methanol (M), ethanol (E), hexane (H), chloroform (C), isopropanol (I) and DMSO (D) against tested microorganisms by disc diffusion method

Microorganisms	M	E	H	C	I	D
	Inhibition zones (mm)					
S. faecalis	14	13	-	9	11	-
S. typhimurium	-	-		-	-	-
E. coli	-	-	-	11	15	-
P. aeruginosa	10	10	-	-	11	9
A.hydrophyla	7	8	-	7	10	9
S. epidermidis	-	-	-	-	-	-
K. pneumoniae	9	10	-	8	23	10
B. cereus	-	9	-	12	12	8
B. subtilis	17	17	11	-	12	11
C. albicans	18	11	-	-	12	10

Table 3 : Inhibition zone diameters of the references antibiotics against test organisms

Microorganisms	A	P	E	C	N
	Inhibition zones (mm)				
S. faecalis	28	30	22	25	
S. typhimurium	25	32	27	23	-
E. coli	29	32	24	33	-
P. aeruginosa	28	33	22	28	-
A. hydropyhila	27	31	25	26	-
S. epidermidis	23	30	23	25	-
S. aureus	29	27	24	20	-
K. pneumoniae	28	29	26	25	-
B. cereus	26	30	25	24	-
B. subtilis	32	35	16	30	-
C. albicans	-	-	-	-	12

A: Ampicillin (10 µg.disc^{-1}); P: Penicillin (30 µg.disc^{-1}); E: Erythromycin (10 µg.disc^{-1}); C: Chloramphenicol (30 µg.disc^{-1}) (30 µg.disc^{-1}); N: Nystatin (30 µg.disc^{-1}); (-): No inhibition

Table 4 : MICs of chloroform and DMSO extracts of leaves of lemonbalm and reference antibiotics. MIC ($mg.mL^{-1}$)*

Microorganisms	C	D	E	C	N
Extracts antibiotics					
S. faecalis	2	32	4	2	-
S. typhimurium	16	16	0,016	4	-
E. coli	32	32	2	0.008	-
P. aeruginosa	128	32	4	0.016	-
A. hydropyhila	64	8	2	0.16	-
S. epidermidis	64	8	4	0.16	-
S. aureus	8	8	2	8	-
K. pneumoniae	128	8	2	2	-
B. cereus	32	16	2	4	-
B. subtilis	64	64	32		-
C. albicans	0.16	4	-	-	16

*MIC values are significantly ($p<0.05$) different from the MIC of control (extracts and antibiotics); C: Chloroform; D: %10 DMSO; E: Erythromycin; C: Chloramphenicol; N: Nystatin; (-): No inhibition

Table 5 : Volatile components of dimethyl sulfoxide and chloroform extracts of lemonbalm leaves (GC-MS analysis)

Component	Area (%)	Rt[b]
Dimethyl sulfoxide extracts		
Chloroform	1.04%	5.32
Acetic acid	1.02%	15.81
Dimethyl sulfoxide	98.53%	19.43
Undefined	31.0%	
Chloroform of extract lemon balm leaves		
Ethylene 1, 2 dichloro(Z)	0.22%	5.00
Chloroform	96.6%	5.25
Bromochloromethane	0.36%	5.64
Toluene	0.19%	0.36
1,8 cineol	0.33%	8.81
E-citral	0.16%	24.10
Alpa curcumen	0.28%	25.36
Caryophyllene oxide	0.62%	31.37
Undefined	0.65	

[a]Components listed in order of elution from a HP-1capillary column
[b]Retention time (as min)

monocytogenes). The essential oil exhibited a strong antimicrobial activity against all the strains tested with very low MICs. The inhibition zones ranged between 17 and 18 mm for the Gram-positive bacteria and 14-21 mm for the pathogenic Gram-negative bacteria. In general the essential oils were more active against Gram-positive bacteria than against Gram-negative ones. Abdellatif *et al.* (2014) reported that *Melissa officinalis* oil was more active against the Gram negative ones. The data of antimicrobial activities of methanol, ethanol, hexane, chloroform, isopropanol and DMSO against tested microorganisms by disc diffusion method is given in Table 2. Also, inhibition zone diameters of the reference antibiotics against test microorganisms is shown in Table 3. Tested antibiotics showed 16-35 mm inhibition zones against tested bacteria. Nystatin showed 12 mm inhibition zones against to *C.albicans.* Among the bacteria, ısopropanol extracts showed the best inhibition zone against *K. pneumoniae* (23 mm). Methanolic extracts showed the best inhibition zone (18 mm) against *C. albicans* (Table2). A lot of researchers (Stanojevic *et al*., 2010; Kačániová *et al.*, 2016; Jafari and Sani, 2016) tested Melissa extracts against different pathogens and found moderate inhibitory activity against the pathogens. The differences in the antimicrobial activities with the reported one may be due to different geographical environment, age of the plant, collection time, different method followed for isolation of extracts, cultivar type, seasonality etc.

The results showed that the essential oil presented high antimicrobial activity against all microorganisms targeted mainly against five human pathogenic bacteria and yeast *Candida albicans* tested. The MICs ranged from 1.00 to 5.00 µl ml^{-1}. Among the tested antibiotics, *B. subtilis* showed the maximum inhibition zone to penicillin (35mm) and least to erythromycin (16 mm). Kačániová *et al.* (2016) reported that minimal inhibition concentration of *Mellisa officinalis* ranged from 3.2 in MIC_{50} resp. 3.41 in MIC 90 to 27.36 resp. 66.13 µg ml^{-1}. The results of the antimicrobial activities of *M. officinalis* performed by disk method and MICs against human pathogens *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Salmonella enterica* and *Listeria monocytogenes* showed that the essential oil exhibited a strong activity against all the strains tested with very low MICs.

Khorshidi *et al.* (2015) reported that the results of susceptibility testing of selected bacterial strains to different standard antibiotics via measuring inhibition zone diameter (mm) by the disc diffusion method indicated that *Staphylococcus aureus* and *Klebsiella pnomoniae* were to resistant penicillin (10 µg/disc); *Bacillus cereus* to penicillin (10 µg/disc), sulfamethoxazole/trimethoprim (1.25 µg/23.75 µg/disc) and trimethoprim (5 µg/disc); *Escherichia coli* to erythromycin (15 µg/disc), penicillin (10 µg/disc), sulfamethoxazole/trimethoprim (1.25 µg/23.75 µg/disc) and trimethoprim (5 µg/disc) and Salmonella enteritidis to erythromycin (15 µg/disc) and penicillin (10 µg/disc) respectively. Comparison between the results of antibacterial activity of *M. officinalis* essential oil and susceptibility testing of selected bacterial strains to different standard antibiotics demonstrated that *M. officinalis* essential oil can be used instead of antibiotics erythromycin (15 µg/disc), penicillin (10 µg/disc), sulfamethoxazole/ trimethoprim (1.25 µg/23.75 µg/disc) and trimethoprim (5 µg/disc) against five selected antibiotic resistant bacteria. Jafari and Sani (2016) reported that the diameters of inhibition zones varied from 12-25 mm and 25-30 mm for various concentrations of essential oil and gentamycin respectively. Among the four bacteria, *B. cereus* was most sensitive to essential oil (25 mm). *S. enterica* was most resistant to essential oil (12 mm). Jafari and Sani (2016) reported that the results of disc diffusion method showed inhibition zone from 25.88 to 12.33 mm against tested bacteria. Minimum inhibitory concentrations ranged from 1.04 to 4.42 mg ml^{-1} and minimum bactericidal concentrations

of *Melissa officinalis* essential oil ranged from 1.3 to 25 mg ml^{-1} respectively. Moussaoui and Alaoui (2016) reported that *M. officinalis* showed no activity against *P. putida, P. aeruginosa* (ATCC 27853), *S. enteritidis* and *P. aeruginosa.*

MICs of M. officinalis different extracts obtained by broth serial dilution method this sentence is can be omitted. According to MİC results, the chloroform extracts of lemon balm was most active with MIC values ranging from 0.016-128 mg ml^{-1}. *C. albicans* showed the lowest sensitivity to 0.016 $mgml^{-1}$ concentration of chloroform extracts (Table 4). The MIC values against the microorganisms were significantly ($p<0.05$) different from the MIC produced by antibiotics against the organisms. In the present study, five reference antibiotics *viz.,* Ampicilin, Penicillin G, Erythromycin, Chloramphenicol and Nystatin were used as positive control. *B. subtilis* was inhibited by ampicilin, Penicillin and Chloramphenicol, but *S. typhimurium* and *K. pneumoniae* were inhibited by Erythromycin. Nystatin inhibited the growth of *C. albicans* weakly.

The GC/MS analyses of dimethyl sulfoxide extracts showed the presence of chloroform, acetic acid and dimethyl sulfoxide.The main constituents of lemon balm leaves extract was dimethyl sulfoxide (98.53%) in Table 5.

GC/MS analysis of chloroform extract of lemonbalm leaves showed the presence of ethene 1,2 dicloro, Chloroform, Methane-bromo-chloro-toluene, 1,8 cineol, E-citral, Alpa curcumen, caryophyllene oxide & undefined compards also. (Table 5). Previous studies have reported the antimicrobial activity of 1,8 cineol (Hendry *et al.,* 2009). E-citral (Leite *et al.,* 2014) and acaryophyllene oxideis (Yang et al., 2000) in plants. Thus it can be concluded that the solvent extracts of *Melissa officinalis* in this study demonstrated a broad-spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria and fungi. Isopropanol extracts of *Melissa officinalis* showed the best antibacterial activity against *K. pneumoniae* white. DMSO extracts showed the best anticandidal activity compared to standard antibiotics.

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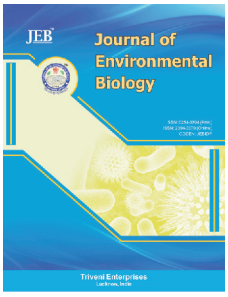

Interactive effects of gibberellic acid and salt stress on growth parameters and chlorophyll content in oat cultivars

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Abstract

Aim: The main objective of this study was to evaluate the possible mode of interaction between salinity and application of gibberellic acid (GA_3) and exploring how GA_3 can mitigate the harmful effects of salinity. On the basis of reduction, growth parameter and chlorophyll contents, the cultivars were categorized as tolerant, moderate and sensitive.

Methodology: To determine the effect of salinity on seed germination, seedling growth and chlorophyll contents pot experiment was carried out. Three different varieties (UPo-212, NDO-2 and UPO-94) were germinated under four different salinity levels (25, 50, 75 and 100mM). After 24 hrs., two sets of each were treated with 100 ppm of GA_3 (reused for 15 days).

Results: The results revealed that when seedling were treated with GA_3 under different salinity conditions, cv. NDO-2 and UPO-212 showed better growth and high chlorophyll content than cv. UPO-94. This study conducts that 100 ppm concentrations of GA_3 will be able to overcome the toxic effects of salt stress in seed germination, seedling growth and chlorophyll contents.

Interpretation: The study reveals that application of GA_3 is useful to mitigate salinity stress and is more effective on salt tolerant cultivars.