JAEFS

International Journal of Agriculture, Environment and Food Sciences

e-ISSN : 2618-5946 DOI: 10.31015/jaefs.2021.3.19 www.jaefs.com

Research Article Int J Agric Environ Food Sci 5 (3):398-404 (2021)

Statistical determination of in-vitro antimicrobial effects of extracts of marjoram (*Origanum majorana* L.) from Muğla, Turkey

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Abstract

To determine the chemical composition and antimicrobial potential of marjoram (*Origanum majorana* L.) leaves extratcs obtained from Muğla, Turkey. The extract of ethanol, methanol, hexane, chloroform, isopropanol and water of marjoram were tested for antimicrobial activity against eleven bacterial strains and one yeast by disc diffusion method. The minimum inhibitory concentrations (MICs) of isopropanolic extract of marjoram leaves were determined using broth serial dilution method. The constitituents were analyzed by gas chromatography-mass spectrometry (GC/MS). The Shapiro-Wilk of Normal Distribution Test, Kruskal-Wallis and Mann-Whitney Tests were applied for statistical analysis in the study. All of the tested extracts, isopropanolic extracts of marjoram leaves showed the best inhibition zones against *Klebsiella pneumoniae* CCM 2318 (24 mm) and *Candida albicans* ATCC 10239 (28 mm), methanolic extracts against *Staphylococcus aureus* ATCC 6538/P (26 mm). In our study, the results of the inhibition zones are varied 6 to 26 mm against tested bacteria. The results of Mann-Whitney Tests showed that isopropanolic extract is a significant different from chloroform, water and hexane extracts, statistically ($P_{isopropanolic\text{-}chloroform}$=0.003, $P_{isopropanolic\text{-}water}$=0.000, $P_{isopropanolic\text{-}hexane}$=0.000). Isopropanolic extracts of *O.marjorana* were among the most active with the MIC values ranging from 0.008-64 mg/mL. MICs of *O.marjorana* L. different extracts obtained by the broth serial dilution method. The GC/MS analyses allowed fifteen compounds to be determined; the main constituents of the of isopropanolic extract of marjoram leaves was carvacrol %86.33. Carvacrol, major constituent of the marjoram isopropanolic leaf extract in our study, had possible antimicrobial activity by testing commercial carvacrol. It has been observed that the extracts can be used in the food industry to inhibit the growth of pathogens or as natural preservatives in foods.

Keywords: *Origanum majorana* L.**,** Leaf extract, Antimicrobial activity, GC/MS analysis, Statistical analysis

Introduction

Studies in plant extracts have attracted in human beings. It can be used for both academic and industrial circles due to a growing interest in green consumerism, world-wide reduction in the composition of salt in food (health reasons), and the need of alternative techniques to assure quality and safety of perishable foods (Burt 2000; Holley and Patel 2005; Hammer et al.,1999; Dorman and Deans 2000; Lambert et al.,2001; Baydar et al.,2004).

Among the members of Lamiaceae family, the genus *Origanum majorana* L. is an aromatic, perennial, herbaceous plant. The plant has been used as a flavouring, herbal spice, perfumery, alsoused in both Ayurveda and folkloric system to cure various human diseases from time immemorial (Kirtikar and Basu., 1985; Farooqi and Sreeramu.,2004; Deans and Svoboda., 1990; Ezzeddine et al.,2001; Leeja and Thoppil.,

Cite this article as:
Ceyhan Guvensen, N., Keskin, D. (2021). Statistical determination of in-vitro antimicrobial effects of extracts of marjoram (*Origanum majorana* L.) from Muğla, Turkey. J. Agric. Environ. Food Sci., 5(3), 398-404.
Doi: https://doi.org/10.31015/jaefs.2021.3.19
Orcid: Nur Ceyhan Guvensen: 0000-0001-8753-2664 and Dilek Keskin: 0000-0003-2479-0070
Received: 29 March 2021 **Accepted:** 09 July 2021 **Published Online:** 11 September 2021 **Revised:** 25 September 2021
Year: 2021 **Volume:** 5 **Issue:** 3 (September) **Pages:** 398-404
Available online at : http://www.jaefs.com - http://dergipark.gov.tr/jaefs

2007; Busatta et al.,2008; Mohamed et al.,2011).

The use of natural preservatives is currently encouraged due to increasing bacterial resistance to chemical preservatives, food safety regulations, and increased awareness of the adverse effects of chemicals.Although various measures have been implemented in the past to control and reduce the prevalence of pathogenic microorganisms, reports of foodborne disease outbreaks as a result of their infection subsists (Pernin et al.,2019).

The objectives of present study were therefore to investigate the antimicrobial activities of six different solvent extracts from West Anatolian marjoramand to determine the chemical compound content to find out the relationship between antimicrobial activity and the compound content. Therefore, we have tested antimicrobial activity against bacteria and fungi, including opportunistic-pathogenic species: Gram negative bacteria; *Klebsiella pneumoniae* CCM 2318, *Salmonella typhimurium* CCM 583, *Aeromonas hydrophila* ATCC 19570, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 35218.Grampositive bacteria; *Bacillus cereus* CCM 99, *Staphylococcus aureus* ATCC 6538/P, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus faecalis* ATCC 8043, *Bacillus subtilis* ATCC 6633. fungus; *Candida albicans* ATCC 10239.

The antimicrobial activity of marjoram extracts was measured by using disc diffusion method and minimal inhibitory concentration (MIC) and the data obtained were evaluated statistically in this study.

Materials and Methods

Plant material

Marjoram leaves samples were obtained from market place in Mugla, Turkey in 2013. The specimens were identified and authenticated by botanist Prof. Dr. Aykut Guvensen from the Department of Biology, Ege University, Turkey. The specimens were air-dried. The leaves were taken and used inthis study to determine its antimicrobial activity against the test microorganisms.

Preparation of plant extracts

Dried leaves of marjoram were mechanically graded, and 2g of plant was extracted with 20 ml of ethanol, methanol, hexane, chloroform, isopropanolor water then was gently heated after rinsed for 24 h atroom temperature (Ates and Erdogrul., 2003; Ozdemir et al., 2004).

Microorganisms and growth conditions

Test microorganisms were as follows: *Salmonella typhimurium* CCM 583, *Aeromonas hydrophila* ATCC 19570; *Escherichia coli* ATCC 35218, *Klebsiella pneumoniae* CCM 2318, *Pseudomonas aeruginosa* ATCC 27853, *Streptococcus faecalis* ATCC 8043, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* CCM 99, *Staphylococcus aureus* ATCC 6538/P, and *Candida albicans* ATCC 10239.

The bacterial strains were inoculated on nutrient broth (Merck) and incubated for 24 h at 37ºC, *C. albicans* was inoculated on potato dextrose broth (Merck) and incubated for 48 h at 25 ºC.

Antimicrobial assays

Disc diffusion method

The disc diffusion method was used to screen the antimicrobial activities. The test were done according to Phadke and Kulkarni., 1989). Penicillin (10 µg/disc) (Oxoid), ampicillin (10 µg/disc) (Oxoid), erythromycin (10 µg/disc) (Oxoid), chloramphenicol (30 µg/disc) (Oxoid) and nystatin (30 µg/disc) (Oxoid) were used as positive controls. Ethanol, methanol, hexane, chloroform and isopropanol were also used as negative controls. The three sterile discs of 6 mm diameter were place ontoeach agar plates containing microorganisms with sterile forceps. Then 30 µl of extracts were absorbed onto discs under sterile conditions. Agar plates containing strains were incubated at 35 ºC at 24 h. All experiments were done inthree times (Phadke and Kulkarni., 1989).

Carvacrol, major constituent of the marjoram isopropanolic leaf extract in our study, was tested to detect the possible antimicrobial activity. For this purpose, 86.33% solution of commercial carvacrol (98%, Sigma-Aldrich) was prepared by dissolving in dimethyl sulfoxide (DMSO) (99.9%, Sigma-Aldrich) and used. DMSO was used as negative control to determine the sensitivity of the tested strains.

Minimum inhibitory concentrations (MICs) tests

MIC tests were carried out according to Kim et al., (2005), using a microplate (96 wells). The stock solutions of the extracts were diluted and transferred into the first well, and serial dilutions were performed so that concentrations in the range of 0.008-128 mg/mL were obtained. Erythromycin, chloramphenicol and nistatin (Oxoid) was used as the reference antibiotic control and pure isopropanol was also tested.

Chromatographic analysis

The steam-distilled components were analysed by Gas Chromatography-Mass Spectrometry(GC/MS) according to Adams,1995.

Statistical analysis

Marjoram leaves, solvents and standard antibiotics data were taken as variables. The Shapiro-Wilk of Normal Distribution Test is applied to variable groups. The variables not having normal distribution were tested by Kruskal-Wallis, non-parametric statistical test. Mann-Whitney Tests were performed for multiple comporisons. In this way difference between zone diameters is tested.

Results and Discussion

In our study, the results of the inhibition zones are varied 6 to 28 mm against tested bacteria (Table 1) with all extracts. Among the tested marjoram extracts, isopropanolic extract showed the best inhibition zones against all bacteria and *C. albicans* (15-28mm) in Table 1. The results of Mann-Whitney Tests showed that isopropanolic extract is a significant different from chloroform, water and hexane extracts, statistically ($P_{isopropanolic\text{-}chloroform}$=0.003, $P_{isopropanolic\text{-}water}$=0.000, $P_{isopropanolic\text{-}hexane}$=0.000). Isopropanolic extract had the highest antibacterial activity on *K. pneumoniae* (24mm) (Table 1). Besides, the inhibition zone diameters of the tested extracts against the test microorganisms were shown Table 2. In this table, all of the tested solvents showed no inhibition zones

against *S.typhimurium* and *S.epidermidis* in Table 2.

Mohamed *et al*., 2011 reported that *Origanum marjorana* L. at the concentration of 170 µl showed a strong growth inhibition effect (11, 10, 10, 9, 10 and 9 mm) against *E. coli, S. enteritidis, S. aureus, B. cereus, Aspergillus* spp. and *Penicillum* spp. respectively (Mohamed et al., 2011). However, water extract at low concentration did not show strong antimicrobial activities against all of investigated microorganism, especially *S.aureus, E. coli, Aspergillus* spp. and *Penicillum spp*. This observation is completely concurrent with the observation confirmed by Adwan et al., 2009. Leeja and Thoppil., (2007) reported that in-vitro microbicidal activity of the methanol extract of *Origanum majorana* L. was tested against seven fungi and six bacteria (Leeja and Thoppil., 2007). The methanol extract of *O. majorana* can be used as an effective herbal protectant against different pathogenic bacteria and fungi. Ramos et al., (2011) reported that it was active against *S.aureus, Enterococcus faecalis, E. coli* and *Klebsiella pneumoniae.* Previous study conducted by Ben *et al*., 2001 suggests that the essential oil of *O. majorana* posses antibacterial activity like Farooqi and Sreeramu., (2004).

Tablo 1. Antimicrobial activity of marjoram (*Origanum majorana* L.) extracts against test microorganisms by disc diffusion method.

Microorganisms	Extracts of marjoram								
	Methanol (mm)			Ethanol (mm)			Hexane (mm)		
	Methanol	Water	% 10 DMSO	Ethanol	Water	% 10 DMSO	Hexane	Water	% 10 DMSO
S. faecalis	16	13	13	20	12	17	12	9	17
S. typhimurium	17	11	18	15	9	11	13	8	13
E. coli	22	11	20	20	20	14	8	8	10
P. aeroginosa	18	9	16	15	15	8	8	9	15
A. hydrophila	13	7	14	14	8	7	8	7	14
S. epidermidis	21	9	16	16	8	6	8	11	13
S.aureus	26	23	21	22	18	15	10	7	16
K. pneumoniae	16	10	14	24	11	15	9	10	10
B. cereus	17	7	20	19	12	11	8	8	18
B.subtilis	20	11	21	23	11	20	12	11	15
C. albicans	25	13	25	24	15	14	19	11	21

-: no inhibition

Tablo 1. Antimicrobial activity of marjoram (*Origanum majorana* L.) extracts against test microorganisms by disc diffusion method (continuation).

Microorganisms	Extracts of marjoram							
	Chloroform (mm)			Isopropanol (mm)			Water (mm)	
	Chloroform	Water	% 10 DMSO	Isopropanol	Water	% 10 DMSO	Water	% 10 DMSO
S. faecalis	20	19	8	21	-	13	15	18
S. typhimurium	14	6	9	21	8	10	13	11
E. coli	14	20	8	23	9	12	10	20
P. aeroginosa	7	9	9	19	9	9	12	9
A. hydrophila	9	9	8	16	8	10	8	12
S. epidermidis	6	10	9	15	15	11	8	13
S.aureus	-	14	8	21	11	11	13	19
K. pneumoniae	16	12	19	24	14	13	8	9
B. cereus	12	7	8	22	10	8	13	9
B.subtilis	21	19	14	23	9	17	20	19
C. albicans	22	12	24	28	12	24	9	20

-: no inhibition

Table 2. Antimicrobial activity of solvents against tested bacteria by disc diffusion method.

Microorganisms	Solvents					
	Methanol(mm)	Ethanol(mm)	Hexane(mm)	Chloroform(mm)	Isopropanol(mm)	DMSO(mm)
S. faecalis	14	13	-	9	11	-
S. typhrium	-	-	-	-	-	-
E. coli	-	-	-	11	15	-
P. aeroginosa	10	10	-	-	13	9
A. hydrophyla	7	8	-	7	10	9
S. epidermidis	-	-	-	-	-	-
S.aureus	17	14	-	-	21	16
K. pneumoniae	9	10	-	8	23	10
B. cereus	-	9	8	-	12	8
B.subtilis	17	17	11	-	12	11
C. albicans	18	11	-	-	12	10

In this study, 5 standard antibiotics were used as positive control. These include, ampicillin (10µg/disc), chloramphenicol (30µg), nystatin (30µg/disc), erythromycin (15µg/disc) and penicillin G (10µg/disc). Ampicillin, penicillin and chloramphenicol very strongly inhibited the growth of *B.subtilis* whereas, erythromycin exhibited a very big zone of inhibition against *S. typhimurium* and *K.pneumoniae. E. coli*. Nystatin weakly inhibited the growth of *C. albicans* in Table 3.

The lowest MIC (2 mg/mL) was recorded for the isopropanolic extract with *E. coli* ATCC 35218, *K. pneumoniae* CCM 2318, *B.subtilis* ATCC 6633 (Table 4). Isopropanolic extracts of *O.marjorana* were among the most active with the MIC values ranging from 0.008-64 mg/mL.

The GC/MS analyses allowed fifteen compounds to be determined; the main constituents of the of marjoram leaves extract was carvacrol %86.33 in Table 5. Previous studies showed that carvacrol is well known antimicrobial compounds isolated from different plant species (Lambert et al.,2001; Ben et al.,2006; Bnyan et al.,2014).

Ramos et al., (2011) reported that the major constituents of the oil were: cissabinene hydrate (30.2%), terpinen-4-ol (28.8%). French and Italian studies reported similar results Novak et al., (2004), but the oil from Turkey (Baser et al., 2014) was reported to have a completely different composition, because *O.majorana* from Turkey contained 78% carvacrol like our study. Many researchers reported that chemical composition of essential oils of *O.majorana* from Cuba (Pino et al., 1997). Brazil (Busatta et al., 2008), Hungary (Vági et al., 2005) and Tunisia (Ben et al., 2001) and Egyptian (Selim et al., 2013). Helal et al., (2006) reported that *O.majorana* oil contain mainly 51.5% 3-cyclohexen-1-01,4-methyl-1(1-methylethyl)-(CAS). It would also be worthy to be cited here that the composition of any plant essential oil is influenced by the presence of several factors such as; local, climate, plant species, methodology and experimental conditions (Mishra et al., 1994; Prudent et al., 1995; Daferea et al., 2000). These factors may alter the biological and antimicrobial activities of the oils produced (Shu and Lawrence., 1997; Vardar-Ünlü et al., 2003).Distillation time and temperature can also significantly affect the oil constituents (Janssen et al., 1987).

Table 3. Inhibition zone diameters (mm) of the standard antibiotics against test microorganisms.

Microorganisms	Standard antibiotics				
	Ampicillin	Penicillin G	Erythromycin	Chloramphenicol	Nystatin
S. faecalis	28	30	22	25	NT
S. typhimurium	25	32	27	23	NT
E. coli	29	32	24	33	NT
P. aeruginosa	28	33	22	28	NT
A.hydropyhila	27	31	25	26	NT
S. epidermidis	23	30	23	25	NT
S. aureus	29	27	24	20	NT
K. pnemuoniae	28	29	26	25	NT
B. cereus	26	30	25	24	NT
B. subtilis	32	35	16	30	NT
C. albicans	NT	NT	NT	NT	12

NT: not tested

Table 4. MICs of isopropanolic extract of marjoram leaves, isopropanol and standard antibiotics against test microorganisms.

Microorganisms	MIC(mg/mL)				
	Isopropanolic extract	Isopropanol	Antibiotics		
			Eritromycin	Chloramphenicol	Nystatin
S. faecalis	8	128	4	2	NT
S. typhimurium	8	128	0,016	4	NT
E. coli	2	64	2	0,008	NT
P. aeruginosa	16	128	4	0,016	NT
A.hydrophila	32	128	2	0,16	NT
S. epidermidis	64	128	4	0,16	NT
S.aureus	8	64	2	8	NT
K. pneumoniae	2	128	2	2	NT
B. cereus	8	128	2	4	NT
B.subtilis	2	64	32	0,008	NT
C. albicans	0,16	32	NT	NT	16

Table 5. Volatile components of the isopropanolic extract of marjoram leaves (GC-MS analysis).

Component[a]	Area(%)	Rt[b]
n-Decane	%0.38	5.13
Methane, oxy bis (Dichloro)	%0.24	5.31
1-Propanol (CAS)	%0.23	5.46
Toluene	%0.30	5.67
2- Pentanol ,2 methyl	%0.31	6.34
Trans sabinenen hydrate	%0.37	15.87
Dimethyl sulfoxide	%0.42	19.04
Trans Caryohyllene	%1.13	20.19
Endo-Borneol	%1.09	23.08
Beta- bisabolene	%0.45	23.99
Caryophyllene oxide	%0.34	31.37
S-2-methlene- 1-cyclohexanol	%1.22	35.75
Thymol	%0.94	36.03
Carvacrol	%86.33	36.76
18-Crown 6-ether	%0.43	40.80
Undefined	%5.83	

[a] Components listed in order of elution from a HP-1capillary column

[b] Retention time (as min).

Table 6**.** Antimicrobial activity of carvacrol against tested microorganisms by disc diffusion method.

Microorganisms	*S.f*	*S.t*	*E.c*	*P.a*	*A.h*	*S.e*	*S.a*	*K.p*	*B.c*	*B.s*	*C.a*
Inhibition zones (mm)	16	15	16	16	12	15	21	20	14	15	22

S.f: Streptococcus faecalis ATCC 8043, *S.t*: *Salmonella typhimurium* CCM 583,*E.c*:*Escherichia coli* ATCC 35218, *P.a*:*Pseudomonas aeruginosa* ATCC 27853, *A.h*:*Aeromonas hydrophila* ATCC 19570, *S.e*:*Staphylococcus epidermidis* ATCC 12228, *S.a*:*Staphylococcus aureus* ATCC 6538/P, *K.p:Klebsiella pneumoniae* CCM 2318, *B.c*:*Bacillus cereus* CCM 99, *B.s: Bacillus subtilis* ATCC 6633, *C.a*:*Candida albicans*

The results of inhibition zones of carvacrol showed 12 to 22 mm against tested microorganisms like tested extracts in Table 6. It can be suggested that antimicrobial activity of marjoram leaves based on carvacrol.

Conclusion

Based on these results, it is possible to conclude that six different extracts of marjoram from Muğla, Turkey had different level antibacterial and anticandidal activity. Carvacrol which is the main compound of isopropanolic extract of marjoram exhibited high levels of antimicrobial activity against all of the tested strains. Our results support that carvacrol have antimicrobial activity against the most important opportunistic pathogenic bacteria which leads to infection of human and food contaminants. It is clear that there is a significant correlation between the chemical compositions and antimicrobial activity of marjoram extracts. The marjoram extracts studied have the ability to inhibit growth of pathogens for the food industryor as flavoring agent and natural preservative and the use of this plant in traditional medicine for the search for new drugs. These data demonstrate the effectiveness of marjoram extractstested, at the projected concentrations, against the opportunistic-pathogenic microorganisms investigated and, as such, may allow the formulation of new antimicrobial products for potential use as food additives.

As for the statistic test results, it was tested whether there is a statistically significant difference between groups by applying Kruskal-Wallis Test. According to the test results, there is a statistically significant difference between groups. To see differences between variables, pairwise comparisons were performed by applying Mann-Whitney Test. As a result, it can be said that isopropanolic marjoram leaf extract and carvacrol can be used instead of the standard antibiotics such as erythromycin. In our findings, there is no statistically a significant difference between isopropanolic extract and standard antibiotic erythromycin (P=0.176).

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

Funding

This project financially supported by Adnan Menderes University Research Fund (BAP, Project Number: BAP-CMYO 13-001).

Data availability

Not applicable.

Consent for publication

Not applicable.

Acknowledgements

Authors are thankful to Prof. Dr. Aykut Guvensen for the taxonomic identification of plant material and thanks to Serdar Demir for his valuable contribution to statistical analysis.

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