

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

Nur Ceyhan Guvensen<sup>1,\*</sup> 

Dilek Keskin<sup>2</sup> 

<sup>1</sup>Tire Kutsan Vocational High School, Ege University, İzmir, Turkey

<sup>2</sup>Köşk Vocational High School, Adnan Menderes University, Aydın, Turkey

\*Corresponding Author: [nur.ceyhan.guvensen@ege.edu.tr](mailto:nur.ceyhan.guvensen@ege.edu.tr)

### Abstract

To determine the chemical composition and antimicrobial potential of marjoram (*Origanum majorana* L.) leaves extracts 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 constituents 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 ( $P_{\text{isopropanolic-chloroform}}=0.003$ ,  $P_{\text{isopropanolic-water}}=0.000$ ,  $P_{\text{isopropanolic-hexane}}=0.000$ ). Isopropanolic extracts of *O. majorana* were among the most active with the MIC values ranging from 0.008-64 mg/mL. MICs of *O. majorana* 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, also used in both Ayurveda and folkloric system to cure various human diseases from time immemorial (Kirtikar and Basu., 1985; Farooqi and Sreeram.,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>

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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 marjoram 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. Gram positive 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 in this 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, isopropanol or water then was gently heated after rinsed for 24 h at room temperature (Ates and Erdogru., 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 37°C, *C. albicans* was inoculated on potato dextrose broth (Merck) and incubated for 48 h at 25 °C.

## 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 placed on each 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 °C at 24 h. All experiments were done in three 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 comparisons. 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 ( $P_{\text{isopropanolic-chloroform}}=0.003$ ,  $P_{\text{isopropanolic-water}}=0.000$ ,  $P_{\text{isopropanolic-hexane}}=0.000$ ). 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 majorana* 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 *Penicillium* 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 *Penicillium* 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).

Table 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
<i>S. faecalis</i>	16	13	13	20	12	17	12	9	17
<i>S. typhimurium</i>	17	11	18	15	9	11	13	8	13
<i>E. coli</i>	22	11	20	20	20	14	8	8	10
<i>P. aeruginosa</i>	18	9	16	15	15	8	8	9	15
<i>A. hydrophila</i>	13	7	14	14	8	7	8	7	14
<i>S. epidermidis</i>	21	9	16	16	8	6	8	11	13
<i>S.aureus</i>	26	23	21	22	18	15	10	7	16
<i>K. pneumoniae</i>	16	10	14	24	11	15	9	10	10
<i>B. cereus</i>	17	7	20	19	12	11	8	8	18
<i>B.subtilis</i>	20	11	21	23	11	20	12	11	15
<i>C. albicans</i>	25	13	25	24	15	14	19	11	21

-: no inhibition

Table 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
<i>S. faecalis</i>	20	19	8	21	-	13	15	18
<i>S. typhimurium</i>	14	6	9	21	8	10	13	11
<i>E. coli</i>	14	20	8	23	9	12	10	20
<i>P. aeruginosa</i>	7	9	9	19	9	9	12	9
<i>A. hydrophila</i>	9	9	8	16	8	10	8	12
<i>S. epidermidis</i>	6	10	9	15	15	11	8	13
<i>S.aureus</i>	-	14	8	21	11	11	13	19
<i>K. pneumoniae</i>	16	12	19	24	14	13	8	9
<i>B. cereus</i>	12	7	8	22	10	8	13	9
<i>B.subtilis</i>	21	19	14	23	9	17	20	19
<i>C. albicans</i>	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)
<i>S. faecalis</i>	14	13	-	9	11	-
<i>S. typhrium</i>	-	-	-	-	-	-
<i>E. coli</i>	-	-	-	11	15	-
<i>P. aeruginosa</i>	10	10	-	-	13	9
<i>A. hydrophyla</i>	7	8	-	7	10	9
<i>S. epidermidis</i>	-	-	-	-	-	-
<i>S. aureus</i>	17	14	-	-	21	16
<i>K. pneumoniae</i>	9	10	-	8	23	10
<i>B. cereus</i>	-	9	8	-	12	8
<i>B. subtilis</i>	17	17	11	-	12	11
<i>C. albicans</i>	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
<i>S. faecalis</i>	28	30	22	25	NT
<i>S. typhimurium</i>	25	32	27	23	NT
<i>E. coli</i>	29	32	24	33	NT
<i>P. aeruginosa</i>	28	33	22	28	NT
<i>A. hydrophyla</i>	27	31	25	26	NT
<i>S. epidermidis</i>	23	30	23	25	NT
<i>S. aureus</i>	29	27	24	20	NT
<i>K. pneumoniae</i>	28	29	26	25	NT
<i>B. cereus</i>	26	30	25	24	NT
<i>B. subtilis</i>	32	35	16	30	NT
<i>C. albicans</i>	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
<i>S. faecalis</i>	8	128	4	2	NT
<i>S. typhimurium</i>	8	128	0,016	4	NT
<i>E. coli</i>	2	64	2	0,008	NT
<i>P. aeruginosa</i>	16	128	4	0,016	NT
<i>A. hydrophila</i>	32	128	2	0,16	NT
<i>S. epidermidis</i>	64	128	4	0,16	NT
<i>S. aureus</i>	8	64	2	8	NT
<i>K. pneumoniae</i>	2	128	2	2	NT
<i>B. cereus</i>	8	128	2	4	NT
<i>B. subtilis</i>	2	64	32	0,008	NT
<i>C. albicans</i>	0,16	32	NT	NT	16

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

Component <sup>a</sup>	Area(%)	Rt <sup>b</sup>
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 sabinen hydrate	%0.37	15.87
Dimethyl sulfoxide	%0.42	19.04
Trans Caryophyllene	%1.13	20.19
Endo-Borneol	%1.09	23.08
Beta- bisabolene	%0.45	23.99
Caryophyllene oxide	%0.34	31.37
S-2-methylene- 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	

<sup>a</sup> Components listed in order of elution from a HP-1capillary column

<sup>b</sup> Retention time (as min).

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

Microorganisms	<i>S.f</i>	<i>S.t</i>	<i>E.c</i>	<i>P.a</i>	<i>A.h</i>	<i>S.e</i>	<i>S.a</i>	<i>K.p</i>	<i>B.c</i>	<i>B.s</i>	<i>C.a</i>
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 industry or 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 extract tested, 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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