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In-vitro Antimicrobial Activities of Different Extracts of Grapevine Leaves (*Vitis vinifera* L.) from West Anatolia against some Pathogenic Microorganisms

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The extract of ethanol, methanol, aqueous grapevine leaves (*Vitis vinifera* L.) were screened *in vitro* for their antimicrobial activity against Gram-positive, Gram-negative bacteria and one yeast. Disk diffusion method was used for antimicrobial activity of extracts and the microwell dilution assay was used for determination of minimal inhibitory concentration (MIC) of each extract. Ethanolic extracts of grapevine leaves showed various antimicrobial activity (0-25 20μL^{-1} inhibition zone) to the microorganisms tested. The methanolic extracts showed antimicrobial activity (0-16 20μL^{-1} inhibition zone) to the microorganisms tested. The aqueous extracts showed no inhibition zone five out of ten microorganisms. The ethanolic extract displayed the best activity (MIC 6.25 μg/ml) against *S. typhimurium* CCM 583. Other microorganisms (*S. aureus* ATCC 6538/P, *P. aeruginosa* ATCC 27853, *K. pneumoniae* CCM 2318, *C. albicans* ATCC 10239) were showed between MIC 12.5-200 μg/ml. The methanolic and aqueous extracts were between MIC 50-400 μg/ml and between MIC 100-400< μg/ml, respectively. In the study, chemical composition of ethanolic grapevine leaves extract was analysed by GC/MS analysis. The GC/MS analyses allowed 15 compounds to be determined; the main constituents of the grapevine leaves extract were ethanol (91.82), cyclotrisiloxanehexamethyl (1.25 %) and diethoxydimethylsilane (1.14 %). The results of the present in vitro work indicate that grapevine leaves extracts, especially ethanolic, could be used as natural antimicrobial agents in the food preservation and human health for microorganisms.

Key words: *Vitis vinifera* L., West Anatolia, leaf extract, antimicrobial activity, Pathogenic microorganisms, Chemical composition.

In nature there are a large number of different types of antimicrobial compounds that play an important role in the natural defence of all kinds of living organisms. Extracts from herbs, spices and their derivatives are the most common plant materials used for this purpose[1-3]. Indeed, plants use a huge, mainly unknown reservoir of substances for their defence against microorganisms, insects, and herbivors. Consumer concern about the safety of food containing preservatives has stimulated interested in identification of new natural antimicrobials as food preservation systems. Some plant family have a good antibacterial effect, especially Lamiaeceae, Vitaceae, Cyperaceae and Juglandeaceae[4].

Grapevine (*Vitis vinifera* L.) is considered as the world's largest fruit crops, with an

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approximate annual production of 58 million metric tonnes[5]. It's widely also grown in Turkey for the manufacturing of wine and other grape products. Leaves are also of commercial importance because they are traditionally included in the diet and in various herbal medicine formulations[6]. Grapevine leaves with mainly constituting mainly of triterpenoids, sterols, faty acids, esters and heterocyclic compounds[7]. These compounds have also antimicrobial properties. There have been many studies in antimicrobial properties of *Vitis vinifera* L. plants in Turkey and other countries[3,8-13]. However, no data are available in the literature about the grapevine (*Vitis vinifera* L.) leaf extract from Cine region in West Anatolia, Turkey.

The objectives of this work were therefore to investigate the antimicrobial activities of three different solvent extracts from West Anatolian grapevine leaves and to determine the chemical compound content to find out the relationship between antimicrobial activity and the compound content. Therefore, we have tested antimicrobial effect against some microorganisms including opportunistic pathogens: (*Staphylococcus aureus* ATCC 6538/P, *Bacillus cereus* CCM 99*, Bacillus subtilis* ATCC 6633*, Streptococcus faecalis* ATCC 8043*, Klebsiella pneumoniae* CCM 2318, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* CCM 583, *Aeromonas hydrophila* ATCC 19570*, Escherichia coli* ATCC 35218) and *Candida albicans* ATCC 10239. The antimicrobial activity was measured by using disk diffusion method and minimal inhibitory concentration (MIC).

MATERIALS AND METHODS

Collection of leaves

Grapevine (*Vitis vinifera* L.) leaves were obtained from Cine region that were collected on September West Anatolian of Turkey (N:37-32' 30.1", E:28° 08' 35.6 altitude: 520m).

Preparation of leaf extracts

They were sun dried to a constant weight and milled to a fined powder using a porcelain muller. The powdered leaf (25 g) was soaked in 150 ml of distilled deionizer water to prepare the aqueous extract and in 150 ml of absolute ethanol (96º, Fluka Chemical) and in 150 ml of absolute methanol (97º, Fluka Chemical) to prepare the ethanolic and methanolic extracts. The suspension was stirred at 200 rpm at room temperature for 4 days after which it was filtered with the aid of a Whatman No 1 filter paper. The residue was re-extracted with 150 ml of the solvent as described. The combined extract were then evaporated to dryness at 40 ºC, re-dissolved in the corresponding solvent to obtained extracts (400 mg/ml) and stored at 4 ºC prior to use[14].

Microorganisms and media

The bacteria (*Staphylococcus aureus* ATCC 6538/P, *Bacillus cereus* CCM 99*, Bacillus subtilis* ATCC 6633*, Streptococcus faecalis* ATCC 8043*, Klebsiella pneumoniae* CCM 2318, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* CCM 583, *Aeromonas hydrophila* ATCC 19570*, Escherichia coli* ATCC 35218) and *Candida albicans* ATCC 10239 were obtained the stock culture collection of the Basic and Industrial Microbiology Section of Ege University, Izmir, Turkey. Cultures of these bacteria were grown in brain heart infusion broth (Merck) at 37 ºC for 24 h and *C. albicans* was incubated in sabouraud dextrose broth (Merck) at 30 ºC for 48 h.

Screening of antimicrobial activity of extracts

The antimicrobial activity of the grapevine (*Vitis vinifera* L.) leaf extracts against the selected microorganisms was evaluated by the disc diffusion method[14]. A 20 ml of the molten medium was seeded with 0.2 ml of broth cultures of the test organisms in sterile petri dishes. The petri dishes were rotated slowly to ensure a uniform distribution of microorganisms. The Mueller Hinton Agar (Merck) was left to solidify in the dish for bacterial strains, the sabouraud dextrose agar (Merck) for *C. albicans*. 20 µl of each extract (400 mg of extract/ml) were inoculated into the 6.0 mm diameter sterile discs with the aid of a sterile pipettes. The discs were take placed onto the dishes. The plates were allowed to stand for 30 min at room temperature to allow for proper diffusion of the extract to take place. The bacteria were then incubated at 37 °C for 24 h and *C. albicans* was incubated at 25 ºC for 48h. At the end of the incubation period, inhibition zones formed on the medium were measured in mm. In addition, commercial antibiotic discs such as tobramycin (10 µg/disc) (Oxoid), ampicillin (10 µg/disc) (Oxoid) and nystatin (30 µg/disc) (Oxoid) were

used as positive control and water, ethanol, methanol were used as negative control to determine the sensitivity of the tested strains. Whole studies were performed in three times and the results were expressed as average values.

Determination of minimal inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined for five of test microorganisms that determined the best inhibition on. The microwell dilution assay with slight modification was performed by using the CLSI standards[15,16]. A sterile 96 microtiter well plate was labeled. A volume of 100 µL of extract solution was pipetted into the first row of the plate. To all other wells 50 µL of double strength mueller hinton broth or potato dextrose broth was added. Serial dilutions were performed using a micropipette (A1-A10). Tips were discarded after use such that each well had 50 µL of the test material in serially descending concentrations. Then, 50 µL of broth containing bacterial suspension (5×10^6 cfu/mL) or *C. albicans* (5×10^5 cfu/mL) was added to each well. Each column of wells contained a single antimicrobial extract in progressive dilutions and was inoculated with a single microorganism. This analysis was performed at final concentrations of each extract (400, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 mg of extract/ml).

Each plate had a set of both a growth (A11) and sterility control (A12). Plates were sealed with clean film to ensure that microorganisms did not become dehydrated. The plates were prepared and placed in an incubator set at 37°C for 18-24 h and at 25ºC for 48 h, respectively for bacteria and *C. albicans*. After incubation, added 10 µL of 0.2% 2,3-5 Triphenyl tetrazolium chloride (TTC) solution to each well of microtitre plate. The plates containing TTC were incubated one h at 37°C for reaction. The color change was then assessed visually. Any color changes from purple to pink, which showed the growth of organism. MIC concentration does not exhibit reduction of TTC into formazan so the MIC in mg/ml was defined as that the lowest inhibitory concentration of each extract contained in the microtiter well in which the absence of visual color change (colorless) first observed. The average of five values was calculated and that was the MIC for the test extract and microorganism.

GC/MS analysis

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 (0.5µm coating) was employed for the GC analysis. GC/MS analysis was performed on a HP-5973 mass selective detector coupled with a 6890 gas chromatograph, equipped with a HP 6890 gas chromatograph, equipped with HP-1capillary column. The column temperature was programmed from an initial temperature of 60 ºC to a final temperature of 250 ºC at 15 ºC/min. The carrier gas was helium (14.1mL/min). Identification of the individual components was performed by comparison of mass spectra with literature data and by a comparison of their retention time (Rt) relative to a C_{8}-C_{32} *n*-alkanes mixture[17]. A computerized search was carried out using the Wiley 7n.l GC/MS library and ARGEFAR GC/MS library created with authentic samples.

RESULTS AND DISCUSSION

In the study, ethanol and methanol were analysed as negative control and they did not show inhibition against tested microorganisms. Ethanolic extracts of grapevine leaves showed various antimicrobial activity (0-25 $20\mu L^{-1}$ inhibition zone) to the microorganisms tested. The methanolic extracts showed antimicrobial activity (0-16 $20\mu L^{-1}$ inhibition zone) to the microorganisms tested. The aqueous extracts showed no inhibition zone five out of ten microorganisms. The ethanolic extracts was found to be the most effective antimicrobial agent as compared to the methanolic and aqueous extract. All these results were shown in table 1.

Similarly, Parekh and Chanda[11] reported that the ethanolic as well as the aqueous extract of *V.vinifera* L. was active against more than 85 and 65 per cent of the bacterial strains respectively. And also, Oskay and Sari[18] reported that the ethanol extracts of *V.vinifera* L. leaves showed broad spectrum antimicrobial activity against gram positive and gram negative bacteria, using the agar-well diffusion method. Deliorman-Orhan *et al.*[13] studied the fractions of different polarity, namely chloroform, ethylacetate, n-butanol and reamaining water fractions, were fractioned from an aqueous extract of *V.vinifera* leaves. All of the fractions displayed a little more antibacterial activity against

gram positive bacteria than gram negative bacteria.

When we compared to MIC value of the ethanolic, methanolic and aqueous extracts, the ethanolic extract displayed the best activity (MIC 6.25 µg/ml) against *S. typhimurium* CCM 583. Other microorganisms (*S. aureus* ATCC 6538/P, *P. aeruginosa* ATCC 27853, *K. pneumoniae* CCM 2318, *C. albicans* ATCC 10239) were showed between MIC 12.5-200 µg/ml. The methanolic and aqueous extracts were between MIC 50-400 µg/ml and between MIC 100-400< µg/ml, respectively (Table 2). The results of MIC showed that ethanolic extracts were the best activity compared to methanol and aqueous like disc diffusion method. Methanol is a toxic solvent, so it must not be used in food systems.

In another similar work, *Aeromonas hydrophila, Bacillus cereus, Enterobacter aerogenes, Streptococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium*, *Staphylococcus aureus, Yersinia enterocolitica* tested were also inhibited by grape pomace extracts concentrations of 2.5, 5, 10 and 20%, except for *Y enterocolitica* which was not inhibited by the 2.5% concentration[9].

Jayaprakasha *et al.*,[8] tested for antibacterial activity of Bangalore grape seed extract by the pour plate method against six bacteria

Table 1. Antimicrobial activity of of grapevine (*Vitis vinifera* L.) leaves by the disc diffusion method

Microorganisms		*Vitis vinifera* L leaves			Standard antibiotics		
		Ethanol extract	Methanol extract	Aqueous extract	TOB	AMP	NYS
E. coli ATCC 35218	G(-)	15	0	9	10	12	NT
S. aureus ATCC 6538/P	G(+)	16	0	0	13	15	NT
A. hydrophila ATCC 19570	G(-)	9	14	9	11	10	NT
S. typhimurium CCM 583	G(-)	22	14	0	10	12	NT
P. aeruginosa ATCC 27853	G(-)	22	16	0	11	9	NT
K. pneumoniae CCM 2318	G(-)	25	12	9	11	9	NT
B. subtilis ATCC 6633	G(+)	10	11	10	17	10	NT
B. cereus CCM 99	G(+)	10	9	9	18	12	NT
S. faecalis ATCC 8043	G(+)	0	0	0	9	14	NT
C. albicans ATCC 10239	Y	18	13	0	NT	NT	20

Results (mean of three replicates) indicate zone of inhibition in mm and include filter paper disc diameter (6 mm), TOB: Tobramycin (10 µg/disc), AMP: Ampicillin (10 µg/disc), NYS: Nystatin (30 µg/disc), G: Gram reaction, Y: Yeast, NT: Not tested.

Table 2. The MIC values (µg/ml) of grapevine (*Vitis vinifera* L.) leaves against microorganisms tested in the microwell dilution assay

Microorganisms		Concentration range (400-0.39 mg of extract/ml) *Vitis vinifera* L.leaves			Concentration range (400-0.39 µg/ml) Standard antibiotics		
		Ethanol extract	Methanol extract	Aqueous Extract	GN	ERY	NYS
S. aureus ATCC 6538/P	G(+)	200	400	400<	0.78	1.56	NT
S. typhimurium CCM 583	G(-)	6.25	50	400<	3.12	0.78	NT
P. aeruginosa ATCC 27853	G(-)	100	100	400<	1.56	0.39	NT
K. pneumoniae CCM 2318	G(-)	25	200	100	1.56	0.78	NT
C. albicans ATCC 10239	Y	12.5	100	400<	NT	NT	6.25

GN: Gentamycin, ERY: Erythromycin, NYS: Nystatin, G: Gram reaction.

and determined that Gram-positive bacteria were completely inhibited at 850-1000 ppm while gram negative bacteria were inhibited at 1250-1500 ppm concentrations of extract. Ozkan *et al*.[9] reported that the antibacterial effects of extracts change according to cultivar, extraction method, concentration of extracts and the method used for antibacterial effect determination. And also Batosvka *et al*.[19] reported season affect leaf surface composition. In their study, the leaf surface layers of 16 grapevine plants (*Vitis vinifera* L.) are the source of metabolites typical of cuticular plant wax, which indicate certain interactions between the plant and the environment. Differences in their composition during two consecutive seasons, the summer and the autumn of 2007, were statiscally significant. It is suggested that these differences were mainly due to the specific insects available in the two seasons and to the adaptation of grapevine to lower temperatures.

The ethanol extracts of grapevine leaves were also evaluated for their chemical composition by GC/MS in this study. Ethanol is our solvent as shown in Table 3. The GC/MS analyses allowed 15 compounds to be determined; the main constituents of the grapevine leaves extract were ethanol (91.82), cyclotrisiloxanehexamethyl (1.25 %) and diethoxydimethylsilane (1.14 %) (Table 3). Previous studies showed that, cyclotrisiloxane hexamethyl is well known antimicrobial compunds isolated from different plant species[20].

Table 3. Volatile components of the ethanol extracts of grapevine leaves extracts (GC-MS analysis)

Component[a]	Area(%)	Rt[b]
Acetaldehyde	0.45	3.79
Butanal	0.05	4.24
Cyclotrisiloxane hexamethyl	1.25	4.34
Diethoxydimethylsilane	1.14	4.53
Ethyl acetate	0.22	4.79
Etanol	91.82	5.28
1,3,diethoxy-1,1,3,3 Tetramethyldisiloxane	0.83	6.64
Chloroform	0.60	6.95
2-Mehoxycarbonyl	0.25	7.51
Eucalyptol	0.03	14.41
Pentadecane	0.04	14.53
1,2,3,4 Tetramethylbenzene	0.02	16.20
Phenacyl thiocyanate	0.88	16.27
Methoxyphenyl oxime	0.92	18.31
1,3 Bis trimethylsilyl) benzene	0.21	19.17
Tanýmsýz	0.72	19.31
Total	**99.43**	**-**

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

[b] Retention time (as min).

According to the results from this study there are hopes that ethanolic extract of grapevine leaves may be used for treatment of some resistant types of microorganisms such as *S. typhimurium* CCM 583 and *C. albicans* ATCC 10239. Also it can be used as preserving material for food stuff in food industries. We reported that environmental factors such as geography, temperature, day length, nutrients, etc, were considered to play a key role in the chemical composition of grapevine leaves.

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