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Antioxidant and Antimicrobial Activities of Methanol and Water Extracts of Fruits, Leaves and Seeds of *Vitis vinifera* L. cv. Karaerik

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Antioxidant and antimicrobial studies were performed on methanol and water extracts of leaf, fruit and seed of *Vitis vinifera* L. cv. Karaerik. Lipid peroxidation inhibition, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and the amount of total phenolic compounds present in the extracts were determined. All parts of the plant have antioxidant potential. While seeds have the highest antioxidant potential, fruits have the lowest one. Antimicrobial activities of the above extracts were also tested against 96 clinical isolates of bacteria strains (*Enterobacter aerogenes, Escherichia coli, Proteus miribalis, Pseudomonas aeroginosa, Staphylococcus aureus*) and 90 *Candida* strains (*Candida albicans, Candida glabrata, Candida guillermondii, Candida kefyr, Candida krusei, Candida parapisilosis, Candida pseudotropicalis, Candida tropicalis, Geotricum candidum*) by disc-diffusion method and minimal inhibitory concentration (MIC) values of each active extract were determined. Water extracts of the grape leaves had shown the anticandidal activity against 3 *Candida* spp. (*C. albicans, C. glabrata, C. tropicalis*) and 1 bacteria species (*S. aureus*), and methanol and water extract of it have shown antibacterial activity against only *Staphylococcus aureus* with 13 mm inhibition zone and 0.625 mg/mL MIC value amoung the tested microorganisms. The highest anticandidal activity of leaves extract was found to be in their water extract against *Candida albicans* with 20 mm inhibition zone and 1.25 mg/mL MIC. Each one of the water and methanol extracts of seeds had shown antibacterial activity against three bacteria with inhibition zone range of 10-30 mm and 0.312- 2.5 mg/mL MIC values. The highest activities were shown by water and methanol extracts of the seed against *Staphylococcus aureus* with 30 mm inhibition zone and 0.312 to 0.156 mg/mL MIC values, respectively. Unlike leaves and seeds extracts, none of the fruits extracts had shown antimicrobial activity.

Key Words: *Vitis vinifera*, Antioxidant activity, Antimicrobial activity, DPPH radical scavenging, Total phenolic compounds.

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INTRODUCTION

Modern chemistry and biochemistry studies in nutrition have strongly supported and further developed the idea that components in food can serve as medicine, particularly when the foods are given in extraphysiological dosages[1]. Ethnomedicinal literature contains a large number of plants that can be used against disease, in which reactive oxygen species and free radicals and also some microorganisms play a major role.

Grape (*Vitis vinifera* L.) is one of the most important horticultural fruit crops in the world. The plant is grown for wine, juice, raisins and as fresh fruit[2]. *Vitis vinifera* is a part of the Vitaceae family, which comprises 17 genera, mostly woody or herbaceous lianas primarily inter-tropical in their distribution. It is the only species originating from Eurasia and it has been spread throughout the world by human cultivation[3]. The leaves of the grape are used to stop bleeding, healing wounds and skin diseases[4]. The grape leaves are also used in making a traditional food, called dolma, in which leaves are filled with minced beef, rice and onions then are wrapped with the same leaves and cooked.

Grapes have been recognized as beneficial to human health for a long time[5-8]. Reports concerning the effect of grape on human health have been numerous in the literature. Grapes have antiulcer[9], antihypertensive[10], anti-mutagenic[11], spare vitamin E[12], antistress[13], antiinflammatory activities[14] and inhibitive enzymatic activities[15,16]. These health beneficial effects of grape are based on its antioxidant activity[17,18]. The pharmacological properties of grape seeds are believed that flavonoids (from polyphenols) are its most potent constituents[19]. The mature seeds of *Vitis vinifera* are an important natural source of oligomers and polymers of catechin and epicatechin, which are also denominated procyanidins[20]. Although much work has been done on the antioxidant effects of grape, especially on grape seeds[21-24] there is no more information about the grape leaves. And also there are a few reports on the antimicrobial activity of grapes against clinical isolates of human origin[25-27].

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are various forms of activated oxygen and nitrogen that include free radicals such as superoxide anion ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$) and nitric oxide radicals ($NO^{\bullet}$) as well as non-free-radical species such as hydrogen peroxide (H_2O_2) and nitrous acid (HNO_2). In living organisms, various ROS and RNS can be formed by different mechanisms. Normal aerobic respiration, stimulated polymorph nuclear leukocytes and macrophages and peroxisomes appear to be the main endogenous sources of most of the oxidants produced by the cells[28]. Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents and pesticides[29]. Free radicals can also cause lipid peroxidation in foods that leads to their deterioration.

Oxidation does not only affect lipids. ROS and RNS may cause DNA damage that may lead to mutation[30]. All aerobic organisms, including human beings, have antioxidant defenses that protect against oxidative damage[31]. However, these natural antioxidant mechanisms can be inefficient and hence dietary intake of antioxidant compounds becomes important[32]. Although there are some synthetic antioxidant compounds, there are also some concerns about the side effects of these compounds[33,34]. Therefore, studies on the determination of the natural antioxidants sources are important.

The aim of the present work was to investigate the antioxidant activities of the methanol and water extracts of the Karaerik grape cultivar leaves, fruits and seeds. It was also interesting to find out antimicrobial activities of these grape parts on some human pathogen clinical isolates.

EXPERIMENTAL

The grapes were collected at their optimum commercial maturity when oenologically ripe in Uzumlu Town, Erzincan, Turkey. The fresh fruit samples were packed on ice while being transported to the laboratory. The seeds were removed manually. Fruit samples were frozen at -20 ºC until extraction. The seeds and leaves were dried in shade and powdered with a blender. The plant powdered parts were extracted with methanol in a Soxhlet apparatus for 24 h. Then methanol was evaporated with rotary evaporator. Water extracts were also prepared by adding boiling water to 20 g of powdered material in a glass flask and incubated at room temperature for 2 h. on a rotating shaker (200 rpm). Mixture was filtered using Whatmann No. 1 filter paper and then filtrate was lyophilized. All extracts were stored in freezer at -24 ºC until use.

Antimicrobial activity tests were carried out against clinical isolates of 96 bacterial strains and 90 *Candida* strains. Microorganisms were provided by Department of Clinical Microbiology, Medicine Faculty, Erzurum, Turkey. Microorganism species, isolation origins and numbers are shown for bacteria and *Candida* in Table-1.

Antimicrobial activity

Disc-diffusion assay: The dried methanol and water extracts were dissolved in the extraction solvent (methanol and sterile distilled water). Final concentration was 30 mg/mL. Antimicrobial test were than carried out by disc-diffusion method[35] using suspension containing 10^8 colony forming unit (CFU)/mL of bacteria, 10^6 CFU/mL of yeast spread on nutrient agar (NA; oxoid). The disc (6 mm in diameter) were impregnated with extracts and placed on the inoculated NA. Negative controls were prepared using the same solvents employed to obtain extracts. Ofloxacin (oxoid) for gram-positive bacteria, cefaperazone-sulbactam (oxoid) for gram-negative bacteria and amphotericin B (Sigma) for *Candida* spp. were used as positive controls.

TABLE-1
BACTERIA AND *Candida* SPECIES AND
ISOLATION ORIGINS AND NUMBERS

Microorganisms	Blood	Urine	Wound	Ear	Throat	Mouth	Total
Enterobacter aerogenes	-	12	6	-	-	-	18
Escherichia coli	2	15	4	2	-	-	23
Proteus miribalis	1	9	1	1	-	-	12
Pseudomonas aeruginosa	3	3	10	2	-	-	18
Staphylococcus aureus	10	4	5	3	3	-	25
Candida albicans	8	3	1	-	1	3	16
Candida glabrata	-	5	-	-	3	-	8
Candida guilliermondii	-	-	-	-	8	-	8
Candida kefyr	-	-	-	-	3	5	8
Candida krusei	2	-	3	-	2	-	7
Candida parapsilosis	7	2	-	-	-	2	11
Candida pseudotropicalis	5	4	-	-	-	2	11
Candida tropicalis	7	2	2	-	-	2	13
Geotricum candidum	3	2	2	-	-	1	8
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The inoculated plates were incubated at 37 ºC for 24 h for clinical bacterial strains and at 35 ºC for 48 h for yeast. The antimicrobial activity was then evaluated by measuring the inhibition zone against test microorganisms.

Minimal inhibitory concentration (MIC): The minimal inhibitory concentration (MIC) values were also studied for the microorganisms which were determined as sensitive to the methanol and/or water extracts of plant parts (seed, leaf and fruits) in disc-diffusion assay. MIC values of extracts against microbial strains were determined based on a micro-well dilution method[36]. The inoculations of microorganisms were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Firstly, the extracts dissolved in 10 % dimethyl sulfoxide (DMSO) were diluted to 10 mg/mL and then serial two fold dilutions were made in a concentration range (0.078-10 mg/mL) in a sterile test tube containing nutrient broth (NB). The 96-well plates were prepared by dispensing into each well 95 µL NB and 5 µL of the inoculums. A 100 µL of extracts initially prepared at the concentration of highest concentration was added the first well, then 100 µL from serial dilutions was transferred into other consecutive wells. The plates covered with a sterile plate sealer and then incubated for 24 h (for bacterial strains) and 48 h (for fungal strains). The MIC was defined as the lowest concentration of the extracts to inhibit the growth of microorganisms.

Antioxidant activity: The antioxidant activity was determined according to the thiocyanate method[37]. Briefly, stock extracts solutions were prepared at 2 mg/mL concentration. Required stock solutions were mixed with 2.5 mL of 0.02 M linoleic acid (Fluka) emulsion [contains an equal weight of Tween-20 (Sigma) in pH 7.4 phosphate-buffered saline (Sigma)] and the final volume was adjusted to 5 mL with phosphate-buffered saline (0.02 M, pH 7.4) in a test tube and incubated in darkness at 40 °C. Final concentrations of the extracts were 100 µg/mL. BHT (Sigma) was used as positive control (100 µg/mL). The amount of peroxide was determined by measuring the absorbance at 500 nm after colouring with $FeCl_2$ and thiocyanate after 24 h incubation. Lower absorbance indicates higher antioxidant activity. To eliminate the solvent effect, the same amount of solvent used to prepare the solutions of test samples was added into the control test sample, which contains the linoleic acid emulsion. Measurements of antioxidant activity were carried out for three sample replications and values are the average of three replicates. This activity is given as per cent lipid peroxidation inhibition which is calculated as follows:

$$\text{Lipid peroxidation inhibition (\%)} = \left(\frac{\text{Control Abs.} - \text{Sample Abs.}}{\text{Control Abs.}}\right) \times 100$$

DPPH Radical-scavenging activity: Experiments were carried out as described previously[38]. Briefly, 0.5 mM DPPH (Fluka) radical solution in methanol was prepared and then 1 mL of this solution was mixed with 3 mL of the sample solution. Final concentrations of essential oils were 100 and 300 µg/mL. BHT was used as a positive control at the same concentrations. After incubation for 0.5 h in the dark, the absorbance was measured at 517 nm. Decreasing the absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity. This activity is given as per cent DPPH radical scavenging, which is calculated with the following equation:

$$\text{Activity \%} = \left(\frac{\text{Control Abs.} - \text{Sample Abs.}}{\text{Control Abs.}}\right) \times 100$$

Control contains 1 mL of DPPH solution mixed with 3 mL of ethanol. The measurements of DPPH radical scavenging activity were carried out for two sample replications and values are an average of two replicates.

Determination of total phenolic compounds: Antioxidant compounds generally contain phenolic group(s). Because of this, amounts of phenolic compounds in each of the extracts were compared to obtain more information about the extract(s) which posses(s) antioxidant potential. This was carried out as described previously[37]. Briefly, extract solution was transferred into a tube and then final volume was adjusted to 4 mL by addition of distilled

water. Afterward, 0.25 mL of Folin-Ciocalteu Reactive (FCR) (Fluka) was added into this mixture and after 3 min 0.75 mL of Na_2CO_3 solution was added. Subsequently, mixture was shaken on a shaker for 2 h at room temperature and then absorbance was measured at 760 nm. Amount of total phenolic compounds were carried out for two sample replications and values are an average of two replicates. Gallic acid was used as the standard for a calibration curve. The phenolic compound content was expressed as gallic acid equivalent using the following equation based on the calibration curve:

$$Y = 0.2582X$$

where Y is the absorbance of the sample and X is the gallic acid equivalent (μg mL^{-1}).

Statistical analysis: Statistical analysis was carried by using SPSS 12.0. Values at $p < 0.05$ were considered to be significant and significant at $p < 0.01$.

RESULTS AND DISCUSSION

Antioxidant activities: All extracts were able to inhibit lipid peroxidation (Fig. 1). The most effective one was the water extract of grape seeds with 90 % of inhibition. This was followed by the methanol extract of grape seeds with 75 % inhibition. The lowest activities were measured in both of the methanol and water extracts of fruit with about 22 and 23 % inhibition, respectively. Unlike seed extract, methanol extract of the leaf showed higher inhibition activity then the water extract of leaf with 60 and 42 % inhibition, respectively. In the light of these results (Fig. 1) one could say that the most effective part of the grape is seed. However, any generalization about the extraction solvents could be made.

The highest DPPH radical scavenging activity was measured in the methanol extract of the leaf with 90 % scavenging. This extract effectiveness was followed by leaf water extract and water and methanol seed extract (Fig. 2). The less effective part of the grape, like peroxidation inhibition, was fruit. DPPH scavenging activities were higher in 300 μg/mL leaf then 100 μg/mL leaf or fruit extracts. However, there was no noticeable extract concentration effect in seed extracts.

Similar to antioxidant activity, the highest amount of the phenolic compounds were present in seed extracts (Fig. 3). Like peroxide inhibition and DPPH radical scavenging activities, fruit extracts contain the lowest amount of phenolic compounds.

From the results given in Figs. 1-3, it appears that there is a relation between each of the phenolic compounds amount, DPPH scavenging activity and lipid peroxidation activities.

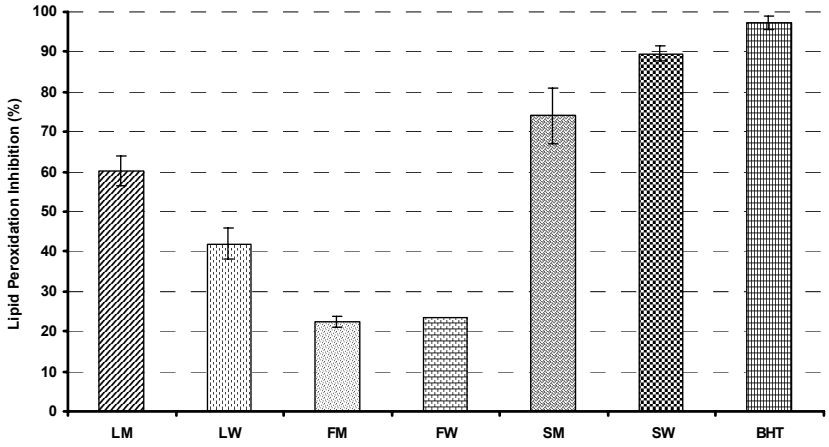

Fig. 1. Inhibition of lipid peroxidation by 100 µg/mL extract and BHT. Measurements were carried out after 24 h incubation at 37 ºC (S = seed, L = leaf, F = fruit, M = methanol extract, W = water extract; BHT = butylated hydroxytoluene)

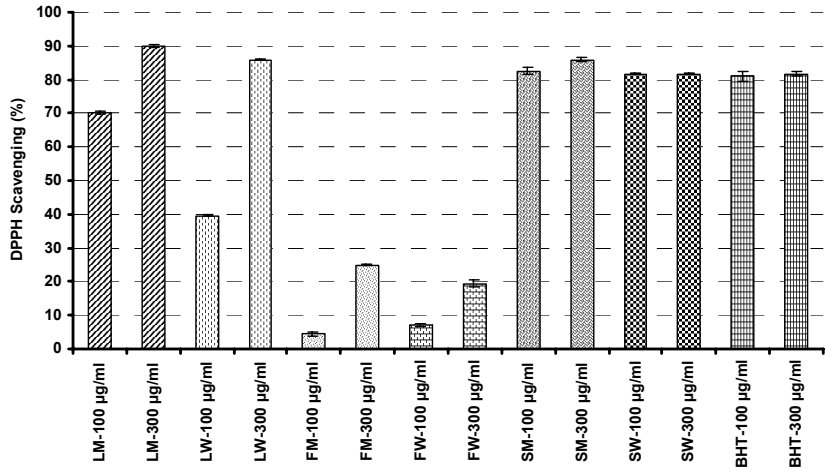

Fig. 2. DDPH radical scavenging activity (S = seed, L = leaf, F = fruit, M = methanol extract, W = water extract; BHT = butylated hydroxytoluene)

In fact according to Pearson correlation test, there is statistically significant correlation between DPPH radical scavenging and phenolic compounds ($r = 0.927$; $p < 0.01$); between peroxide inhibition and phenolic compounds amount ($r = 0.832$; $p < 0.01$); between peroxide inhibition and DPPH radical scavenging ($r = 0.919$; $p < 0.01$).

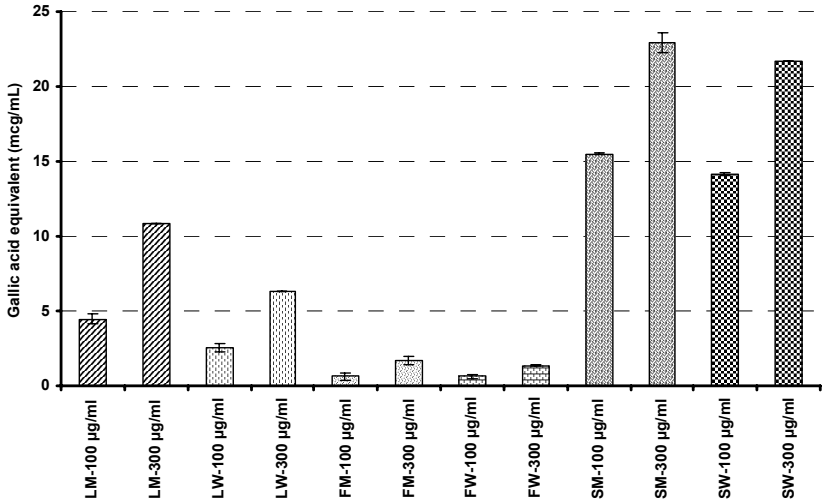

Fig. 3. Amount of total phenolic compounds (S = seed, L = leaf, F = fruit, M = methanol extract, W = water extract)

It has been previously reported that phenolic compounds are called high-level antioxidants because of their ability to scavenge free radicals and active oxygen species such as singlet oxygen, superoxide free radicals and hydroxyl radicals[39]. In addition, these compounds act as antioxidants by metal ion chelating[37]. Therefore, the present results are compatible with the previous ones.

Antimicrobial activities: Antimicrobial activity tests were carried out against clinical isolates of 96 bacterial strains and 90 *Candida* strains, the list of which given in Table-2.

Disc diffusion assay is a standard method widely used for quick screening of natural products for antimicrobial activity. Grape extracts were screened using this method. Then, minimal inhibitory concentration assays were used to determine the concentration at which the extracts are effective. In the present study, minimum inhibitory concentration was determined for extracts, which have ≥ 10 mm inhibition zone diameters by disc diffusion method. The results of the disc diffusion and the minimal inhibitory concentration (MIC) are given in Table-2.

None of the fruit extracts showed any antimicrobial activity against any tested microorganisms. As fruit extracts showed lowest antioxidant potential, this is very interesting. Water extracts of the grape leaves have shown anticandidal activity against 3 *Candida* spp. (*C. albicans, C. glabrata, C. tropicalis*) and 1 bacteria species (*S. aureus*). Methanol and water extracts of it have shown antibacterial activity against only *Staphylococcus aureus* with 13 mm inhibition zone and 0.625 mg/mL MIC value among the tested

TABLE-2
ANTIMICROBIAL ACTIVITIES OF METHANOL AND WATER EXTRACTS OF SEEDS, LEAVES AND FRUITS OF *Vitis vinifera* cv. KARAERIK GRAPE CULTIVARS

Microorganisms	Plant part	Extracts	Inhibition zones (mm)	MIC (mg/mL)
Candida albicans	Leaf	Methanol extract	NA	-
	Fruit		NA	-
	Seed		NA	-
	Leaf	Water extract	20	1.250
	Fruit		NA	-
	Seed		NA	-
Candida glabrata	Leaf	Methanol extract	NA	-
	Fruit		NA	-
	Seed		NA	-
	Leaf	Water extract	15	2.500
	Fruit		NA	-
	Seed		NA	-
Candida tropicalis	Leaf	Methanol extract	NA	-
	Fruit		NA	-
	Seed		NA	-
	Leaf	Water extract	15	2.500
	Fruit		NA	-
	Seed		NA	-
Escherichia coli	Leaf	Methanol extract	NA	-
	Fruit		NA	-
	Seed		15	1.250
	Leaf	Water extract	NA	-
	Fruit		NA	-
	Seed		15	1.250
Pseudomonas aeruginosa	Leaf	Methanol extract	NA	-
	Fruit		NA	-
	Seed		10	2.500
	Leaf	Water extract	NA	-
	Fruit		NA	-
	Seed		10	2.500
Staphylococcus aureus	Leaf	Methanol extract	13	0.625
	Fruit		NA	-
	Seed		30	0.156
	Leaf	Water extract	13	0.625
	Fruit		NA	-
	Seed		30	0.312

NA=no activity, inhibition zone was no greater than 6 mm, - = extract not tested. Negative controls (methanol and water) showed no inhibiting effect. Inhibition diameters and MIC values of positive controls were ranging to 18-20 mm and 0.12-1 µg/mL for ofloxacin, 19-22 mm and 0.12-0.5 µg/mL for cefaperazone and 12-15 mm and 0.5-1 µg/mL for amphotericin B, respectively.

microorganisms. The highest anticandidal activity of leaves extract was found to be water extract against *Candida albicans* with 20 mm inhibition zone and 1.25 mg/mL MIC. Each one of the water and methanol extracts of seeds had shown antibacterial activity against three bacteria with inhibition zone range of 10-30 mm and 0.312-2.5 mg/mL MIC values. The highest activities were shown by water and methanol extracts of the seed against *Staphylococcus aureus* with 30 mm inhibition zone. However, MIC was 0.156 mg /mL for the methanol extract of it and it was 0.312 mg /mL for the water extract.

The same extracts showed some antibacterial activity against *P. aeruginosa* (10 mm inhibition zone; 2.5 mg/mL MIC value) and *E. coli* (15 mm inhibition zone; 1.25 mg/mL MIC value). But there is no activity on other bacteria and fungi (data is not shown). These results were supported by the other researchers in their studies[27]. They reported that 4-20 % grape seed acetone extracts useful for antibacterial agents against some gram-positive and gram-negative bacteria. The leaf extracts have antibacterial activity against only gram-positive bacteria *S. aureus* (13 mm) (Table-2).

The present experiments revealed that grape seed extracts posses a significant antibacterial activity against some testing bacteria. The results are similar to those obtained by Jayapraskha *et al.*[26]. In some studies, the seed extracts of some plant species showed higher antibacterial activity than did other parts of the plant[40,41].

Another issue of interest is none of the extracts of grape seeds produced inhibition zone on *Candida* spp., which have activity on bacteria. Similar results were reported by Palma and Taylor[25]. Only the water extract of leaves have anticandidal activity against three *Candida* spp. (Table-2) while the methanol extracts of leaves have no any effect. This might have resulted from the lack of solubility of the active constituents against *Candida* in methanol solutions. The leaf aqueous extract had the highest activity against *Candida albicans* (1.25 mg/mL MIC). Among the *Candida* species the most sensitive species was *Candida albicans*. Similar results were reported by other investigators[42,43]. Methanol and water fruit extracts had no inhibitory effects on tested organisms, which is in agreement with other reports[27].

Conclusion

In conclusion, the results obtained show strong antimicrobial activity of methanol and water extracts of grape parts (except fruits) against bacteria and fungi used as test organisms. The results demonstrate a correlation between the antioxidant activity and antimicrobial activity of different parts of grape. Seed extracts posses a significant antibacterial activity and leaf extracts also show antifungal activity, which have high antioxidant activities at the same time. Neither methanol nor water extracts of grape fruit had shown antimicrobial activities, which have also weak antioxidant activities.

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