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Research Article

Phytochemical Screening and *In-vitro* Evaluation of Antioxidant and Antimicrobial Activities of the Entire Khella Plant (Ammi visnaga.L.) A member of Palestinian Flora

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ABSTRACT

Objectives: In the developing countries, a large number of people depend on the traditional folk medicine as primary substances in their healthcare systems especially rural areas healers and patients from centuries. An estimated 35,000 to 70,000 plant species are used for medicinal and therapeutic purposes in the world. The objectives of this study were to screen the phytochemical constituents and antibacterial activities also to evaluate antioxidant property of the *Ammi visnaga* (L.) Lam. entire plant. Methods: The *Ammi visnaga* plant was phytochemically screened (acetone, methanolic and aqueous) for the presence of phytogenic chemicals and, their effect on 2,2-Diphenyl-1-picryl-hydrazyl radical (DPPH) was used to determine their free radical scavenging activity. Broth microdilution method was applied to detect antibacterial activity and determine minimal inhibitory concentration (MIC) of aqueous and organic extracts of *Ammi visnaga* (L.). The antimicrobial activity was examined against 3 reference strains namely: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). Moreover, MIC against *Acinetobacter baumannii* clinical strain was included. Results: The results showed that *Ammi visnaga* methanolic extract contains a mixture of phytochemical classes as proteins, tannins, flavonoids, glycosides and steroids and revealed that this plant has high antioxidant activity (IC50=6 µg/ml). While aqueous extract exhibited antimicrobial activity against *S. aureus* (MIC= 25 mg/ml), *E. coli* (25 mg/ml), *P. aeruginosa* (25 mg/ml) and *A. baumannii* (50 mg/ml). Organic extract of *Ammi visnaga* (L.) showed stronger inhibition of bacterial growth, where MIC values for *S. aureus*, *E. coli*, *P. aeruginosa* and *A. baumannii* were 0.35, 2.78, 5.56 and 5.56 mg/ml, respectively. Conclusions: The results of the present study indicated that the *Ammi visnaga* (L.), especially organic extract, exhibited strong antibacterial activity against Gram-positive and Gram-negative bacterial isolates and has high antioxidant activity and therefore it can provide natural source of antibacterial drugs and antioxidants and can be useful in preventing various diseases including cancer

Keywords: *Ammi visnaga* (L.) Lam. Phytochemical screening, Antioxidants, antibacterial activity.

INTRODUCTION

Medicinal plants primary and secondary metabolite constituents have a long history of use in old and modern medicines and in certain systems of traditional medicines, and are considered the sources of important medications such as vincristine, codeine, quinine, morphine, digoxin, atropine and others[1].

Ammi visnaga (L.) Lam. has many English common names, including Khella, Visnaga, Bisnaga, and Toothpick weed. It is a member of the *Apiaceae* (*Umbelliferae*) family; widely and wildly distributed in Asia, Europe, and North Africa[2-4]. Botanically *A. visnaga* is biennial or annual herbaceous plant, growing about 1 m height. Flowers are tetracyclic pentamerous with radial symmetry, bearing inferior ovary and five stamens composed from two united carpels. The inflorescence is a compound umbel of white flowers and highly swollen at the base, later on it becomes woody and used as toothpicks (Fig. 1). Leaves are about 20 cm long with oval triangular shape. The fruit is a compressed oval-shaped structure consisting of two mericarpes and around 3 mm in length. Stems are erect and highly branched[5,6].

Centuries ago, Arabs and other Mediterranean nations discovered that the Khella fruits could relieve a number of ailments, such as acute pain caused by angina pectoris. Further, Khella considered diuretic, antiasthmatic, antipsoriasis, vasodilator and an effective smooth muscle relaxant[7]. In addition to that it was used by Egyptions from ancient times, to relief the severe pain caused by kidney stones[8]. *A. visnaga* has been used in the traditional Jordanian and Palestinian folk medicines for treatment of gastrointestinal cramps, liver disorders and for relieving painful menstruation and inflamed gall bladder[9,10]. Also, *A. visnaga* is used as a supportive treatment in the respiratory conditions such as cough, bronchitis, asthma and whooping cough[11,12], and used in the cardiovascular

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Figure 1: *Ammi visnaga* flower

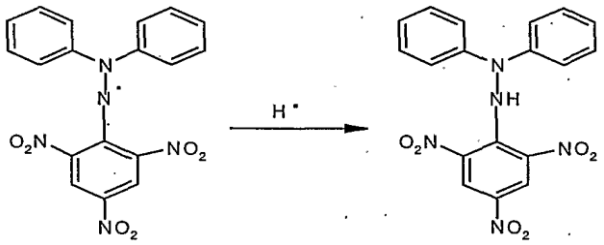

Figure 2: DPPH reduced to DPHH-H

disorders for congestive heart failure, hypertension, cardiac arrhythmias, hypercholesterolemia and atherosclerosis[13,14]. Topically, *A. visnaga* has been found useful in the recovery of psoriasis, vitiligo, skin inflammation conditions wound healing, and poisonous bites[15,16]. *A.visnaga* contains various groups of chemical compounds such as pyrones, saponins, flavonoids and essential oils[17,18]. The major constituents of *A. visnaga* are γ-pyrones (furanochromone derivatives) as khellin and visnagin, visnaginone, ammiol, Khellinol, khellol, visammiol, , khellinin, khellinone.

Another important group of major constituents are coumarins as visnadin, ammoidin, visnagans, , samidin, dihydrosamidin and xanthotoxin[19-22]. Flavonoids flavonol group as kaempferol and quercetin were identified in *A. visnaga* growing in Iraq[23]. Eleven flavonols were isolated from the aerial *A. visnaga* parts. There were four monoglycosides, four aglycones, one triglycoside and two diglycosides. Among the aglycones flavonoids, one was quercetin hydroxylated and three methoxylated (rhamnazin, rhamnetin and isorhamnetin). The mono-glycosides were actually modified rhamnetin, isorhamnetin and rhamnazin with 3-O-glucosides and one isorhamnetin-7-O-glucoside. The two diglycosides were quercetin-3-O-rutin and isorhamnetin-3-O-rutin while the single one was quercetin-7,3,3'-O-triglucoside[17]. The main essential oils of *A. visnaga* were amyl isobutyrate , linalool , methyl-2-isoamyl butyrate and amyl valerate , non-terpene esters and oxygenated isopentyl isovalerate[24]. The current study, DPPH method, which relies on the reduction of 2, 2-diphenylpicrylhydrazyl (DPPH) radical was used. This method is simple, fast and inexpensive for measuring the antioxidant capacity. Furthermore, it is not specific to any particular antioxidant component and could be applied to either sol id or liquid samples. The DPPH with free radical has a purple color and a strong absorption maximum at 517nm. As shown in Figure 2, when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant it will be reduced to DPPH-H, the color becomes yellow and the molar absorptivity of the DPPH radical at 517nm reduces from 9660 to 1640[25]. The anti antioxidants activity is usually compared with a reference standard and a common example is Trolox. Trolox is a (Hoffman-La Roche) trade name for (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid); a water soluble vitamin E analogue used in this research as an antioxidant standard[25,26].

MATERIAL AND METHODS

Instrumentation & Reagents

Reagents: The following reagent were used in evaluation of antioxidant activity: Methanol, n- hexane, Trolox ((s)-(-)-6 hydroxy -2 ,5 ,7 ,8 –tetramethychroman – 2 carboxylic acid), (DPPH) 2 ,2-Diphenyl-1-picrylhydrazyl. For phytochemical screening included the following reaggetns: Millon's reagent , Ninhydrain solution, Benedict's reagent, Molish's reagent , H_2SO_4, iodine solution ,NaOH ,chloroform HCl, magnesium ribbon, acetic acid, $FeCl_3$. Moreover, Nutrient broth (HIMEDIA, India), API 20E (biomerieux, France), Dimethyl sulfoxide (DMSO) and Antibiotics (OXOID, UK) were used in the evaluation of antimicrobial activity.

Instrumentation: Shaker device (Memmert Shaking Incubator, Germany), rotary evaporator (Heidolph OB2000 Heidolph VV2000, Germany), spectrophotometer (Jenway 7135), freeze dryer (Mill rock technology ,model BT85 , Danfoss ,china), grinder (Moulinex model , Uno, China), balance (Rad wag, AS 220 / c/2 ,Poland), filter paper (Machrery-Nagel ,MN 617 and Whatman no.1), Micropipettes (Finnpipette, Finland), Incubator (Nuve, Turkey), syringe filter 0.45 μm pore size (Microlab, China) and micro broth plate (Greiner bio-one, North America).

Collection and preparing plant materials

Ammi visnaga entire plant was collected in July 2014 by Reem Speih from the mountains of Jenin region of Palestine. The plant was botanically identified by Dr. Nidal Jaradat from the Pharmacy Department of the An-Najah National University. Voucher specimen was deposited in the Herbarium of the Pharmaceutical Chemistry and Technology Division (Laboratory of Pharmacognosy) and the plant herbarium code is (Pharm-PCT-139).

The entire plant was washed and then dried in the shade at room temperature until all the plants parts became well dried. After drying process, the plants materials were ground well into fine powder using mechanical blender and transferred into special containers with proper name labeling for future use.

Preparation of plant extracts for phytochemical analysis

The phytochemical extraction was performed using organic solvent extraction as well as aqueous extraction. The organic extraction was performed by Soxhlet extraction method. This extraction was done by taking

Table 1: Phytochemical constituents of Khella plant

Plant	Proteins	Carbo-hydrate	Phenols/ Tannins	Flavo -noids	Saponins	Glyco-sides	Steroids	Terpenoids	Alkaloids
Khella aqueous extract	+	–	+	+	–	+	+	+	–
Khella methanolic extract	+	–	+	+	–	+	+	+	–
Khella acetone extract	–	–	+	+	–	+	+	+	–
Khella ethanolic extract	+	–	+	+	–	+	+	+	–

20gm of dried plant powder and was uniformly packed into a thimble and extracted with 250ml of different solvents separately (ethanol, acetone, and methanol). The extraction process continues till the solvent in siphon tube of an extractor become colorless. After that the extract was heated on hot water bath at 30-40ºC till all the solvent evaporated. Dried plant crude extract was kept in refrigerator at 2-8ºC for their future use.

The aqueous extraction was done by taking five grams of the powdered plant and was placed in a beaker with 200ml of distilled water. The mixture was heated on a hot plate at 30º-40ºC and mixed with continuous stirring for 20 minutes. The mixture was filtered using whatman filter paper filter and the filtrate was used for the phytochemical analysis.

Preparation of plant extracts for biological tests

A total of 25 g of the powder of Khella plant was weighed and exhaustively extracted by adding 50 ml of n-hexane and 125 ml of 50% ethanol in triple distilled water. The mixture was placed in the shaker for 72 hours at 25^0 C with continuous shaking at (200rpm) then filtered by using suction flask and Buchner funnel filtration. The resulting liquid filtrate was separated by separatory funnel into 2 phases. The lower phase, which is the aqueous phase representing the first aqueous extract and the upper phase, which is the organic phase representing the organic extract. The remaining solid filtrate was extracted again by adding 125ml of 50% ethanol in triple distilled water and was placed in the shaking incubator for 72 hours at 25^0C with continuous shaking at (200rpm) as before. After that it was filtered to obtain the second aqueous extract. Both first and second aqueous extracts were pooled together and placed in the rotary evaporator for 1 hour at 40^0C to evaporate any leftover organic solvents. Then they were dried completely in for 24 hours and stored in refrigerator at 4^0C.

Preparation of plant extracts for antioxidant evaluation

About 10g of the grounded plant were soaked in 1 Liter of methanol (99%) and put in a shaker device at 100 rounds per minute for 72 hours at room temperature and stored in refrigerator for 4 days. The extracts were then filtered using filter papers. The extract was then concentrated under vacuum using a rotatory evaporator. The crude extract was stored at 4ºC for further use.

Antioxidant activity

Trolox standard and plant working solutions

A stock solution of a concentration of 1mg/ml in methanol was firstly prepared for the plant extract and trolox. The working solutions of the following concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, 100µg/ml) were prepared by serial dilution with methanol from the stock solution.

Spectrophotometric measurements

DPPH was freshly prepared at a concentration of 0.002% w/v. The DPPH solution was mixed with methanol and the above prepared working concentration in a ration of 1:1:1. The spectrophotometer was zeroed using methanol as a blank solution. The first solution of the series concentration was DPPH with methanol only. The solutions were incubated in dark for 30 minute at room temperature before the absorbance readings were recorded at 517nm.

Table 2: inhibition activity for Trolox standard and Khella entire plant

Concentrations	% by inhibition of Trolox	% by inhibition of Khella
0	0	0
1	43.0284	30.638
2	53.0234	31.064
3	65.174	34.468
5	84.079	36.595
7	88.059	39.148
10	92.039	40.425
20	92.537	45.106
30	93.532	51.915
40	94.029	67.659
50	94.527	71.915
80	95.025	87.659
100	96.019	90.638
IC50	1.542017	6.077668
SD	0.100677	2.149774

Percentage of inhibition of DPPH activity

The percentage of antioxidant activity of the plants and the trolox standard were calculated using the following formula:

Percentage of inhibition of DPPH activity (%) = (A-B)/A ×100%

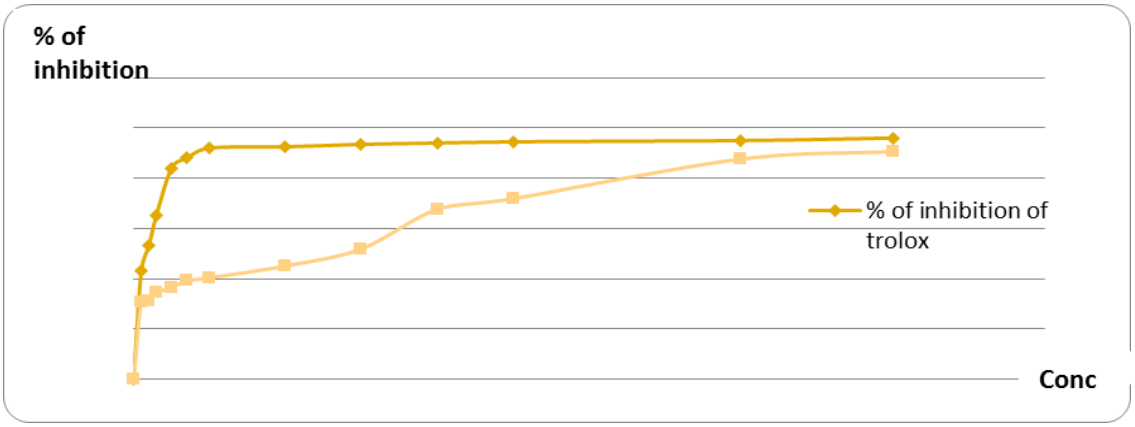

Figure 3: Inhibition activity of Trolox standard and Khella entire plant

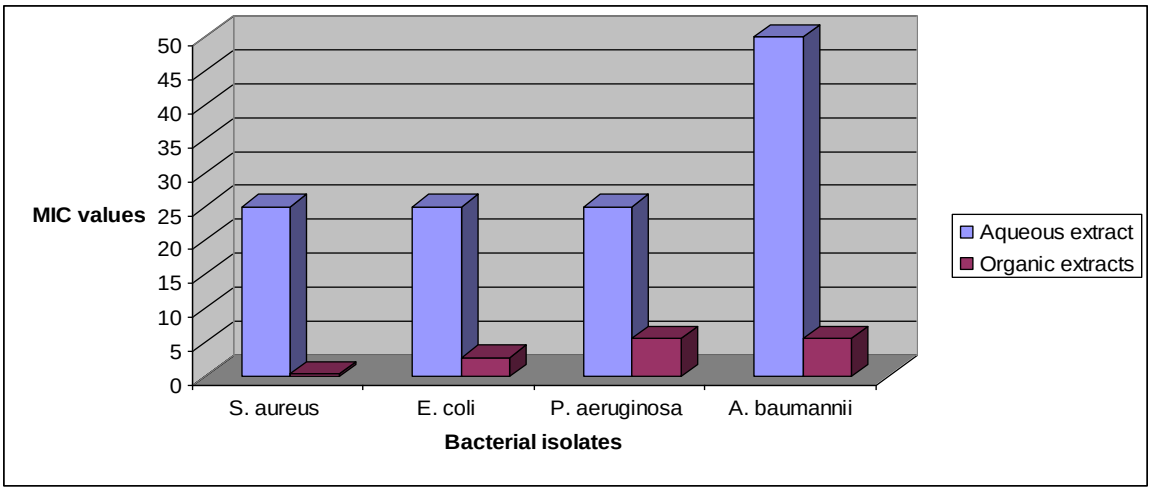

Figure 4: Antibacterial activity of aqueous and organic extracts of *Ammi visnaga* (L.)

where: A = optical density of the blank,
B = optical density of the sample.

The antioxidant half maximal inhibitory concentration (IC50) for the plant samples and the standard were calculated using BioDataFit edition 1.02 (data fit for biologist).

Data analysis

The antioxidant activity was reported as percentage of inhibition. The inhibition of Khella plant and Trolox standard at different concentration were plotted and tabulated and the IC50 for each of them was calculated using the BioDataFit fitting program.

Qualitative phytochemical analysis

The crude extract was tested for the presence of bioactive phytochemical compounds by using following standard identification methods.

Test for proteins

Millon's test: 2ml of Millon's reagent mixed with the entire plant crude extract, appeared white precipitate, which turned into red color upon gentle heating that result confirmed the presence of protein in the plant.

Ninhydrin test: Boil 2ml of 0.2% Ninhydrin solution with the entire plant Crude extract , appeared violet color indicate the presence of proteins and amino acids.

Test for carbohydrates

Fehling's solutions test: Fehling solutions A and B were mixed together in equal volumes and 2ml of it was added to crude plant extract and gently boiled. A red brick precipitate indicated the presence of reducing sugars.

Benedict's reagent test: Boil 2ml of Benedict's reagent with crude entire plant extract, a reddish brown color precipitate formed, which indicated the presence of the carbohydrates.

Table 3: Antibacterial activity of aqueous and organic extracts of *Ammi visnaga* (L.)

Bacterial Isolates	MIC value (mg/ml)	
	Aqueous extract	Organic extracts
S. aureus (ATCC 25923)	25	0.35
E. coli (ATCC 25922)	25	2.78
P. aeruginosa (ATCC 27853)	25	5.56
A. baumannii	50	5.56

Molisch's solution test: Shake 2ml of Molisch's solution with crude plant extract then add 2ml of H_2SO_4 concentrated and poured carefully along the side of the test tube. a violet ring appeared at the inter phase of the test tube indicated the presence of carbohydrate.

Iodine test: 2ml of iodine solution mixed with crude plant extract. Purple or dark blue color indicated the presence of the carbohydrate.

Test for tannins and phenols

2ml of 2% solution of $FeCl_3$ mixed with crude extract. Black or blue-green color indicated the presence of tannins and phenols.

Flavonoids tests

Shinoda test: fragments of magnesium ribbon and concentrated HCl were mixed with crude plant extract after few minutes pink colored scarlet appeared which indicated the presence of flavonoids.

Alkaline reagent test: 2ml of 2% NaOH solution was mixed with plant crude extract, intensive yellow color was formed, which turned on addition of few drops of diluted acid to colorless solution, which indicated the presence of flavonoids.

Saponins test

5ml of distilled water added to crude plant extract in a test tube and it was shaken vigorously. The foam formation indicated the presence of saponins.

Glycosides test

Liebermann's test: 2ml of chloroform and 2ml of acetic acid mixed with entire plant crude extract. The mixture was then cooled to ice. Carefully added H_2SO_4 concentrated. A color change from violet to blue to green indicated the presence of steroidal nucleus, i.e., aglycone portion of glycoside.

*Salkowski's test:*2ml of concentrated H_2SO_4 was added carefully to the entire plant crude extract and shaken gently. A reddish brown color indicated the presence of steroidal ring, i.e., aglycone portion of the glycoside.

Test for steroid

2ml of chloroform and concentrated H_2SO_4 were mixed with the entire plant crude extract. in the lower chloroform layer produced red color that indicated the presence of steroids.

Another test was performed by mixing 2ml of each of acetic acid with H_2SO_4 concentrated and crude extract with 2ml of chloroform. Green color indicated the presence of steroids.

Test for terpenoids

Crude entire plant extract was dissolved in 2ml of chloroform and evaporated to dryness then 2ml of H_2SO_4 concentrated was added to the mixture and heated for about 2 minutes. A grey color indicated the presence of terpenoids.

Antibacterial Test

Antibacterial activities of aqueous and organic extracts of *Ammi visnaga* (L.) were examined against 3 reference bacterial strains obtained from the American Type Culture Collection (ATCC), which were *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). Morover, in the current study we included a mutidrug-resistant clinical isolate of *Acinetobacter baumannii* obtained from Rafedia hospital in Nablus district in Palestine. Identification of *A. baumannii* was confirmed by Gram-stain and API 20E. Using disc diffusion method[27], *A. baumannii* was found to be resistant to Ampicillin, Ceftazidime, Cefotaxime, Cefpodoxime, Nalidixic acid and Ciprofloxacin. The detection of *Ammi visnaga* (L.) antibacerial activity for both the organic and aqueous extracts of *Ammi visnaga* (L.) were determined using broth microdilution method. The applied method was similar to that of CLSI[27,28]. Briefly, 100 mg/ml aqueous extract solution was prepared in sterile distilled water. Organic extract was dissolved in 100% Dimethyl sulfoxide (DMSO) achieving a concentration of 178 mg/ml. The resulting solutions were sterilized by syringe filter with 0.45 μm pore size. Plant extract solutions were serially diluted (2-fold) 11 times with nutrient broth. Well number 11 was considered negative control of bacterial growth, while well number 12 contained nutrient broth only and was used for positive control of bacterial growth. The achieved 10 concentrations of aqueous and organic plant extract were from 0.098 to 50 mg/ml and 0.174 to 89 mg/ml, respectively. For detection of any possible antibacterial activity of DMSO in broth microdilution method condition, a serial 2-fold dilution of DMSO with nutrient broth was prepared with concentration form 0.098% to 50%. The final bacterial concentration in each well (except negative control) was adjusted to 5×10^5 CFU/ml. After inoculation of bacteria, the plates were covered and incubated at $35^{\circ}C$ for 18 hours. Each bacterial isolate was examined in duplicate. The lowest concentration of plant extract that did not allow any visible bacterial growth in the test broth was considered minimal inhibitory concentration (MIC).

RESULTS

Phytochemical screening

The phytochemical characteristics of Khella plant were summarized in Table 1. It could be seen that proteins, phenols, tannins, falvonoids, glycosides and steroids were present in khella plant. In the other hand carbohydrates, terpenoids and saponins were absent.

Antioxidant capacity

The free radical scavenging activity of the methanolic extract of Khella has been tested by DPPH radical method using Trolox as a reference standard. The concentration ranged from 1–100µg/ml. The zero inhibition was considered for the solution, which contained only DPPH without any plant extract. The result revealed a high antioxidant activity with IC50 =6µg/ml which comparable to Trolox stanrd which has an IC50 of 1.5 µg/ml The detailed results are shown in Table 2 and Figure. 3

Antibacterial activity

The MIC values of aqueous and organic extracts of *Ammi visnaga* (L.) against examined bacterial isolates are shown in Table 3 and Figure 4.

Aqueous extracts of *Ammi visnaga* (L.) possessed antibacterial activity (MIC = 25mg/ml) against *S. aureus* reference isolate, which is Gram-positive bacterium. In addition *Ammi visnaga* (L.) aqueous extract was found to exhibit similar antimicrobial activities against Gram-negative bacterial isolates of the present study. MIC values of aqueous extracts against *E. coli* and *P. aeruginosa* were identical, which were equal to 25mg/ml. Aqueous extract of *Ammi visnaga* (L.) had lower level of activity (MIC= 50mg/ml) against *A. baumannii*. Antimicrobial activity of organic extract of *Ammi visnaga* (L.) was stronger than the aqueous extract and

variations among species were obvious. Among examined isolates, the strongest activity of organic extracts was against Gram-positive bacteria (*S. aureus*). Lower level of inhibition was observed when Gram-negative isolates were examined. However, the activity of organic extract against Gram-negative bacteria was much higher than the aqueous extract. The organic extract MIC values for *E. coli* , *P. aeruginosa* , and *A. baumannii* were 2.78, 5.56 and 5.56 mg/ml, respectively. Variation of antimicrobial activity of organic extracts against bacterial isolate of the current study may be related to the presence (and degree of permeability) of outer membrane in Gram-negative bacteria and not Gram-positive bacteria. DMSO was used for dissolving organic extract. MICs of organic extract were found in wells of microbroth dilution that contained very low concentration of DMSO (0.195 - 3.125%), which did not possess any inhibition of bacterial growth. DMSO inhabited bacteria growth at 6.25-12.5% concentration depending on bacterial species examined.

CONCLUSION

In conclusion, *Ammi visnaga* (L.) has a high content of phenolic compounds and a high antioxidant activity, therefore they can be used to treat several diseases in which there is an increase in free radical production also *Ammi visnaga* (L.) extract, possess antibacterial activity against Gam-positive (*S. aureus*) and Gram-negative bacterial (*E. coli*, *P. aeruginosa* and *A. baumannii*) isolates of the present study. In addition, antibacterial activity of *Ammi visnaga* (L.) organic extract was stronger than the aqueous extract. However, further studies are needed to identify which phytogenic phenols are responsible for the antioxidant activity of the entire plant, and assess the way in which the phytogenic substances contribute to this activity. In addition to that *in vivo* antioxidant assays are needed to confirm the potential use of this plant in the treatment un curable diseases as Alzheimer.

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