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Original Research Article

Fungal contamination of *Ammi visnaga* seeds, antimicrobial activity of the plant seeds secondary metabolites and detection of alkaloids and non-alkaloids compounds

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K e y w o r d s

Ammi visnaga; mycoflora; antmicrobial activity; secondary metabolite; active components; microorganisms.

A B S T R A C T

In the present study, we describe the mycoflora of the *Ammi visnaga* where forty-seven species in addition to 2 species varieties belonging to 23 genera were isolated from the plant seeds on glucose and cellulose-Czapek□s agar at 28°C by using dilution-plate method. The most frequently encountered fungal species on the two types of media were: *Aspergillus flavus, A. nigers, A.sydowii , A.terreus* var.*aureus* and *Emericella nidulans* . Aqueous, methanol, ethyl acetate and n-butanol extracts of the plant seeds were tested at different concentrations against 17 pathogenic and non-pathogenic fungi. These extracts were also evaluated for their activity against some pathogenic bacteria .The aqueous Khella extract lost approximately 20% of its relative activity after one month storage at 4°C and also at 25°C. Most of the studied microbes showed high sensitivity to all tested fractions. The active components of methanolic khella extract was screened by using TLC technique as it showed high antimicrobial activity against the tested microorganisms. The developed TLC plate revealed that possible presence of 6 compounds of alkaloids with different Rf values in case of using chloroform system and also 2 compounds in case of using chloroform: methanol system and staining with Dragendroff's reagent and also 3 compounds of non-alkaloids by using chloroform: methanol: H_2O system with different Rf values.

Introduction

Ammi visnaga, a member of the family Umbeliferae (Apiaceae). It has slightly aromatic odor and a very bitter taste. Portions of the plant are made into toothpicks (Mabberley *et al*, 1987). The fruits have been used in Egyptian folk medicine as diuretics and for the treatment of kidney and bladder stones (Franchi *et al*, 1985). Khella also has been used for the traditional management of diabetes in Israel (Yaniv *et al*, 1987). Visnagin, which is found in *Ammi visnaga*, has biological

activity as a vasodilator and reduces blood pressure by inhibiting calcium influx into the cell (Lee *et al*., 2010). *Ammi visnaga* contains coumarins and furocumarins (psoralens), the most important of which are khellin and visnagin. Khellin is present in fruits in a concentration of approximately 1% and visnagin in a concentration of approximately 0.3% (Martelli *et al.*, 1984). Generally, natural medicinal products have a wide fungal bioburden higher than raw materials of synthetic origin or substances of biological origin isolated in pure state. Some of these fungal contaminants can be toxinogenic species that involve potential hazard to human and animal health (Rizzo *et al*. 1998). Bugno *et al.* (2006) evaluated ninety-one samples of medicinal plants for the fungal contamination and mycotoxigenic potential of *Aspergillus and Penicillium* isolated from the samples. Results indicated that the predominant mycoflora was distributed in 10 genera. From these 89.9% of the isolates corresponded to genera *Aspergillus and Penicillium*. Medicinal plants have been employed for centuries as remedies for human diseases, as they harbour components that have been recognized for their therapeutic value. Recently, the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics have compelled researcher to evaluate the antimicrobial activities of certain medicinal plants (Hammer *et al*., 1999; Maoz and Neeman, 1998). Maoz and Neeman (1998) studied the inhibitory effect of aqueous extracts of 10 plants among them *Ammi visnaga* against *Trichyophyton rubrum* and *Microspoum canis*, the aetological agents of dermal fungal infections in humans. Aqueous extracts were also evalulated for their activity against some bacteria. He found that aqueous extracts from the leaves of *Inula viscola* produced detectable antifungal activity against these dermatophytes. The extract of *Ammi visnaga* was also effective against these dermatophytes. In Egypt, Mahmoud (2002) tested the effect of five different concentrations (2, 4, 6, 8, 10 mg ml^{-1}) of an aqueous extracts of *Lipunus albus*, *Ammi visnaga* and *Xanthium pungens* on growth and aflatoxin production by *Aspergillus flavus*. He found that all the plants inhibited mycelial growth and aflatoxin formation. The inhibitory effect of these extracts was proportional with their concentrations. Low concentrations of *Ammi visnaga* had no or slightly effect on fungal growth and aflatoxin production, whereas high concentrations inhibited fungal growth and, consequently aflatoxin formation. Maximum inhibitory effect was recorded at 10 mg ml^{-1} concentration of extract. El-Mougy and Abdel-Kader (2007) studied the antifungal effect of 20 powdered spice and their extracts among them *Ammi visnaga* and *Cymbopogon proximus* at concentrations of 2, 4, 8 and 1, 3, 6 respectively against soilborne fungi causing damping-off disease. He found that concentration of 8% powdered spices and 6% of their extract were able to cause complete inhibition of tested fungi.

Materials and Methods

Collection of plant samples

Twenty samples of *Ammi visnaga* seeds were collected from different locations in Qena Governorate. Samples were put in a sterile polyethylene bag, sealed and kept in another bag which was also sealed. Storage in a double-bag container minimizes the loss of water content and gives sufficient aeration. Samples transferred immediately to the laboratory

and kept in a cool place (5°C) for fungal determination.

Determination of Mycoflora

The dilution- plate method was used for the estimation of fungal flora associated with *Ammi visnaga* seeds as described by Christensen (1963) and employed by Moubasher *et al*., (1972; 1980). Ten g of each sample was suspended in 100ml sterile distilled water inside 500ml conical flasks. Preliminary trials showed that dilution 1:100 was suitable to obtain reasonable number of fungal colonies in agar cultures of *Ammi visnaga*. Fifteen ml of melted glucose- and cellulose-Czapek□s agar medium, cooled at 45°C, were poured over the suitable suspension in petriplates which were swirled to distribute the suspension. Four replicates were prepared and cultures were incubated at 28°C, for 7days. The developing fungi were counted, examined and identified (based on macro-and microscopic characteristics) and the numbers were calculated as colonies per gram samples.

Antimicrobial Activity

Tested Micro-organisms

The micro-organisms used in this study consisted of: five common fungal species (*A.flavus, A.niger, C.spicifer, F.dimerum, M.circinelloides*) as well as four crop threatening pathogenic fungi, (*Alternaria alternata*, *Cochliobolous spicifer*, *Stachybotrys atra* var.*microspora*, and *Ulocladium botrytis*) isolated from *Vicia faba*. Fungal species were incubated on Potato glucose agar (PGA) (Potato, 200 g; Glucose, 20 g; Agar, 20 g and 1000 ml distilled water) for 7 days at 28C.

Eight dermatophytic fungi (*Candida albicans*, *Candida tropicalis*, *Candida krusei*,*Epidermophyton floccosum*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Trichophyton verrucosum* and *Microsporium canis*) collected from Assuit fungal centre. Dermatophytic fungi employed in the screening on Sabouroud glucose agar medium at 28C. Sabouroud glucose agar (SGA) is composed of Glucose, 40 g; Peptone; 10 g; Cyclohexamide, 0.5 g; Agar, 20 g per 1L distilled water for 15 days.

Three pathogenic bacteria (*Staphylococcus aureus* MARSA, *Escherichia coli* and *Salmonella typhi*) collected from Bacteriology laboratory at Qena were grown on nutrient agar at 37C (Nutrient agar composed of (peptone, 5 g; beef extract, 3 g; agar, 15 g).

Preparation of Plant Aqueous Extract

Powdered samples (100gm) of *Ammi visnaga* seeds were macerated with 1000 ml sterile distilled water in a blender for 10 min. The macerate was first filtered through double –layered muslin cloth followed by centrifugation at 4000rpm for 30 min. at room temperature. The supernatant was filtered through Whatmann No. 1 filter paper and sterilized, which served as the mother extract (Satish *et al*. 2007). For evaluation of antifungal activity of the extracts, percentage dilutions i.e. 15%, 20%, 30% and 40% of extract were obtained by adding appropriate of standard basic stock solution to stock media.

Antimicrobial Activity Assay

For screening of antimicrobial activity of powdered ingredients of *Ammi visnaga* poisoned food technique was followed (Sinha *et al*. 1993). Potato Dextrose Agar (PDA) medium was prepared and

sterilized. The medium was supplemented with different serial dilutions of aqueous extracts i.e. 15, 20, 30, & 40% (stock solution). About 15ml of this medium was poured into each petriplate and allowed to solidify. Ten mm disc of 7-day-old culture of each fungus were placed at the centre of the each petriplate and incubated at 28°C for 3, 4, & 5 days . After incubation, the colony diameter was measured in millimeter (mm). For each treatment group (for a given percentage of extract) four replicates were maintained. PDA medium without the aqueous extract was taken as control but in case of dermatophytes we used Sabouroud Dextrose Agar (SDA) medium. For pathogenic bacteria we used Nutrient Agar (NA) medium. The fungitoxicity of the extracts was taken in terms of percentage inhibition of mycelial growth was calculated by using the following formula:

$$\% \text{ inhibition} = dc\text{-}dt/dc \times 100$$

Where dc= Average increase in mycelial growth in control, dt = Average increase in mycelial growth in treatment (Singh and Tripathi, 1999)

Stability test

Stability test was carried out on the aqueous extract of *Ammi visnaga.* The extract was divided into two parts, and stored at 4 and 25°C (ambient temperature) for one month. The extract was then assayed for the antimicrobial activity.

Preparation of Organic Extract

Ten grams of dried plant material was successively extracted with 100 ml of methanol, 100 ml n-butanol and100 ml ethylacetate kept on a rotary shaker for 24 h at room temperature (Al-Fatimi *et al*. 2007). The solvent concentrated under vaccum, weighted and kept in frozen (-20C) for antimicrobial assays.

Antimicrobial Activity Assay

Antimicrobial activity was studied using filter paper disk diffusion method (Benson, 1990). The degree of growth inhibition was evaluated after 24hr for bacteria and 48hr for fungi and compared with the growth inhibition results obtained from the controls.

Identification of the active components in methanol extract of *Ammi visnaga*

Bioautography of TLC

Methanol extract of *Ammi visnaga* seeds (5µl/spot) was spotted on a TLC plate. After drying, the plate was running to detect the occurrence of both alkaloids and non-alkaloids. For detection of alkaloids two systems were used, the first one contain chloroform only and the second was chloroform: methanol (9:1). Non-alkaloids compounds were detected using chloroform: methanol: H_2o system (6:3:0.5).

Staining of TLC plates

The developed TLC plates were dried to remove solvents and sprayed with Dragendroff's reagent to detect alkaloid spots (Sreevidya and Mehrotra, 2003). For detection of non-alkaloids, the developed TLC plates were sparyed with *P*-anisaldehyde reagent (15g anisaldehyde in 250 ml ethanol and 2.5ml sulfuric acid) and heated at 60^{o}C.

Results and Discussion

Mycoflora associated with *Ammi visnaga*:

The dilution plate method showed that *Ammi visnaga* seeds were highly contaminated with various types of fungi where thirty-nine species in addition to 2 species varieties belonging to 21 genera were isolated from the plant seeds on glucose-Czapek□s agar at 28°C and twenty-four species in addition to 1 species variety belonging to 15 genera were isolated on cellulose- Czapek□s agar at 28°C. The most frequently encountered fungal species on the two types of media were: *Aspergillus flavus,A. niger,A.sydowii, A. terreus* var. *aureus,,* and *Emericella nidulans.* Data shown in Table (1). The results agreed with that obtained by Bungo *et al*., 2006. reported that the predominant mycoflora on ninety-one samples of medicinal plants was distributed in 10 genera. From these 89.9% of the isolates corresponded to genera *Aspergillus and Penicillium*. Moharram *et al*. (1989) and Regina and Roman, (1992) reported that the fungi of *Aspergillus* spp., *Penicillium* spp, *Rhizopus* spp. and *Fusarium* spp. are contaminated (anise, cumin, coriander, carawy and fennel) which the most important medicinal and aromatic seeds in Egypt and in the world.

Antimicrobial Activity

The aqueous extract of Khella (*A.visnaga)* showed antimicrobial activity against the tested fungi and bacteria (14/20 species), where the activity varied between high, moderate and low. *A.visnaga* extracts showed strongest inhibition of *C.spicifer* mycelium followed by *A. niger*, *M. circinelloides* and *A. flavus* with an average of inhibition of 70.39, 54.6, 38.3 and 42.23%, respectively. For the other recipient species, the inhibition average was 22.17% for *A. alternata* and only 15.1% for *E. coli* (fig.1). The results obtained also in agreement with Maoz and Neeman (1998) studied the inhibitory effect of aqueous extracts of 10 plants among them *Ammi visnaga* against *Trichophyton rubrum* and *Microspoum canis*, the aetological agents of dermal fungal infections in humans. Aqueous extracts were also evalulated for their activity against some bacteria. He found that aqueous extracts from the leaves of *Inula viscola* produced detectable antifungal activity against these dermatophytes. The extract of *Ammi visnaga* was also effective against these dermatophytes. The storage of aqueous Khella extract for one month at 4°C or 25°C resulted in a loss of 20% activity against tested organisms but the fresh extract has been reported to be effective against tested organisms 70% of tested isolates. This decrease in activity after storage for one month at 4 or 25°C.destroyed some of inhibitory components of it (fig. 2), similar results obtained by Arora and Kaur(1999), phytopathogenic fungus *C.spicifer* was sensitve only to Khella aqueous extract after storage at 4°C(24.5mm) for one month and this increase in activity may due to preserve their antifungal activity or formation of a new antimicrobial agent. Similar results obtained by Lee *et al*. (2007).

The organic extracts (Methanol, Ethylacetate and N-butanol) were more effective than the aqueous extract where it showed high antifungal activity against organisms growth (16/20 species) where the activity varied between high, moderate and low. Results showed in figures (3).

Table.1 Average total counts , maximum values(calculated per g dry seeds in all samples), number of cases of isolation (NCI, out of 20 samples) and occurrence remarks (OR) of fungal genera and species recovered from 20 samples of *Ammi visnaga* seeds on glucose- and cellulose- Czapek's agar at 28°C

Genera and specie	Glucose		Cellulose	
	ATC±SD (MV)	NCI&OR	ATC±SD (MV)	NCI&OR
Acremonium	125 ± 1.892969 (4)	3 L	50 ±0.57735 (1)	2 R
A.furactum	-	-	25± 0.5 (1)	1 R
*A.fusidiode*s	100 ±1.414214 (3)	2 R	25 ±0.5 (1)	1 R
A.strictu	25 ±0.5 (1)	1 R	-	-
Alternaria	150 ± 1.914854 (4)	2 R	-	-
A.alternata	75 ± 0.957427 (2)	1 R	-	-
A citri	75± 1.5 (3)	1 R	-	-
Aspergillus	19275 ±12.44655 (204)	20 H	17975 ±10.24288 (195)	20 H
A.clavatus	25 ±0.5 (1)	2 R	625± 1.892969 (9)	3 L
A.flavus	1675 ± 3.774917 (20)	18 H	2025± 3.201562 (23)	13 H
A.fumigatus	1875± 2.5 (22)	L 4	2025± 3.201562 (23)	5 M
A.niger	9400± 11.74734 (104)	20 H	9750± 7.325754 (108)	19 H
A.ochraceus	300± 1.414214 (5)	3 L	-	-
A.sydowii	5400 ±6.831301 (63)	6 M	4225± 8.261356 (54	5 M
A.tamarii	50± 0.57735 (1)	1 R	-	-
A.terreus var.*africanu*s	200 ±1.825742 (4)	2 R	-	-
A.terreus var. *aureus*	350± 2.081666 (6)	7 M	500± 2.94392 (8)	5 M
Circinella	-	-	25± 0.5 (1)	1 R
C. muscae	-	-	25± 0.5 (1)	1 R
Cochliobolus	325± 2.217356 (6)	6 M	50± 1 (2)	1 R
C.bicolor	100± 1.414214 (3)	1 R	-	-
C.lunatus	50 ± 1 (2)	1 R	-	-
C.spicifer	175 ± 0.957427 (3)	5 M	50± 1 (2)	1 R

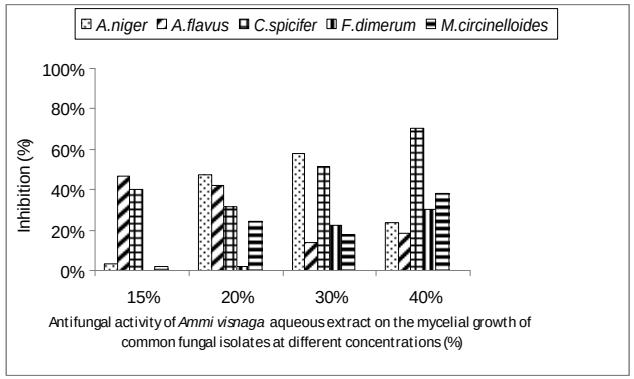
Table (1): Cont.

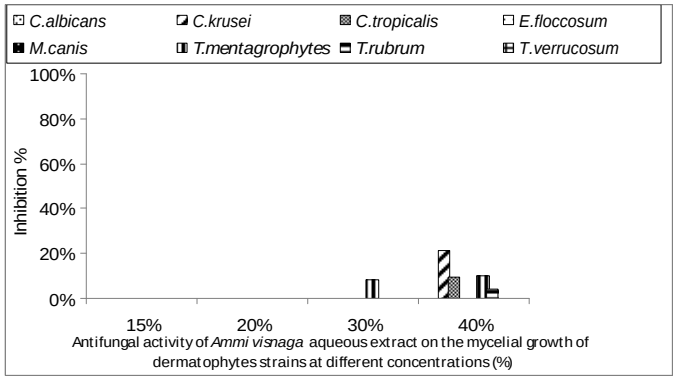
Genera and specie	Glucose		Cellulose	
	ATC±SD (MV)	NCI&OR	ATC±SD (MV)	NCI&OR
Coleophoma cylindrospora	-	-	75 ±1.5 (3)	1R
Curvularia	25± 0.5 (1)	1R	25 ± 0.5 (1)	1R
C. ovoidea	25± 0.5 (1)	1R	25 ± 0.5 (1)	1R
Drechslera	100± 0 (1)	2R	-	-
D. biseptata	25± 0.5 (1)	1R	-	-
D. fugax	50 ± 0.57735 (1)	1R	-	-
D. indica	25 ± 0.5 (1)	1R	-	-
Emericella	575 ± 1.707825 (8)	7 M	1350± 2.886751 (17)	7 M
E. nidulans	575± 1.707825 (8)	7 M	1350± 2.886751 (17)	7 M
Eurotium	1025± 4.856267 (13)	4 L	25 ±0.5 (1)	1 R
E. chevalieri	1025± 4.856267 (13)	4 L	25 ±0.5 (1)	1 R
Fusarium	175± 1.5 (4)	5M	175± 1.5 (4)	1 R
F. dimerum	-	-	175± 1.5 (4)	1 R
F. oxysporum	25± 0.5 (1)	1 R	-	-
Gibberella	150 ±1.732051 (3)	3L	-	-
G. fujikuroi	25± 0.5 (1)	1 R	-	-
G. gordonii	125± 1.5 (3)	2 R	-	-
Humicola	50 ±0.57735 (1)	1 R	-	-
H. grisea	50 ±0.57735 (1)	1 R	-	-
Hypocrea	25± 0.5 (1)	1 R	-	-
H. semiorbis	25± 0.5 (1)	1 R	-	-
Monographella nivalis	25± 0.5 (1)	1 R	-	-
Mucor	25± 0.5 (1)	1 R	50± 0.57735 (1)	2 R
M. circinelloides	-	-	50± 0.57735 (1)	2 R

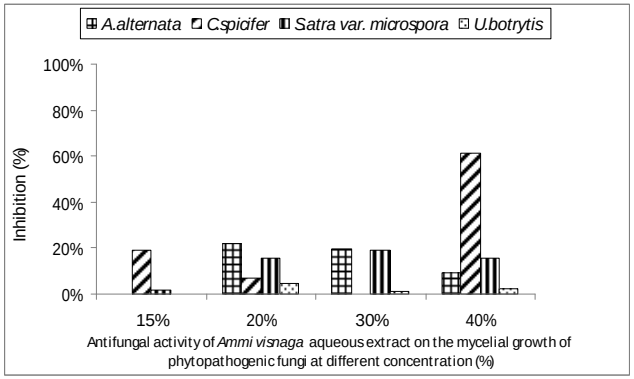
Table (1): Cont.

Genera and specie	Glucose		Cellulose	
	ATC±SD (MV)	NCI&OR	ATC±SD (MV)	NCI&OR
M. hemalis	25± 0.5 (1)	1 R	-	-
Penicillium	250± 1.732051 (4)	7 M	400 ±2.94392 (8)	3 L
P. camembertii	50± 0.57735 (1)	1 R	25 ±0.5 (1)	1 R
P. chrysogenum	75 ±0.957427 (2)	3L	25 ±0.5 (1)	1 R
P. duclauxii	-	-	200± 1.414214 (4)	1 R
P. funiclosum	25± 0.5 (1)		-	-
P. griseofulvum	-	-	100± 0.816497 (2)	1 R
P. jensenii	50± 0.57735 (1)	1 R	-	-
P. oxalicum	25± 0.5 (1)	1 R	-	-
P. viridicatum	25± 0.5 (1)	1 R	50± 1 (2)	1 R
Phoma	100± 1.414214 (3)	1 R	100± 0.816497 (2)	2 R
P. eupyrena	-	-	25± 0.5 (1)	1R
P. exigua	100± 1.414214 (3)		-	-
P. medicaginis	-	-	75± 0.957427 (2)	1R
Plectosphaerella cucumerina	25± 0.5 (1)	1 R	-	-
Scopulariopsis	25± 0.5 (1)	1 R	-	-
S. brevicaulis	25± 0.5 (1)	1 R	-	-
Setosphaeria rostrata	100 ±1.414214 (3)	3 L	-	-
Stachybotrys	75± 0.957427 (2)	3 L	125 ±1.258306 (3)	2 R
S. melanopasamma pomiformis	75± 0.957427 (2)	3 L	125 ±1.258306 (3)	2 R
Sterile mycelia (white & dark colour)	150± 0.57735 (2)	5 M	225± 2.061553 (4)	4 L
Gross total count	22775±17.4045(240)		20700 ± 10.24695(221)	
Number of genera =23	21		15	
Number of species=47+2var.	39+2var.		24 +1var.	

Fig. 1 Antimicrobial activity of *Ammi visnaga* aqueous extracts on the growth of tested fungi and bacteria at different concentrations (%).







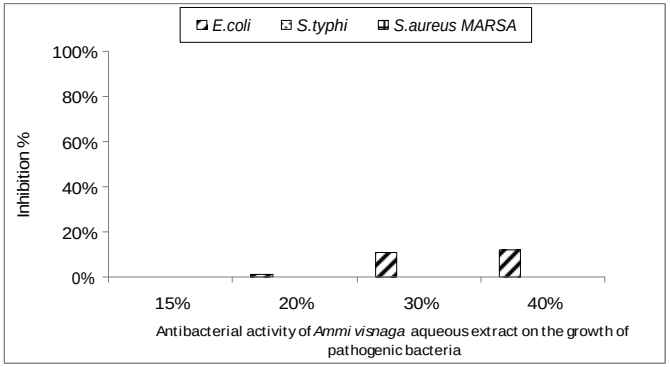
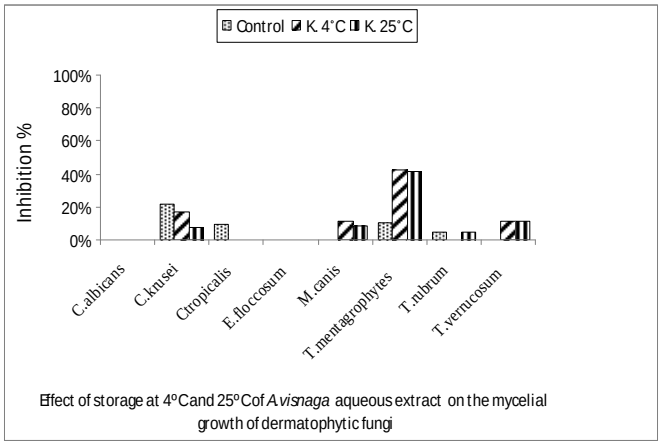
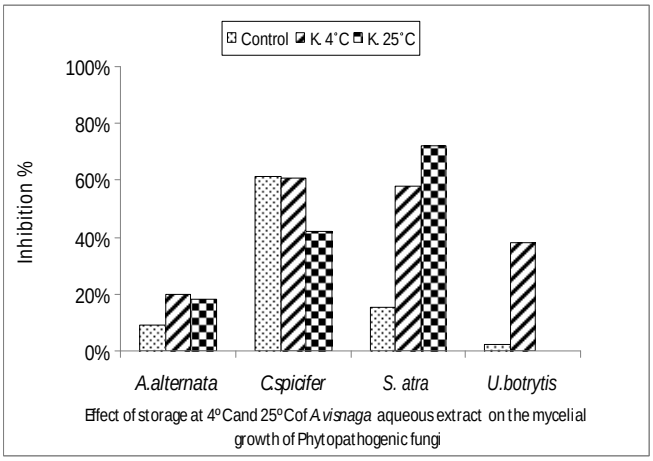


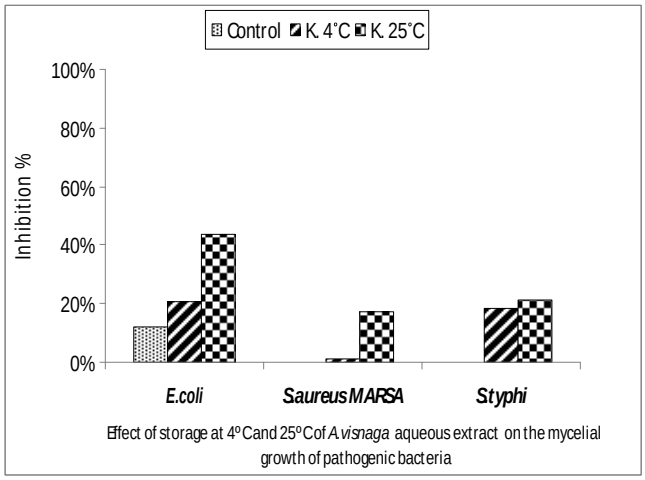
Fig.2 Effect of storage at 4□C and 25□C on the aqueous extract of *Ammi visnaga*.



Effect of storage at 4ºC and 25ºC of *A.visnaga* aqueous extract on the mycelial growth of dermatophytic fungi

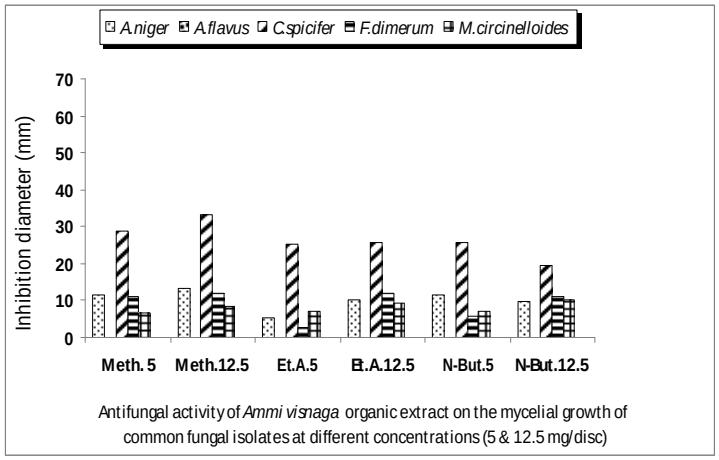


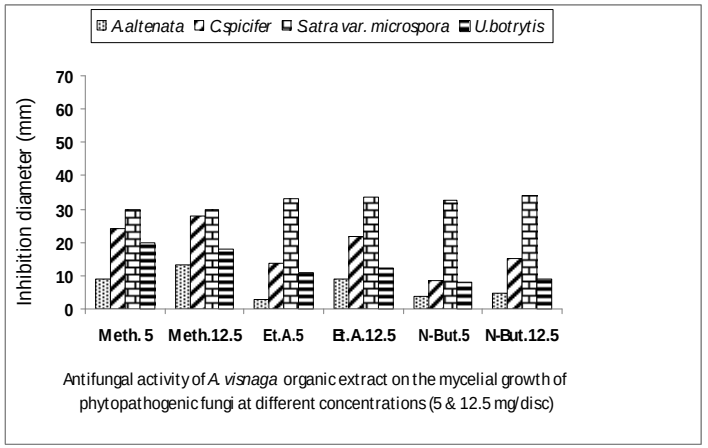
Effect of storage at 4ºC and 25ºC of *A.visnaga* aqueous extract on the mycelial growth of Phytopathogenic fungi

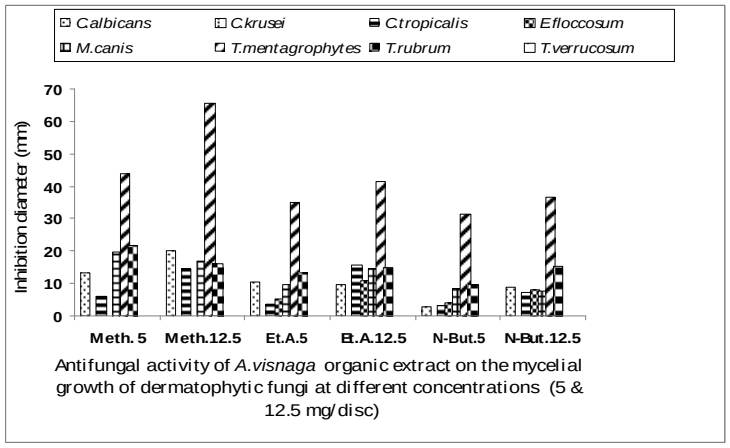


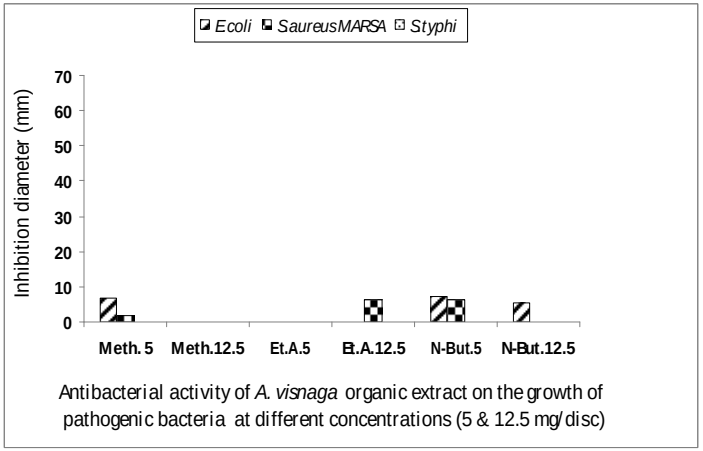
Effect of storage at 4ºC and 25ºC of *A.visnaga* aqueous extract on the mycelial growth of pathogenic bacteria

Fig.3 Antimicrobial activity of *Ammi visnaga* organic extract on the growth of tested fungi and bacteria at different concentrations (mg/disc)



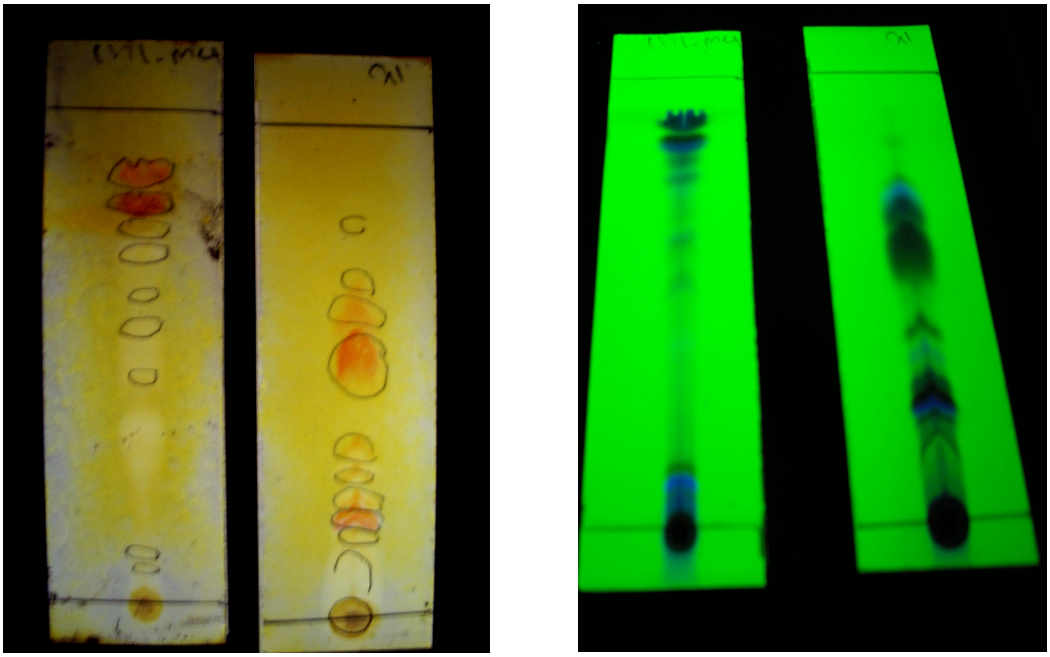






The present results are inconformity with other studies: El- Mougy and Abdel - kader (2007) studied the antifungal effect of 20 powdered spices and their extracts among them *Ammi visnaga* at concentrations 2,4 and 8% against soilborne fungi causing damping-off disease .He found that concentration of 8% powdered spices of its extract was able to cause complex inhibition of tested fungi .Moderate inhibitory effect was observed with other tested species among them *Ammi visnsga.* Dababneh *et al.* (2008) studied the antimicrobial activity of crude extracts from five commonly used medicinal plants in Jordan among them *Ammi visnsga.* He found that *A.visnaga* extract was effective against *Candida albicans* and possessed the highest inhibitory effect on *E.coli* at MIC 800 ppm (DIZ=9mm). The developed TLC plate revealed that possible presence of 6 compounds of alkaloids with different Rf values in case of using chloroform system (0.21, 0.25, 0.3, 0.3625, 0.5375, 0.6375) and also 2 compounds in case of using chloroform: methanol system (0.8125, 0.8625) and staining with Dragendroff's reagent and also 3 compounds of non-alkaloids by using chloroform: methanol: H_2O system with different Rf values (1, 0.825, 0.675) and staning with *P*-anisaldehyde reagent (Plate 1. A). Visualization of TLC spots under UV and compared to those sprayed with Dragendroff's reagent shows that other compounds apart of alkaloids are present in the extract. Using these developing systems mentioned above, the other UV absorbed spots may be terpenoidal

Plate.1 Detection of alkaloids compounds in methanol extraction of *Ammi visnaga* seeds using two running systems chloroform: methanol (9:1) (left plate) and chloroform (right plate) and sprayed with Dragendroff's reagent (A) , and alkaloids and non- alkaloid compounds in methanol extraction of *Ammi visnaga* seeds using two running systems chloroform: methanol: water (6:3:0.5) (left) and chloroform (right) under UV.



A **B**

compounds where phytochemical screeing tests confirmed the presence of such compounds (Plate 1.B).

Preliminary phytochemical (TLC) analysis of methanol extract of *A. visnaga* showed that the antifungal principles are alkaloids (Farnsworth and Fong, 1969) (Plate. 1 A) and also non-alkaloids (Plate. 1 B) (many be terpenoidal compounds). This is not surprising, since saponine have known to posses antifungal activity in many plant species (Osbourn, 2003). Further work is progressing on the purification of active antifungal compounds in *Ammi visnaga*.

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