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CHEMICAL COMPOSITION, ANTI-INFLAMMATORY AND ANTIMICROBIAL ACTIVITY OF ALGERIAN *AMMI VISNAGA* ESSENTIAL OIL

Amira Amraoui[1]*, Fouad Bahri[1] and Juergen Wanner[2]
[1]Laboratory of Microbiology and Plant Biology, Faculty of Nature and Life Sciences, Abdelhamid Ibn Badis University, Mostaganem, Algeria
[2]KurtKitzing Co., HintermAltenSchloss, Wallerstein, Germany
Email*: amiraamraoui@yahoo.com

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ABSTRACT

The essential oil obtained by hydrodistillation from the aerial part of *Ammi visnaga* L., harvested in the northwest of Algeria was analyzed and its antimicrobial and anti-inflammatory activities were studied. 26 compounds were identified by GC-FID and GC-MS representing 95.1% of the total oil. The major components found were limonene (27.8%), p-cymene (19.9%), carvacrol (18.7%), thymol (14.2%) and gamma-terpinene (10%). The EO was slightly toxic with an LD_{50} of 500 mg/kg. The antimicrobial activity showed that all strains tasted were sensitive to the EO, with diameter halos ranging from 17.73 to 36.1 mm in the disc diffusion method, the results of MIC and MBC-MFC showed that the activity was microbicidal against all the tasted strains. Concerning the anti-inflammatory activity, *Ammi visnaga* EO reduced the carrageenan induced paw edema in rats by 56.35% after 6 hours.

Keywords **:** *Ammi visnaga*, Essential oil, Chemical composition, acute toxicity, Antimicrobial activity, Anti-inflammatory activity.

Introduction

Currently, antibiotics impact and chronic inflammatory diseases are considerable and become one of the main health problems of the world's population (Balouiri *et al*., 2016; Olajide *et al.,* 1999), There is a need to develop new antimicrobial and anti-inflammatory agents with minimum side effects. Essential oils (EOs), aromatic oily liquids extracted from plants, are known especially for their antibacterial properties (Amatiste *et al*., 2014) and for their significant anti-inflammatory activities (Riella *et al*., 2012). *Ammi visnaga* L. also called the Khella or Noukha in the Maghreb is widely distributed in the North of Algeria (Brahmi *et al.*, 2014), is a short annual or biennial herb indigenous to the Mediterranean region of North Africa, Asia, and Europe (Khalil *et al*., 2020). Since ancient times, preparations of *A. visnaga* were used in Middle East as a diuretic and to treat urinary tract pain (Günaydin and Beyazit 2004), they have been used in Egyptian folk-medicine against kidney and bladder stones, and in western medicine against asthma and angina pectoris (franchi *et al*., 1987). Another important effect of this plant is relaxation of the smooth muscle, but this function is limited due to its side effect (Koriem *et al*., 2019), and it's well known for its antispasmodic activity in the coronary vessels of the heart and bronchi (Sellami *et al*., 2013). Our objective through this study is to determine the chemical composition of the essential oil obtained from the aerial part of *Ammi visnaga* L., widely growing in the Béni-saf region in the north-west of Algeria, and to evaluate its acute toxicity in vivo, its antimicrobial activity against seven reference strains which are responsible for nosocomial infections and its anti-inflammatory activity.

Materials and Method

Plant Material

The aerial part of *A. visnaga* were collected in May 2019 from Béni-saf region in the north-west of Algeria (longitude 1°23'1''O, Latitude 35°18'8''N and altitude 25m).The samples were identified by Mr Hachemi Benhassaini, professor at Sidi Bel Abbess University, A voucher specimen was deposited (A.v-A.A 27. 2019) at the laboratory of Microbiology and Plant biology, Mostaganem.

Essential oil extraction

The aerial part of *A. visnaga* was subjected to hydrodistillation (200g) for 3h using a Clevenger-type apparatus (Khalfallah *et al*., 2011). The essential oil was stored at freezing temperature until further analysis and bioassays.

GC-FID and GC-MS analyses

1 µl of EO sample was injected in the GC at a split ratio of 1/100. Enriched fiber was placed in the GC injector and the fiber desorbed for 1 minute at 250ºC. GC-FID and GC-MS analyses were performed in one run and one GC with the help of a MS-FID-splitter consisting of a quartz Y-splitter and a short (ca. 20 cm) 0.1 mm ID fused silica restrictor column as an inlet to the GC-MS interface and a ca. 1 m x

0.25 mm deactivated fused silica column serving as a transfer line to the FID detector. The restriction column brakes the flow into the MS vacuum and prevents entering combustion gases from the FID which is operated at atmospheric pressure. The flow in the analytical column must be greater than the inflow to the MS detector which is restricted to about 1 ml/min by means of the restriction line. The GC column flow must be held constant otherwise the FID-MS split ratio changes with temperature. This configuration gives a FID and a MS chromatogram with similar retention times. A Thermo Fisher Scientific ISQ Mass Spectrometer was used for substance identification, with GC-MS interface warming at 250°C, ion source 230°C. The oven temperature program was 60°C for 1 Min, then warmed up to 230°C at a rate of 3°C/Min. Thermo Xcalibur 2.2 software was operated for identifying the compounds by matching up mass spectra to databases of NIST 08, Wiley 8th ed, Adams library (Adams 2007), MassFinder terpenoids library and our own library. Retention indices are determined according to the method of van den Dool and Kratz (Van den dool and Kratz 1963). Quantification was carried out by normalized peak area calculations of the FID chromatogram without relative FID-response factors.

Pharmacological tests

(i) Animals

Wistar rat (female) were obtained from Pasteur institute (Algeirs-Algeria). They were maintained at 22±3°C and on 12h light/dark cycle and they were acclimatized to laboratory conditions for one week (OECD, 2001).

(ii) Acute oral toxicity

The acute oral toxicity was tasted according to the OECD Guidelines N°423 (OECD, 2001). Rats were fasted for 18h with access to water, the EO was solubilized in tween 80 (1%) and then suspended to provide a 100 mg/ml solution in water (Faria *et al*., 2011), to be administered orally to groups of rats (n=3) a doses of 50, 300 and 2000mg/kg. Animals were continuously observed during 2 hours to detect changes in autonomic or behavioral responses and monitored for any mortality for the following 48 hours and then for 7 days, if no death occurred, the test was repeated at higher doses until 5000mg/kg.

(iii) Anti-inflammatory activity

The anti-inflammatory activity was studied by the inhibition of hind paw edema induced by a single sub-plantar injection of carrageenin (Landucci *et al*., 1995). Rats were fasted for 18h with access to water; they were divided into four groups of five animals each (n=5). Group 1, control, received the vehicle (Tween 80, 10mL/kg), Group 2, standard received reference drug (diclofenac, 10 mg/kg). Groups 3 and 4 received EO of *Ammi visnaga* at doses of 50 and 100 mg/kg respectively. Edema was induced by injecting carrageenan (0.1mL) 30 min after oral drug administration into the sub-plantar region of the right hind paw. Using digital vernier calipers, paw volume was measured before injection and 1, 2, 3, 4, 5 and 6 hours after (Vesudevan *et al*., 2006). Edema inhibition percentage was calculated as follows:

$$\%\text{inhibition} = [(V_t - V_0)_{\text{control}} - (V_t - V_0)_{\text{tasted}}] / V_t - V_0)_{\text{control}}$$

Statistical analysis

The results were statistically analyzed with one-way ANOVA followed by Tukey's HSD test, using R software. Values with $p < 0.05$ were considered statistically significant.

Antimicrobial activity of essential oil

(i) Microbial strains

The EO of *A. Visnaga* was tasted against four gram negative bacteria (*Escerichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 35659 and *Klebsiella pneumoniae* ATCC 700603), two gram positive bacteria (*Bacillus cereus* ATCC 10876 and *Staphyllococcus aureus* ATCC 6538) and one fungal strain (*Candida albicans* ATCC 10231), we chose these strains because they are responsible for nosocomial infections, they were provided by the Institute Pasteur of Algiers (Algeria).

(ii) Disc diffusion method

The antimicrobial activity of *A. Visnaga* EO was rated by disc diffusion method (aromatogram); the aromatogram is a qualitative method to explore the antimicrobial activity of a substance (Amatiste *et al*., 2014). Little sterilized disks of blotting paper (6mm) saturated with with 15 µL of crude EO were placed on the surface of a Müeller Hinton plate count agar previously spreaded with 100 µL of microbial suspensions prepared in sterile 0.9% NaCl solution and adjusted to McFarland 0.5. After a latency period at 37°C for 24 h, the diameter of the inhibition halo was measured with a caliber (Amatiste *et al*., 2014; Bismarck *et al*., 2019). Antibiotic and antifungal discs served as a positive antibacterial control to check the growth of different strains. The measurements of inhibition zones were carried out in triplicates.

(iii) Determination of minimum inhibitory concentration (MIC)

The MIC was determinated by the broth macrodilution method. The EOs were prepared by emulsion then two fold serial dilutions in agar solution (0.2%) (Ousso *et al*., 2004). Final concentrations ranging from 0.781 to 50µL/mL were tested against the selected microbial strains.0.2 mL of oil dilution was added to 1.8 mL of bacterial culture in Mueller Hinton broth and 0.1 mL of each EO dilution was added to 0.9 ml of PDA broth inoculated with fungal culture, the tubes were incubated at 37°C/24 h for bacteria and at 25°C/48 h for yeast (Koné *et al*., 2007). A tube containing nutrient broth and inocula but no EO was used as negative control, while tubes containing sterile broth and each EO concentration were used as positive control. Each oil concentration was assayed in triplicate. The lowest one, which did not show any visual growth, was determined as MIC.

(iv) Determination of minimum bactericidal and fungicidal concentration (MBC- MFC)

The tubes which showed no visible growth during the determination of the MIC were subcultured on Muller Hinton agar plate and incubated at 37°C for 24h for bacteria and on PDA agar plate and incubated at 25°C for 48h for yeast. The low concentration of EO showing no growth on the agar surface is noted as MBC/MFC (Ousso *et al*., 2004).The experiment was performed in triplicate.

Results and Discussion

Essential oil extraction

The EO of *Ammi visnaga* was obtained by steam distillation with a yield of 1.78%. This yield is higher than those obtained from the same species in Algeria, collected in Constantine with 1.3% (Khalfallah *et al.*, 2011) and in boumerdes with 0.16, 0.32 and 0.48 % (mL/100 g of dried plant before flowering, flowering and after flowering respectively (Brahmi *et al.*, 2014). It's also higher than those obtained in morocco and Tunisia with 0.27% essential oil (mL/100g of dried material) (Satrani *et al*., 2004), 0.4 to 1.5 % (mL/100 g of dried material (Zrira *et al*., 2008), and 0.2% yields (V/W), based on dried weight of samples (Khadhri *et al*., 2011).

Essential oil analyses (GC-FID and GC-MS)

The *Ammi visnaga* EO was analyzed by GC-FID and GC-MS methods. The compounds obtained, their concentrations and retention indices are given in Table 1. In total, 26 compounds were identified in the aerial part, accounting for 95.1% of the total oil. The major component found was limonene (27.8%), followed by p-cymene (19.9%), carvacrol (18.7%), thymol (14.2%) and gamma-terpinene (10%), the other components are present with small amounts (Table 1). There were considerable differences qualitatively and quantitatively in comparison to the results previously reported in the *Ammi visnaga* essential oils analyses, in Algeria Brahmi *et al.* (2014), have indentified 49 components in essential oils obtained by hydrodistillation of *Ammi visnaga* from Boumerdes, the main one was 2-methylbutyl 2methyl butanouate (28.56%). Khadhri *et al.* (2011) studied the essential oil of *Ammi visnaga* from tunisia and indentified 41 compounds. Moroccan *Ammi visnaga* essential oil is characterized by the presence of amyle isobutyrate, linalol, 2-methyl-butyrate, isoamyle and du amyl valerate (Satrani *et al*., 2004; Zrira *et al*., 2008). khalil et al (2020) state that Variation in the chemical composition of essential oils and their yields may be associated with variations in the environment, geographic origins and differences in biotypes.

Table 1 : chemical composition (%) of *Ammi visnaga* essential oil from Algeria.

Compound	IR	%Area
alpha-thujene	931	0,19
alpha-pinene	940	1,02
Sabinene	978	0,04
beta-pinene	984	0,07
Myrcene	990	0,33
Verbenene	1009	0,24
delta-3-carene	1016	0,06
alpha-terpinene	1021	0,23
p-cymene	1028	19,94
Limonene	1034	27,84
gamma-terpinene	1063	9,96
p-cymenene	1093	0,26
Linalool	1098	0,04
alpha-thujone	1111	0,15
beta-thujone	1122	0,02
trans-limonene oxide	1143	0,05
Camphre	1153	0,02
Menthol	1172	0,04
terpinene-4-ol	1185	0,97
alpha-terpineol	1196	0,08
dihydro carvone	1202	0,05
cis-p-mentha-1(7),8-dien-2-ol	1232	0,23
Carvone	1249	0,09
Thymoquinone	1253	0,28
Thymol	1283	14,23
Carvacrol	1290	18,71
Somme		95,14
tr. = trace ; RI=Retention index		

Acute oral toxicity

Concerning the acute toxicity study of *A.visnaga* EO, mortality was observed in all rats 18 hours after treatment at the dose of 2000 mg/kg. Sedentary and salivation were also noted during the first hours after treatment with this dose and with the dose of 300 mg/kg. According to the guidelines (OECD, 2002), the lethal dose 50 of *A.visnaga* EO is LD50=500mg/kg, and according to the Hodge and Sterner Toxicity Scale (Ahmed and Azmat, 2014), we conclude that the EO is slightly toxic.

Anti-inflammatory activity of essential oil

The results of the evaluation of anti-inflammatory activity by carrageenan-induced paw edema in rats are given in Table 4 and Figure 1. The paw volume increased progressively after carrageenan injection reaching its peak at four hours in the control group. In the standard group, treated with diclofenac, the inflammation started to decrease after 2 hours, and compared to the control group, it has very significantly reduced the paw volume ($p<0.001$), with a percentage of inhibition of 68.80%. While, the *Ammi visnaga* EO have significantly reduced the paw volume ($P <0.05$) at the highest dose tested(100mg/kg), with a percentage of inhibition of 56.35%.The development of carrageenin-induced oedema is bi-phasic, the first phase (1-2 hours) is attributed to the release of histamine, 5-HT and kinins, while, the second phase (3-6 hours) is sustained by the release of prostaglandins, leukotrienes, lysozymes, proteases, nitric oxide (NO) and also by local neutrophil infiltration (Banerjee *et al*., 2000; Abdelli *et al*., 2018). COX-2, is an inducible enzyme found in activated inflammatory cells, plays a crucial role in cytokine production and prostanoid mediator release (Fachini-queiroz *et al*., 2012). Thymol has an important action on the COX enzymes (Veras *et al*., 2013), and a study made by Riella and her collaborators, showed the important role of thymol on inflammatory response with an inhibition of 35.3% ($p<0.001$) on the edema response (Riella *et al*., 2012). Previous research has indicated that carvacrol suppresses the LPS-induced of COX-2, which can cause repression of inflammation (Tsai *et al*., 2011). Thus, the *Ammi visnaga* EO (consist of 14.2% thymol and 18.7% carvacrol) exhibited a significant anti-inflammatory activity in this study.

Table 2: Anti-inflammatory activity of *Ammi visnaga* essential oil on paw edema induced by carrageenan in rats.

Treatment		Control	Standard	HE 50	HE 100
Paw thickness (mm)	1H	6,08 ± 0,14	5.94 ± 0,19	6,15 ± 0,14	6,31 ± 0,08
	2H	6,17 ± 0,11	5,69 ± 0,21	5,93 ± 0,16	5,76 ± 0,24
	3H	6,05 ± 0,23	5,4 ± 0,13	6,10 ± 0,26	5,37 ± 0,15
	4H	6,42 ± 0,17	5,54 ± 0,15	6,43 ± 0,33	5,79 ± 0,09
	5H	6,41 ± 0,23	4,89 ± 0,25***	6,51 ± 0,29	5,83 ± 0,19
	6H	6,27 ± 0,07	4,66 ± 0,27***	5,56 ± 0,14	5,02 ± 0,15*

Control: vehicle (tween 80, 1%); Standard: Diclofenac.*p<0.05; **p<0.01; ***p<0.001

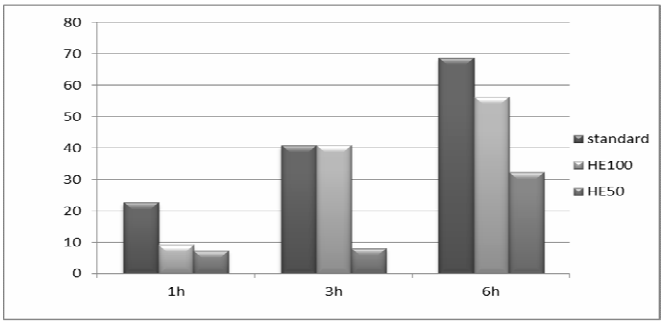

Fig. 1 : Inhibition (in %) of paw edema by *Ammi visnaga* EO and diclofenac during 6 hours after carrageenan injection.

Antimicrobial activity of essential oil

The results of antimicrobial activity of *Ammi visnaga* essential oil using disc diffusion method are given in table 2. All strains tasted were sensitive to the EO with diameter halos ranging from 17.73 to 36.1 mm. the EO showed the best antimicrobial activity against *Bacillus cereus* ATCC10876, *Staphylococcus aureus* ATCC6538 and *Candida albicans* ATCC10231 with 33.97 mm, 36.1 mm and 32.57 mm inhibition zone diameters respectively. The antibacterial effect of *A. visnaga* EO was significantly greater than the antibacterial effect of antibiotics and the antifungal. We compared our results with those of Khalfallah *et al.*, 2011 who studied the antimicrobial activity of *Ammi visnaga* EO on several strains, the oil showed low activity for *Staphylococcus aureus* ATCC 43300 (25mm) and better activity for *Escherichia coli* ATCC 25922 (29mm) and *Pseudomonas aeruginosa* ATCC 27853 (25mm). On the other hand, Brahmi *et al*., 2014 obtained a better result for *Escherichia coli* ATCC25922 (30mm) but low activity for *Staphylococcus aureus* ATCC43300 (15mm), *Pseudomonas aeruginosa* ATCC27853 (16mm) and *Proteus mirabilis* ATCC49565 (15mm) and *Klebsielle pneumoniae* ATCC 33495 (14mm). Our essential oil showed better growth inhibition against Gram-positive strains than Gram-negative ones (Table 2), the observed difference in the sensitivity was due to the difference in the cell wall structure (Trombetta *et al*., 2005), the presence of an outer membrane in Gram-negative bacteria hinders the diffusion of the essential oil through the membrane to the cytoplasm of the cell, making them more resistant to the action of the oil (Teneva *et al*., 2019).

Table 3: Antimicrobial activity of *Ammi visnaga* essential oil and standard drugs using disc diffusion method (inhibition zone diameters in mm)

Strains	*A.visnaga EO*	Céfalexine	Doxycycline	Econazole
B. cereus ATCC10876	33.97 ± 0.34	24.5 ± 0.40	18.56 ± 0.41	–
E. coli ATCC 25922	22.53 ± 0.37	23.83 ± 0.84	0	–
P. mirabilis ATCC 35659	24.93 ± 0.33	21.83 ± 0.23	08.66 ± 0.62	–
P.aeruginosa ATCC 27853	19.47 ± 0.50	22.4 ± 0.40	10.16 ± 0.23	–
S. aureus ATCC 6538	36.1 ± 0.22	15.5 ± 0.40	14.06 ± 0.32	–
K. pneumoniea ATCC 700603	17.73 ± 0.54	14.16 ± 0.84	15.13 ± 0.18	–
C. albicans ATCC 10231	32.57 ± 1.44	–	–	24.16 ± 0.62

The results of MIC and MBC-MFC of *A.visnaga* EO are showed in Table 3, based on these results we notice that the activity was microbicidal against all the tasted strains. MBC/MIC or MFC/MIC ratio of an antimicrobial substance inferior or equal to 4, can be considered as bactericidal or fungicidal, but if the ratio is superior to 4, then it is bacteriostatic or fungistatic (Marmonier, 1990). The lowest MIC and MBC values were determined to be 1.56 µl/ml. concerning *Pseudomonas aeruginosa* ATCC27853, the antimicrobial activity was a little weak than the other strains. Several studies have focused on the antimicrobial effects of *Ammi visnaga* EO, showing their effectiveness against various microorganisms such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* strains (Khalil *et al*., 2020).

Table 4: Antimicrobial parameters values of *Ammi visnaga* EO and standard drugs.

Strains	***Ammi visnaga EO*** (µL/mL)	
	MIC	***MBC-MFC***
B. cereus ATCC10876	1.56	1.56
E. coli ATCC25922	3.12	6.25
P. mirabilis ATCC35659	3.12	3.12
P. aeruginosa ATCC27853	12.5	50
S. aureus ATCC6538	12.5	12.5
K. pneumoniae ATCC700603	12.5	25
C. albicans ATCC10231	3.12	3.12

The antimicrobial activity of *Ammi visnaga* EO could be attributed to its main components, the mechanism of action of terpenes is not fully understood but it is speculated to involve a damage of the plasma membrane stability with subsequent membrane disruption by the lipophilic compounds (Teneva *et al*., 2019). Limonene could demolish the cell wall morphology, and cell membrane could lead to the leakage of some intracellular substances, such as extravasation of protoplasms, thusly leading to cell death. (Han *et al*., 2019). Zahi *et al*., 2017, found that the spherical structure of *Staphylococcus aureus* and *Saccarimyces cerevisiae* and the bacilliform structure of *Escherichia coli* and *Bacillus subtilis* show different forms of distortion and deformation after being treated with limonene. Carvacrol and Thymol, phenolic monoterpenoids structurally very similar (Rua *et al*., 2019), their antimicrobial effect occurs when the

hydrophobic moiety of these molecules interacts with the hydrophobic domain of the cytoplasmic membrane of bacterial cells, and the presence of free hydroxyl groups of these compounds disrupting the ion gradients of bacterial cells (Rua *et al*., 2019; Sim *et al*., 2019). Carvacrol interacts with the lipid bilayer of the cytoplasmatic membrane and itself aligns between the fatty acid chains causing the expansion and destabilization of the membrane structure and increasing its fluidity and permeability (Nastro and Papalia, 2012). P-cymene improves the antimicrobial properties of other substances, such as carvacrol, through synergism, antagonism and additive effects (Marchese *et al*., 2017). It's possible that the components in lower percentage might be involved in synergism with the other active compounds (Shunying *et al*., 2005).

Conclusion

To conclude, the present work aims to enhance the value of the Algerian flora, and more particularly, *Ammi visnaga* L., growing spontaneously in the northwest of Algeria, by evaluating some of its biological properties, such as antimicrobial and anti-inflammatory activity. The extraction by hydrodistillation of the EO of *A. visnaga* gave a yield of 1.78%. This oil was analyzed by GC-FID and GC-MS methods, 26 compounds were identified in the aerial part, representing 95.1% of the total oil. The major component found was limonene (27.8%), p-cymene (19.9%), carvacrol (18.7%), thymol (14.2%) and gamma-terpinene (10%). According to the guidelines (OECD), our EO was slightly toxic and the lethal dose 50 was 500mg/kg. The evaluation of the antimicrobial activity, by the aromatogram method, showed a great inhibitory effect of the essential oil on all the reference strains tested with diameter halos ranging from 17.73 to 36.1 mm, the results of the MIC and MBC/MFC showed that the activity was microbicidal. In regards to the anti-inflammatory activity, the EO reduced the carrageenan induced paw edema in rats by 56.35% ($p<0.05$) after 6 hours.

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