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CHEMICAL COMPOSITION, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF *ALLIUM HIRTIFOLIUM* ESSENTIAL OIL

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ABSTRACT

Allium hirtifolium belongs to Alliaceae family is traditionally used as flavoring agent and as natural remedy for treatment of infectious diseases.
In this study, we analyzed *A. hirtifolium* essential oil by GC and GC-MS; the antioxidant and antimicrobial activity of *A. hirtifolium* essential oil were evaluated *in vitro* condition.
5-chloroorcylaldehyde (55.1%), methyl methylthiomethyl disulfide (24.6%) were the major components of oil. The antioxidant activity of oil (IC_{50} = 1.59%) were compared with BHT (IC_{50} = 0.002%). In *β*-carotene test, the oil (1.59%) showed 32.3% inhibition, while 0.002% BHT showed 99.3% inhibition. The MIC and MLC values of *A. hirtifolium* oil were in the ranges of 0.06-2 and 0.25-2 µl/ml, respectively. *Aspergillus flavus* was more sensitive to oil than that of other microorganism. *A. hirtifolium* oil exhibited high antimicrobial activity against bacteria, yeast and fungi. So, *A. hirtifolium* can be added to foods as antimicrobial or antioxidant agent in addition of its flavoring effect.

Keywords: *Allium hirtifolium* antimicrobial activity antioxidant essential oil

INTRODUCTION

Allium hirtifolium or Persian shallot from *Alliaceae* family with indigenous name of ‘‘Mooseer’’ is one of the important edible alliums in Iran. It is native and endemic from northwestern to southern of Iran and grows as a wild plant in the Zagross Mountains at high elevations of different provinces with the climate of very cold to moderate cold. It is different from common shallot (*Allium ascalonicum* L.) for many characteristics. Bulbs of common shallot are pear-shaped, reddish-brown and clustered at the base of the plant and its clusters may contain as many as 15 bulbs **(Ebrahimi *et al.*, 2009)**. *A. hirtifolium* produces a cluster of bulbs from a single planted bulb. *A. hirtifolium*, an Iranian traditional herb and condiment spice, is well known in Iranian folk medicine and its bulbs have been widely used for treating rheumatic and inflammatory disorders **(Jafarian *et al.*, 2003)**. Several studies have been reported the pharmaceutical activity of Persian shallot including anti-microtubule activities **(Ghodrati Azadi *et al.*, 2009)**, *in vitro* anti-trichomonas activity **(Taran *et al.*, 2006)**, immunomodulatory effects **(Jafarian *et al.*, 2003)**, nematocidal activity **(Taran and Izaddoost, 2010)**, anti-proliferative activity on tumor cell lines (Hela, MCF7, L929) **(Ghodrati Azadi *et al.*, 2008)** and antioxidant activity **(Souri *et al.*, 2008)**. Other study exhibited that *A. hirtifolium* did not show valuable inhibitory activity on α- amylase enzyme **(Nickavar *et al.*, 2009)**. The effectiveness of *A. hirtifolium* alcoholic extract on reduction of ALP, AST, and ALT is also reported more than Glibenclamide **(Kazemi *et al.*, 2010)**. Antifungal activity of alcoholic and aqueous extracts of *A. hirtifolium* were confirmed against *Aspergillus fumigatus*, *A. flavus*, *A. niger*, *Penicillium gryseogenum*, *Alternaria*, *Microsperum canis* and *Trichophyton mentagrophytes* **(Fateh *et al*, 2010)**. The literature survey revealed no study that showed the chemical composition of the *A. hirtifolium* oil. In this study, we compared the antioxidant and antimicrobial activity of *A. hirtifolium* methanol extract and its essential oil.

MATERIAL AND METHODS

Oil extraction

The air-dried and ground shallot bulb was hydrodistilled in a full glass Clevenger-type for 4 h in order to extract the oil. The obtained essential oil was kept in a sealed vial at 4 °C until analyzed.

Oil chromatographic analysis

The essential oil was analyzed by GC-FID (Varian CP-3800) and GC/MS (TermoQuest-Finnigan apparatus coupled to a trace MS detector) with column of DB-5 (60 m × 0.25 mm i.d., film thickness, 0.25 µm) and detector. Sample was injected by splitting and the split ratio was 1:100. The oven temperature programmed as follows: 60-250 °C at 5 °C/min, then held for 10 min at 250 °C. Helium was used as carrier gas at a flow rate of 1.1 ml/min. The injector and detector temperatures were 250 °C and 260 °C, respectively. The quadrupole mass spectrometer in electron impact ionization mode at 70 eV and interface temperature 250 °C was scanned over the 40-500 m/z. The relative percentage amounts of the oil components were computed by GC-FID. Retention indices were calculated for all components using a homologous series of n-alkanes injected in conditions equal to samples ones. Components of volatile oil were identified by retention indices (RI) relative to n-alkanes and by computer search using libraries of Wiley275.L and Wiley7n.1, as well as comparisons of the fragmentation pattern of the mass spectra with data published in the literature **(Adams, 2001, NIST, 1998).**

Preparation of methanol extract

Fifty grams of the powdered bulbs were extracted with 200 ml methanol using a Soxhlet apparatus for 8 h. Solvent was removed under vacuum and dried. Yield was 1.2 g (2.8%) of dry extract. The extract were dried and kept at 4 °C prior to further analyses.

Assay for total phenolic content

Total phenolic content of methanol extract was measured using the Folin–Ciocalteu reagent in term of gallic acid standard **(Slinkard and Singleton, 1997).** 0.2 ml of methanol extract was transferred into a 5 ml volumetric flask and swirled with 3 ml of deionised water. 0.25 ml of Folin-Ciocalteu’s reagent was added and swirled. After 3 min, 0.5 ml of 20% (w/v) sodium carbonate solution was added and mixed. This was recorded as time zero. Deionised water was added to make up the volume to 5 ml exactly. The solution was mixed thoroughly and allowed to stand at ambient temperature for 2 h until the characteristic blue color developed. Quantification was done on the basis of the standard curve of gallic acid at 760 nm by a spectrophotometer (Perkin-Elmer Lambda EZ-210

UV/VIS). All tests were conducted in triplicate and averaged. Results were expressed as gram of gallic acid equivalent (GAE) per 100 g of dry weight.

DPPH radical-scavenging activity

The scavenging activity of the stable free radical; 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined. The principle of DPPH assay involves reaction of the antioxidants with the stable DPPH radical, converting the complex from a deep violet color to a colorless complex. The degree of discoloration indicates the scavenging potentials of the samples.

DPPH solution (0.06 mg/ml) was added to varying concentrations of the plant extract and absorbance was read at 715 nm using a blank containing the same concentration of oil or extract or BHT without DPPH for 70 min in the dark at room temperature. Inhibition percentage of the DPPH radicals was calculated using the formula below:

$I\% = [(A_{blank} - A_{sample}) / (A_{blank})] \times 100$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound and sample is the absorbance of the test compound. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and IC_{50} values were reported as means. BHT was used as an antioxidant food additive **(Slinkard and Singleton, 1997)**.

ß-Carotene/linoleic acid bleaching assay

In this assay, antioxidant activity was determined by measuring the inhibition of volatile organic compounds and conjugated diene hydroperoxides arising from linoleic acid oxidation. The method described by **Miraliakbari and Shahidi (2008)** was used with slight modifications. A stock solution of *ß*-carotene and linoleic acid was prepared with 1 mg of ß-carotene in 1 ml chloroform, 50 µl of linoleic acid and 750 mg Tween 20. The chloroform was evaporated under vacuum and 50 ml of aerated distilled water was then added to the residue. The samples (0.5 mg/ml) were dissolved in methanol and 350 µl of each sample solution was added to 2.5 ml of the above mixture in test tubes. The test tubes were incubated in a hot water bath at 50 °C for 2 h; the test tube with BHT maintained its yellow color during the incubation period. The absorbencies were measured at 470 nm on a spectrophotometer (Perkin-Elmer Lambda EZ-210 UV/VIS). Antioxidant activities (inhibition percentage, I%) of the samples were calculated using the following equation:

$I\% = (A_{\beta\text{-carotene after 2h assay}} / A_{\text{initial }\beta\text{-carotene}}) \times 100$

Where $A_{\text{ß-carotene after 2 h assay}}$ is the absorbance of *ß*-carotene after 2 h assay remaining in the samples and $A_{\text{initial }\beta\text{-carotene}}$ is the absorbance of *ß*-carotene at the beginning of the experiments. All tests were carried out in triplicate and inhibition percentages were averaged.

Microbial strains

In this research, the strains were including: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus saprophyticus* ATCC 15305, *Enterococcus faecalis* ATCC29212, *Enterococcus faecium* ATCC 25788, *Streptococcus salivarius* ATCC 9222, *Streptococcus sanguis* ATCC 10566, *Streptocoocus pyogenes* PTCC 1447, clinical isolate of *Streptococcus agalactiae*, *Bacillus cereus* PTCC 1247, *Bacillus subtilis* ATCC 6051, *Escherichia coli* ATCC 8739, *Enterobacter aerogenes* ATCC 13048,*Proteus vulgaris* PTCC 1079, *Salmonella typhimurium* ATCC 14028,*Pseudomonas aeruginosa* ATCC 9027, *Shigella flexeneri* NCTC 8516, *Shigella dysenteriae* PTCC 1188, *Klebsiella pneumioniae* ATCC 10031, *Serratia marcescens* PTCC 1621, *Candida albicans* ATCC 10231, a farm isolate of *Aspergillus flavus*, *Aspergillus niger* ATCC 16404, *Aspergillus parasiticus* ATCC 15517. The bacteria and fungi were cultured on Brain Heart infusion Agar and Sabouraud dextrose Agar, respectively. The microbial suspensions were prepared in normal saline and the turbidity of microbial suspensions was adjusted to 0.5 McFarland by spectrophotometrical method (wave length 600 and 520 nm for bacteria and fungi, respectively).

Antimicrobial evaluation by micro broth dilution assay

Micro broth dilution assay were done as recommended instruction of NCCLS **(NCCLS, 2006)**. We used the standard antibiotics such as vancomycin, gentamycin and amphotericin B as positive controls for gram positive, gram negative bacteria and fungi, respectively. The essential oil and methanolic extract was dissolved in dimethyl sulfoxide and methanol as solvent, then the essential oil and extract was diluted in distilled water as a diluents in two serial dilutions as the essential oil and extract concentration were in the ranges of 16- 0.25 µl/ml and 1.28-0.02 mg/ml, respectively. 100 µl of each dilution from the essential oil and extract were poured in each well, and then the 100 µl of above microbial suspension was added to 20 ml Muller Hinton broth (for non-fastidious bacteria), RPMI 1640 (for fungi) or Todd Hewitt broth (fastidious bacteria) as broth media. Then 100 µl of diluted suspension were added to each serial dilution of oil or extract. The plates were incubated in aerobic condition for non-fastidious bacteria or fungi and in CO_2 Jar for fastidious bacteria at 37 ºC for 24 and 48 h for bacteria and fungi, respectively. After that, the first dilution of essential oil or extract that had not any turbidity is used as Minimal Inhibitory Concentration (MIC), and the first well that had not any growth on solid media as used as Minimal lethal Concentration (MLC).

RESULTS AND DISCUSSION

Chemical composition of oil

The essential oil yield of powdered bulb of *A. hirtifolium* using hydro distillation method was 0.04% (v/w). GC and GC/MS analysis of the plant essential oil led to the identification and quantification of 11 components which accounted for 96.8% of the total oil. Table 1 shows the results of the qualitative and quantitative oil analyses listed in order of retention indices (RI). As shown 5-chloroorcylaldehyde (55.1%), methyl methylthiomethyl disulfide (24.6%), tricosan (6.3%), pentylthiophene (3.8%), and dimethyl trisulfide (3.1%) were the most abundant components and comprised 93.9% of the oil.

Table 1 Chemical composition of the essential oil of the bulb of *A. hirtifolium*

Compound	RI[a]	Percent
dimethyl trisulfide	975	3.1
methylthiomethyl disulfide	1134	24.6
thymol	1295	0.24
carvacrol	1298	0.31
tris(methylthio)-methane	1473	1.6
5-chloroorcylaldehyde	1534	55.1
pentythiophene	1788	3.8
ethyl hexadecanoate	1992	0.23
n-heneicosane	2043	0.45
tricosane	2314	6.3
tetracosane	2379	1.1

[a] Compounds listed in order of retention indices (RI)

Allium a genus belongs to Liliaceae family, including important food plants such as onion, garlic, shallot, chive, leek and rakkyo. They have been used for hundreds of years and have been recognized as significant sources of photochemical with healthful effect. *A. hirtifolium* is widely consumed as flavor enhancers. Chemical composition analysis showed the presence of 5-chloroorcylaldehyde, methyl methylthiomethyl disulfide, tricosan, pentylthiophene, and dimethyl trisulfide as the main components, the major sulfides in *A. hirtifolium* oil were 27.7% while the four major sulfides (53.2%) of *Allium ascalonicum* oil were diallyl monosulfide (1.59%), diallyl disulfide (24.7%), diallyl trisulfide (16.1%) and diallyl tetra sulfide (10.8%) **(Rattanachaikunsopon and Phumkhachorn, 2009)** higher than *A. hirtifolium* oil and was similar to that garlic oil **(Lawson *et al*, 1991; Tsao and Yin, 2001, Rattanachaikunsopon and Phumkhachorn, 2008)**. Ethyl hexadecanoate, another component of *A. hirtifolium* oil is normally used for flavoring in food field and also used as intermediate for organic synthesis.

Amount of total phenolic constituents and antioxidant activity

Based on the measured absorbance value of the methanol extract reacting with Folin–Ciocalteu reagent, and in comparison with absorbance values of gallic acid solutions in the standard curve, the amount of total phenolic in methanolic extract was estimated 21 mg/g.

Table 2 Antioxidant activity of the essential oil and methanol extracts and BHT

Sample	DPPH IC_{50} (%)	*ß*-carotene/linoleic acid inhibition (%)
Methanol extract	0.13	22.8
Essential oil	1.56	32.3
BHT	0.002	99.3

Antioxidant activity is a complex process that is often carried out by more than one test method. In this work, two classical antioxidant tests namely DPPH and *ß*-carotene/linoleic acid tests were applied. The DPPH radical scavenging capacity assay was used to examine the antioxidant activity of essential oil and crude methanol extract. The oil was assayed over a range of dilutions to establish the concentration of each sample required to scavenge 50% of the DPPH radical present in the assay medium, referred to as the IC_{50}. The antioxidant activity of the oil (IC_{50} = 1.59%) and methanol extract (IC_{50} = 0.13%) were compared with that of BHT, the standard commercial synthetic antioxidant (IC_{50} = 0.002%). The essential oil and methanol extract exhibited weaker radical-scavenging activity than the BHT. In *ß*-carotene test, the oil at concentration 1.59% showed 32.3% *ß*-carotene/linoleic retention while 0.002% BHT inhibited 99.3 % (Table 2).

Antimicrobial activity of *A. hirtifolium*

The MIC and MLC values of *A. hirtifolium* oil were in the ranges of 0.06-2 and 0.25-2 µl/ml, respectively. Among gram positive bacteria, the *A. hirtifolium* oil exhibited the same activity against *S. saprophyticus*, *B. cereus* and *B. subtilis* (MIC, MLC=0.5, 1 µl/ml), *S. aureus*, *S. sanguis* (MIC, MLC=1, 1 µl/ml) and *S. epidermidis*, *S. pyogenes*, *S. agalactiae*, *E. faecalis*, *E. faecium* (MIC, MLC= 1, 2 µl/ml). The MIC value for *E. aerugenes* were higher than that of the others (MIC, MLC=2 µl/ml). The oil showed microbiocidal activity against *Sh. flexeneri*, *Sh. dysenteriae* (MIC, MLC=0.5 µl/ml), *A. niger* (MIC, MLC=0.25 µl/ml), *E. aerugenes* (MIC, MLC=2 µl/ml), *S. sanguis* and *S. aureus* (MIC, MLC=1 µl/ml). *A. flavus* with MIC and MLC values 0.06, 0.125 µl/ml, was more sensitive than the others and *A. flavus* was resistant fungi than others. The antimicrobial activity of antibiotics such as vancomycin, gentamycin and amphotericin B (µg/ml) was comparable with *A. hirtifolium* oil in µl/ml (Table 3).

A. hirtifolium methanol extract showed more activity to *A. parasiticus*, *C. albicans* and *A. niger* (MIC, MLC=19.5 and 34 µg/ml) and followed by *A. flavus*, *Sh. dysenteriae*, *S. epidermidis*, *S. saprophyticus* and *S. aureus* (MIC, MLC= 39 and 78 µg/ml), *P. vulgaris* (MIC, MLC=78 µg/ml), *Sh. flexeneri*, *S. marscenscens*, *P. aeruginosa*, *E. coli*, *St. salivarius* (MIC, MLC=78, 156 µg/ml).

The sensitivity of *S. agalactiae*, *S. sanguis*, *S. typhimurium*, *E. aerugenes*, *K. pneumoniae* (MIC, MLC=156, 312 µg/ml) to *A. hirtifolium* methanol extract was more than that of *B. cereus*, *S. pyogenes* (312, 625 µg/ml). *B. subtilis*, *E. faecalis* and *E. faecium* (MIC, MLC= 625, 1250 µg/ml) was more resistant to *A. hirtifolium* methanol extract (Table 3). Protection against the agents of oxidative stress can be achieved by their ability to scavenge free radicals and other oxidants. In this study, the potency of *A. hirtifolium* oil and methanol extract were assessed by comparison with BHT. The amount of total phenolic compounds in methanol extract was 21 mg/g dry extract. The IC_{50} of methanol extract was lower than that of *A. hirtifolium* oil in DPPH and was lower than BHT. There is a correlation between the total phenolic content of methanol extract and its antioxidant activity.

Souri et al showed that the IC_{50} of *A. hirtifolium* in DPPH and linoleic acid peroxidation were 187.8 µg/ml and 30.67 ng/ml for methanolextract **(Souri *et al.*, 2008)**.

Although, the antioxidant activity of *A. hirtifolium* was very lower than BHT in vitro condition, but it has been demonstrated that several component of *Allium* species can induce phase II detoxification enzyme in mammalian tissues **(Rose *et al.*, 2005)**. So, its suitable antioxidant activity maybe appears in human's body.

Table 3 The antimicrobial activity of *Allium ascalonicum* oil by micro broth dilution assay

Microorganisms	shallot oil (µl/ml)		Methanol extract (µg/ml)		Vancomycin (µg/ml)		Gentamycin (µg/ml)		Amphotericin (µg/ml)	
	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC
S. aureus	1	1	39	78	1	2	-	-	-	-
S. saprophyticus	0.5	1	39	78	1	2	-	-	-	-
S. epidermidis	1	2	39	78	4	8	-	-	-	-
B. cereus	0.5	1	312	625	1	2	-	-	-	-
B. subtilis	0.5	1	625	1250	1	16	-	-	-	-
S. pyogenes	1	2	312	625	0.25	0.5	-	-	-	-
S. agalactiae	1	2	156	312	2	4	-	-	-	-
E.faecalis	1	2	625	1250	2	4	-	-	-	-
E. faecium	1	2	625	1250	4	4	-	-	-	-
S. salivarius	1	2	78	156	1	1	-	-	-	-
S. sanguis	1	1	156	312	2	4	-	-	-	-
E. coli	1	2	78	156	-	-	0.25	0.5	-	-
E. aerugenes	2	2	156	312	-	-	2	4	-	-
P. vulgaris	1	2	78	78	-	-	1	2	-	-
S. typhimurium	1	2	156	312	-	-	1	2	-	-
P. aeruginosa	1	2	78	156	-	-	4	8	-	-
S. marcescens	1	2	78	156	-	-	0.5	0.5	-	-
Sh. flexeneri	0.5	0.5	78	156	-	-	0.5	0.5	-	-
Sh. dysentriae	0.5	0.5	39	78	-	-	0.5	0.5	-	-
K.pneumoniae	1	2	156	312	-	-	0. 5	0.5	-	-
C. albicans	0. 25	0.5	19.5	34	-	-	-	-	0.5	1
A. niger	0.25	0.25	19.5	34	-	-	-	-	0.125	0.5
A. flavus	0.06	0.125	39	78	-	-	-	-	0.5	0.5
A. parasiticus	0.5	1	19.5	34	-	-	-	-	0.5	0.5

MIC=Minimal Inhibitory Concentration; MLC=Minimal Lethal Concentration

There is widespread belief in its preventive potential against a range of infectious diseases. Antibacterial, antifungal, antiviral, antiprotozoal and anti helmentic properties of *Allium* genus have been reported **(Cavallito *et al*, 1944; Ariga and Seki, 2006; Ankri and Mirelman, 1999; Yamada and Azuma, 1997, Taran *et al*, 2006)**. Although, there are many scientific studies on different genus of *Allium*, little work has been carried out on *A. hirtifolium*.

A. hirtifolium oil and methanol extract exhibited the best activity against bacteria, yeast and fungi. The sensitivity of microorganisms was different to oil and extract. The research exhibited that *Allium* genus extracts act via decreasing the oxygen uptake, grows of organism, inhibiting the synthesis of lipids, proteins, nucleic acid and damage to membranes **(Borek, 2001)**. Methanol extract of *A. hirtifolium* was suitable for treatment of infectious diseases or food spoilage that is caused by fungi or yeast because these organisms showed the most sensitivity to methanol extract. The antimicrobial properties of *Allium* sp are attributed to sulfur compounds, sulfides especially with three or more sulfur atoms are potent fungicidal agent **(Rose *et al*, 2005)**. Methyl methylthiomethyl disulfideand dimethyl trisulfide was the sulfides in *A. hirtifolium* extracts. Phenolic compounds such as thymol and carvacrol were found in *A. hirtifolium* oil. It is documented that thymol and carvacrol had broad spectrum antimicrobial activity against microorganisms **(Didry *et al*., 1994; Mahboubi and Ghazian Bidgoli, 2010; Ahmad *et al.*, 2011)**. Thymol and carvacrol inhibit the ergosterol biosynthesis and disrupt the membrane integrity **(Ahmad *et al*., 2011)**. *E. faecalis* and *E. faecium* was the least sensitive bacteria to oil and methanol extract.

CONCLUSION

Chemical composition analysis showed the presence of 5-chloroorcylaldehyde, methyl methylthiomethyl disulfide, tricosan, pentylthiophene, and dimethyl trisulfide as the main components, the major sulfides in *A. hirtifolium* oil were 27.7%. The IC_{50} of methanolic extract was lower than that of *A. hirtifolium* oil in DPPH and was lower than BHT.*A. hirtifolium* oil and methanolic extract exhibited the best activity against bacteria, yeast and fungi.

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