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Full Length Research Paper

Gas chromatography-mass spectrometry (GC-MS) analysis of petroleum ether extract (oil) and bio-assays of crude extract of *Iris germanica*

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Iris plants have immense medicinal importance and are therefore used in the treatment of cancer, inflammation, bacterial and viral infections (Nadkarni, 1976). Gas chromatography-mass spectrometry analysis of the petroleum ether extract (oil) of the plant have resulted in the identification of eleven compounds, 9-hexadecanoic acid methyl ester, 9-octadecenoic acid methyl ester, 8-octadecenoic acid methyl ester, 11-octadecenoic acid methyl, 10-octadecenoic acid methyl ester, 13-octadecenoic acid methyl ester, 16-octadecenoic acid methyl ester, 1,2-benzenedicarboxylic acid diisooctyl ester, bis (2-ethylhexyl) phthalate, methyl 6-methyl heptanoate and nonanoic acid, 9-oxo-methyl ester. The bioassay of petroleum ether extract (oil) showed that, it is potent against for antimicrobial and antioxidant activities.

Key words: *Iris germanica*, gas chromatography-mass spectrometry (GC-MS) analysis, identification of compounds, bioassay.

INTRODUCTION

Iris germanica L. is widely distributed in most parts of the world and also found in northern regions of Pakistan (Ali and Mathew, 1993). The essential oils of the plant get use in perfumes and cosmetics (Deshuparabhu, 1959). Different parts of plants are used to cure various diseases in the folk medicine system. The rhizome of the plant is used in the usual preparations. The juice of rhizome is applied to sores and for removal of freckles of skin. Decoction of the roots of the plant is used in dropsy and as anti-spasmodic, emmenagage, stimulants, diuretic, asperient and in gall bladder diseases (Hawana et al., 1991).

The rhizomes contain iridals which exhibited a strong pesticidal activity at a concentration less then 1 µg/ml and also showed effective anti-cancer activity (Hawana et al., 1991; Miyatke et al., 1997). It is also employed as an ingredient for blood purifier and remedy for veneral diseases (Deshuparabhu, 1959). Its leaves are rich sources of ascorbic acid and vitamins (Miyatke et al., 1997). A number of secondary metabolites have been reported for the plant (Papendrof et al., 1998; Newman et al., 2000; Cordell, 2000; Atta-ur-Rahman et al., 2000,2002, 2003). This is the first report of the petroleum ether extraction (oil) from *I. germanica*.

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Instrumentation

Shimadzu-GC-9A gas chromatograph, FID at 220, N_2 at 1.0 ml/min, SPB-5 capillary column (30 m × 0.53 mm ID; 0.3 µmdf), split ratio 1:30 injector temperature 240°C, column temperature maintained at 50°C for the first five minutes and then raised to 235°C (5°C per minute) followed by five minutes at 235°C. GC-MS: Hewlett-Packard 5890 gas chromatograph, combined with a Jeol JMS-HX 110 mass spectrometer with source at 270°C at 70 eV. Injector was set at 270°C with splitting ratio 1:30. The analysis was performed on the aforementioned programme on equivalent column HP-5 (25 m × 0.22 mm

and 0.25 µmdf). A mass spectral survey was performed using the NIST mass spectral search program 1998.

EXPERIMENTAL

Plant material

I. germanica was collected in October 2005 from Attahmaqum, District Neelum, Azad Jammu and Kashmir Pakistan. The plant was identified by a taxonomist at the Department of Botany, University of Azad Jammu and Kashmir, Muzaffarabad. The voucher specimen has been kept in the herbarium of the department.

Extraction and identification

The whole plant was dried under shade. The air dried plant were chopped and cut into small pieces and then grinded to the powdered form. The powdered material (6 kg) of the plant was soaked in MeOH for ten days. The extract was separated by filtration and evaporated to dryness on rotary evaporator under reduced pressure, which afforded 98 g of MeOH extract. The methanol extract was subjected to column chromatography. The fraction eluted with 5% petroleum ether/ chloroform appeared as an oil. It was in good condition when used for gas chromatographic experiments. The gas chromatographic experiments gave gas chromatograms. On the basis of chromatograms the oil sample was subjected to the GC coupled with mass spectrometer. The coupled mass spectrometer convert each appeared chromatogram to mass spectrum of individual compound. We got a total of 60 mass spectra of different compounds from chromatograms which showed that a lot of compounds are present in the sample with different abundance, but for identification we preferred the spectra of those compounds whose abundance appeared more then 90%. The selected mass spectral plots of the compounds were compared with the spectra of reference compounds through library search by prior spectral interpretation. That enabled to identify the eleven substances, 9-hexadecanoic acid methyl ester (1), 9-octadecenoic acid methyl ester (2), 8-octadecenoic acid methyl ester (3), 11-octadecenoic acid methyl (4), 10-octadecenoic acid methyl ester (5), 13- octadecenoic acid methyl ester (6), 16- octadecenoic acid methyl ester (7), 1, 2-benzenedicarboxylic acid diisooctyl ester (8), bis(2-ethylhexyl) phthalate (9), methyl 6-methyl heptanoate (10) and nonanoic acid, 9-oxo-methyl ester (11).

Antimicrobial activity

The antimicrobial activity of microbial strains for oil sample was done by two ways:

(1) Disc diffusion method
(2) Determination of minimum inhibitory concentration

Microbial strains

The oil sample tested against a set of microorganisms, including two Gram-positive bacteria: *Bacillus subtilis*, *Staphylococcus aureus*, two Gram-negative bacteria: *Pasteurella multocida* (local isolate) and *Escherichia coli* and four pathogenic fungi, *Aspergillus niger*, *Aspergillus paracistic* (local isolate), *Rhizopus solani* and *Fusarium solani*. The pure bacterial and fungal strains were obtained from Department of Veterinary Pathology. Purity and identity were verified by the Department of Veterinary Microbiology, University of Agriculture, Faisalabad, Pakistan. Bacterial strains were cultured overnight at 37°C in nutrient agar (NA, Oxoid, Hampshire, UK) while fungal strains were cultured overnight at 28°C using Potato dextrose agar (PDA, Oxoid, Hampshire, UK).

Disc diffusion method: The antimicrobial activity was determined by disc diffusion method. Briefly, 100 µl of suspension of tested microorganisms, containing 10^8 colony-forming units (cfu)/ml of bacteria cells and 10^5 spores/ml of fungi spread on NA and PDA medium, respectively. The disc (6 mm in diameter) was individually impregnated with oil sample, placed on the agar plates which had previously been inoculated with the tested microorganisms. A disc without compound was used as a negative control. Amoxycillin (30 µg/dish) (Oxoid, Hampshire, UK) and Flumequine (30 µg/disk) (Oxoid, Hampshire, UK) were used as positive reference for bacteria and fungi, respectively to compare sensitivity of strain/isolate in analyzed microbial species. Plates, after 2 h at 4°C, were incubated at 37°C for 24 h for bacteria and at 28°C for 48 h for fungal strains in incubator (Synou, Gemany). Antimicrobial activity was evaluated by measuring the diameter of the growth inhibition zones by zone reader (MAS GmbH, Germany) in millimeters for the organisms and comparing to the controls (Rehman et al., 2001).

Determination of minimum inhibitory concentration: For the determination of minimum inhibitory concentration (MIC), which represents the concentration that completely inhibit the growth of microorganisms, a micro dilution broth susceptibility method was used, as recommended by the Clinical and Laboratory Standards Institute (Fritsche et al., 2007). Test was performed in nutrient broth (NB, Oxoid, Hampshire, UK) for bacterial and sabouraud dextrose broth (SDB, Oxoid, Hampshire, UK) for fungal strains supplemented with DMSO (Sigma-Aldrich, Germany) detergent to a final concentration of 0.5% (v/v). Bacterial strains were cultured overnight at 37°C in NB and the fungi were cultured overnight at 28°C in SDB. Dilution was prepared for the compound in a 96-well micro liter plate, 160 µl of NB and SDB for bacteria and fungi respectively were added onto microplates and 20 µl of tested solution. Then, 20 µl of 5×10^5 CFU/ml of standard microorganism suspension were inoculated onto microplates. Plates were incubated at 37°C for 24 h for bacteria, and at 28°C for 48 h for fungi. The same test was performed simultaneously for the growth control (NB + DMSO) and sterility control (NB + DMSO + test compound).

Amoxycillin was used as a reference standard compound for antibacterial and Flumequine for antifungal activities. The growth was indicated by the presence of a white ‘‘pellet’’ on the well bottom.

Antioxidant activity

The antioxidant activity was determined by two ways:

(1) DPPH radical scavenging assay
(2) Percent inhibition in linoleic acid system

DPPH radical scavenging assay

The antioxidant activity of the oil sample was assessed by quantifying the scavenging ability to stable free radical 2, 2′-diphenyl-1-picrylhydrazyl. The DPPH assay was performed as described by Mimica-Dukic et al. (2003). The sample from 5.5 µg/ml was mixed with 1 ml of 90 µM DPPH solution followed by addition of 95% MeOH up to final volume of 4 ml. The absorbance of the resulting solution and the blank was recorded after 1 h at room temperature. Synthetic antioxidant, BHT was used as a positive control.

The disappearance of DPPH was read spectrophotometrically at 515 nm (U-2001, model 121-0032 Hitachi, Tokyo, Japan). Inhibition

of free radical by DPPH in percent (%) was calculated in following way:

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

Where A_{blank} is the absorbance of the control reaction mixture excluding the test sample, and A_{sample} is the absorbance of the test sample. IC_{50} values, which represented the concentration of essential oil that caused 50% neutralization of DPPH radicals, were calculated from the plot of inhibition percentage against concentration.

Percent inhibition in linoleic acid system

The antioxidant activity of oil sample was determined by using inhibition of linoleic acid oxidation, following the method described by Singh et al. (2005) with modification. The test sample (50 µg) was mixed with 1 ml of ethanol (v/v), linoleic acid (2.5%, v/v), 99.5% ethanol (4 ml) and 4 ml of 0.05 M sodium phosphate buffer (pH 7). The solution was incubated at 40°C for 175 h. The extent of oxidation was measured by peroxide value using the colorimetric method. To 0.2 ml sample solution, 10 ml of ethanol (75%), 0.2 ml of an aqueous solution of ammonium thiocyanate (30%) and 0.2 ml of ferrous chloride solution (20 mM in 3.5% HCl) were added sequentially. After 3 min of stirring, the absorbance was measured at 500 nm, using spectrophotometer (U-2001, Hitachi Instruments, Inc., Tokyo, Japan). A control was performed with linoleic acid but without sample. Butylated hydroxytoluene (BHT) was used as positive control. Percent inhibition of linoleic acid oxidation expressed as percent was calculated as follows:

% Inhibition of linoleic acid oxidation = 100 – [(Abs. increase of sample at 175 h/ Abs. increase of control at 175 h) × 100]

RESULTS AND DISCUSSION

The GC/MS spectral results and comparison of results with library search successfully enabled the identification of the eleven compounds: 9-hexadecanoic acid methyl ester (1), 9-octadecenoic acid methyl ester (2), 8-octadecenoic acid methyl ester (3), 11-octadecenoic acid methyl (4), 10-octadecenoic acid methyl ester (5), 13-octadecenoic acid methyl ester (6), 16- octadecenoic acid methyl ester (7), 1, 2-benzenedicarboxylic acid diisooctyl ester (8), bis(2-ethylhexyl) phthalate (9), methyl 6-methyl heptanoate(10) and nonanoic acid, 9-oxo-methyl ester (11). The structures of all compounds are given in Figure 1 and details of identified compounds are given subsequently and also in Table 1.

9-Hexadecanoic acid methyl ester (1)

The mass spectrum of the compound (1) showed the molecular ion peak at m/z 268.361, corresponding to the molecular formula $C_{17}H_{32}O_2$ indicating two degrees of unsaturation in the molecule.

9-Octadecenoic acid methyl ester (2)

The mass spectrum of the compound (2) showed the molecular ion peak at m/z 296.49, corresponding to the molecular formula $C_{19}H_{36}O_2$ indicating two degrees of unsaturation in the molecule.

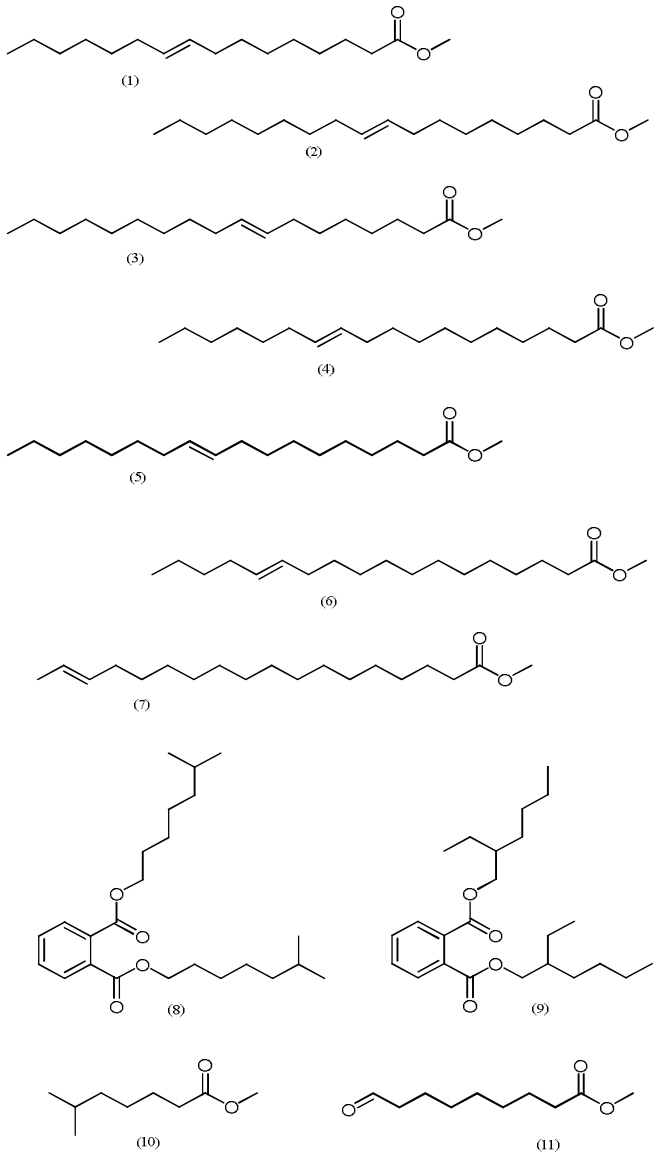

Figure 1. Structures of identified compounds (1) to (11).

8-Octadecenoic acid methyl ester (3)

The mass spectrum of the compound (3) showed the molecular ion peak at m/z 296.49, corresponding to the molecular formula $C_{19}H_{36}O_2$ indicating two degrees of unsaturation in the molecule.

11-Octadecenoic acid methyl ester (4)

The mass spectrum of the compound (4) showed the molecular ion peak at m/z 296.49, corresponding to the molecular formula $C_{19}H_{36}O_2$ indicating two degrees of

Table 1. Identified compounds from petroleum ether extract (oil) of *Iris germanica*.

Compound name	Mol. formula/ Mol. weight	Activities/ Reference
9-Hexadecanoic acid methyl ester (1)	$C_{17}H_{32}O_2$/ 268.36	Effects of the permeability and partition of ions into 1, 2-dimyristoyl-sn-glycero-3-phosphocholine bilayer at the main phase transition (Marek and SekWen, 2000).
9-Octadecenoic acid methyl ester (2)	$C_{19}H_{36}O_2$/ 296.49	Anticarcinogenic activity (Yeong et al., 1989).
8-Octadecenoic acid methyl ester (3)	$C_{19}H_{36}O_2$ / 296.49	Effects of the major serum lipid in lactating women (Alam et al., 2006).
11-Octadecenoic acid methyl ester (z) (4)	$C_{19}H_{36}O_2$/ 296.49/	Absorption and distribution in human plasma and lipoprotein lipids (Emken et al., 1986).
10-Octadecenoic acid methyl ester (5)	$C_{19}H_{36}O_2$/ 296.49	Enhances the immunity of hydroxy unsaturated fatty acid (Yamada et al., 2009).
13-Octadecenoic acid methyl ester (6)	$C_{19}H_{36}O_2$/ 296.49	Fatty acids selectively inhibit eukaryotic DNA polymerase activities *in vitro* (Yoshiyuki et al., 1996).
16-Octadecenoic acid methyl ester (7)	$C_{19}H_{36}O_2$/ 296.49	Selectively inhibit eukaryotic DNA polymerase activities *in vitro* (Yoshiyuki et al., 1996).
1,2-Benzenedicarboxylic acid diisooctyl ester (8)	$C_{24}H_{38}O_4$/ 390.23	Inhibition of human platelet phospholipase A_2 (Labow et al., 1988). A testicular toxicant, an follicle-stimulating hormone binding to membranes from cultured rat sertoli cell (Grasso et al., 1993). Rapidly increases protein phosphorylation in HeLa cells via protein kinase C and casein kinase[1] (Lahousse et al., 2006).
Bis (2-ethylhexyl) phthalate (9)	$C_{24}H_{38}O_4$/ 390.23	Oral toxicity during pregnancy and suckling in the Long-Evans Rat (Arcadi et al., 1998).
Methyl 6-methyl heptanoate (10)	$C_9H_{18}O_2$/ 158.32	Derivatives are useful in the treatment of luteal deficiency (Gastaud, 1985).
Nonanoic acid, 9-oxo-methyl ester (11)	$C_{10}H_{18}O_3$/ 186.01	Exist in human blood and urine and serve as endogenous peroxisome proliferators-activated receptor ligands (Paul et al., 2005).

unsaturation in the molecule.

10-Octadecenoic acid methyl ester (5)

The mass spectrum of the compound (5) showed the molecular ion peak at m/z 296.49, corresponding to the molecular formula $C_{19}H_{36}O_2$ indicating two degrees of unsaturation in the molecule.

13-Octadecenoic acid methyl ester (6)

The mass spectrum of the compound (6) showed the molecular ion peak at m/z 296.49, corresponding to the molecular formula $C_{19}H_{36}O_2$ indicating two degrees of unsaturation in the molecule.

16-Octadecenoic acid methyl ester (7)

The mass spectrum of the compound (7) showed the molecular ion peak at m/z 296.49, corresponding to the molecular formula $C_{19}H_{36}O_2$ indicating two degrees of unsaturation in the molecule.

1, 2-Benzenedicarboxylic acid diisooctyl ester (8)

The mass spectrum of the compound (8) showed the molecular ion peak at m/z 390.23, corresponding to the molecular formula $C_{24}H_{38}O_4$ indicating six degrees of unsaturation in the molecule.

Bis (2-ethylhexyl) phthalate (9)

The mass spectrum of the compound (9) showed the molecular ion peak at m/z 390.53,

Table 2. Disc diffusion method against fungal and bacterial strains for antimicrobial activity: Oil sample used in experiments with amounts: 0.41 g.

Name of sample	P.M	E.C	B.S	S.A	G.L	A.F	A.N	A.A
Petroleum ether extract (oil)	11	10	10	12	10	12	10	12
Rifampicin/ Terbinafen-HCl	16	18	28	26	28	26	22	22
Minimum inhibitory concentration (µg/ml)								
Pet. ether extract (oil)	754	821	811	699	804	706	803	702
Rifampicin/ Terbinafen-HCl	496	447	207	253	213	256	352	356

Bacterial strains: P. M. = *Pasturella multocida,* E. C. = *Escheriachia coli,* B.S. = *Bacillus subtilis,* S. A. = *Staphylus aureus.* Fungal isolates: G. L. = *Ganoderma leucidam,* A.F = *Aspergillus flavus,* A. N.= *Aspergillus niger,* A. A. = *Alternaria alternate.*

Table 3. Antioxidant activities of pet. ether extract (oil) by DPPH radical scavenging and linoleic acid inhibition assay.

Assay method	Petroleum ether extract (oil)	BHT
DPPH inhibition (%)	89.7	98.5
DPPH, IC_{50} (µ/ml)	32.6	19.23
Inhibition in linoleic acid oxidation (%)	58.34	93.04

corresponding to the molecular formula $C_{24}H_{38}O_4$ indicating six degrees of unsaturation in the molecule.

Methyl 6-methyl heptanoate (10)

The mass spectrum of the compound (10) showed the molecular ion peak at m/z 158.32, corresponding to the molecular formula $C_9H_{18}O_2$ indicating one degree of unsaturation in the molecule.

Nonanoic acid, 9-oxo-, methyl ester (11)

The mass spectrum of the compound (11) showed the molecular ion peak at m/z 186.01, corresponding to the molecular formula $C_{10}H_{18}O_3$ indicating two degrees of unsaturation in the molecule.

Bioassay of oil sample

The bioassay of the sample was examined for antimicrobial and antioxidant activities via procedures as stated in the experimental part and detail are given in the Tables 2 and 3, respectively.

Antimicrobial activity

Disc diffusion method: The antimicrobial activity determined by disc diffusion method and evaluated by measuring the diameter of the growth inhibition zones by zone reader (MAS GmbH, Germany) in millimeters for the organisms and comparing to the controls. It was observed that the growth of bacteria (*P. multocida, E. coli, B. subtilis, S. aureus*) and fungi (*Ganoderma leucidam*, *Aspergillus flavus, A. niger, Alternaria alternate*), were decreased in the petroleum ether extract (oil) sample as compared to the reference standards (Rifampicin/ Terbinafen), so oil sample proved to be potent to inhibit the growth of bacteria and fungi.

Determination of minimum inhibitory concentration: For the determination of minimum inhibitory concentration (MIC), which represents the concentration that completely inhibit the growth of microorganisms, a micro dilution broth susceptibility method was used. It was noticed that the minimum inhibitory concentration values were high then reference standard mean that the sample is more potent to inhibit the growth of bacteria and fungi then reference standard compounds. The antimicrobial activities results showed that oil sample is very effective for antibacterial and antifungal activities.

Antioxidant activity

DPPH radical scavenging assay: The antioxidant activity of the oil sample was assessed by quantifying the scavenging ability to stable free radical 2, 2′-diphenyl-1-picrylhydrazyl. The DPPH assay was performed by Mimica-Dukic et al. (2003) and DPPH inhibition (%) is quite significant for sample.

Percent inhibition in linoleic acid system: The

antioxidant activity of oil sample was determined by using inhibition of linoleic acid oxidation by Singh et al. (2005) with modification and sample showed moderate inhibition in linoleic acid oxidation as compared to BHT

Conclusion

This is the first report of isolation and bioassay of oil from *I. germanica* plant and identification of eleven compounds from the isolated oil by GC/MS studies.

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