Radical Scavenging Activities of *Heracleum aquilegifolium* Wight (Apiaceae) Fruit Oils *in vitro*

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The fruits of *Heracleum aquilegifolium* Wight (Apiaceae) were collected from Western Ghats of the Indian Peninsula. The essential oils were extracted by hydrodistillation. The chemical composition of the essential oils was analysed by gas chromatography and gas chromatography-mass spectrometry (GC-MS). β-Pinene (22.3%), 1,8-cineole (20.3%), and β-phellandrene (12.4%) were the main components of *H. aquilegifolium* fruit oils. The antioxidant properties of essential oils of *H. aquilegifolium* were examined by different procedures namely reducing power ability, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, nitric oxide radical scavenging activity, hydrogen peroxide scavenging activity, hydroxyl radical scavenging activity, superoxide anion scavenging activity, and metal chelating activity. The antioxidant activities were compared with those of synthetic antioxidants and standard drugs such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ascorbic acid, α-tocopherol, curcumin, and quercetin. The study confirmed the possible antioxidant potential of essential oils tested with various *in vitro* antioxidant methods. The presence of monoterpenes in combination with other components in the oils could be responsible for the activity.

Key words: Heracleum aquilegifolium, Essential Oil Composition, Radical Scavenging Activity

Introduction

Oxidation is a basic part of the aerobic life and of our metabolism. Thus, radicals are produced either naturally or by some biological dysfunction. Unpaired electrons which are centered in atoms of oxygen or nitrogen are called reactive oxygen species (ROS) or reactive nitrogen species (RNS), and their excess has harmful effects, such as peroxidation of the membrane lipids, aggression to tissue proteins and membranes, and damage to DNA and enzymes (Robinson *et al.,* 1997). Therefore, they can treat some pathologies, such as arthritis, hemorrhagic shock and coronary diseases, cataract, cancer as well as age-related degenerative brain disorders (Halliwell, 2008). Although the antioxidant defense systems include both endogenously and exogenously derived compounds, dietary plants-based antioxidants have recently received great attention (Bravo, 1998). Hence many studies have been performed to identify antioxidant compounds with pharmacological activity and limited toxicity from medicinal plants. In this context, ethnopharmacology plays a significant part in the search for interesting and therapeutically useful plants. In order to contribute to the knowledge on plants from Western Ghats of India, *Heracleum aquilegifolium* Wight (Apiaceae) essential oils were screened in the present study to determine their free radical scavenging and antioxidant activities.

Nowadays, there is great world-wide interest in finding new and safe antioxidants from natural sources, to prevent oxidative deterioration of foods and to minimize oxidative damages of living cells. Recently, chemically synthesized antioxidant compounds, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butylhydroquinone (TBHQ), are used in foods, packaging food items, plastics and medicines in spite of their possible toxicity and due to a suspected action as promoters of carcinogenesis (Pokorny, 1991). However, some of these

Abbreviations: AMU, atomic mass units; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EDTA, ethylenediaminetetraacetic acid; EI, electron impact; Fe^{2+}, ferrous ion; Fe^{3+}, ferric ion; GC-MS, gas chromatography-mass spectrometry; NBT, nitroblue tetrazolium; ROS, reactive oxygen species; RNS, reactive nitrogen species; TBARS, thiobarbituric acid reactive substances; TBHQ, *tert*-butylhydroquinone.

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compounds have been questioned for their safety. Therefore, there is an increasing interest in the investigation of the antioxidative activity of natural compounds. Most of their properties are due to essential oils produced by their secondary metabolism (Sarin and Kapoor, 1963). Volatile oils are very complex mixtures of compounds. The constituents of the oils are mainly monoterpenes and sesquiterpenes which are hydrocarbons with the general formula $(C_5H_8)_n$. Oxygenated compounds derived from these hydrocarbons include alcohols, aldehydes, esters, ethers, ketones, phenols, and oxides. It is estimated that there are more than 1000 monoterpenes and 3000 sesquiterpenes (Svoboda and Deans, 1995). Several of them are qualified as antioxidant and are proposed to replace synthetic antioxidants used in food industry where they do not affect the organoleptic characteristics of the final product. Also, numerous scientific reports have highlighted an important antioxidant activity of essential oils (Ali *et al*., 2005). These biological activities depend on the chemical composition (Chun *et al*., 2005) which varies according to the geographical origin, the environmental and agronomic conditions, the stage of development of the plant material, and the extraction method (Goodner *et al*., 2006). Therefore, the evaluation of the biological activity of an essential oil should be supplemented with the determination of its chemical composition.

Heracleum L. (Apiaceae) includes more than 70 species in the world. In India there are 15 species and 5 are endemic to the Indian Peninsula (Nayar, 1996). *Heracleum aquilegifolium* is growing wildly in grass lands of Western Ghats at 1500 m above sea level. The rhizome and fruits are used as folk medicine. They are reported to be effective in indigestion, sunburn, skin diseases, and external tumours (Heywood, 1971). Many studies reported on the biological activities and essential oil composition of the genus *Heracleum* (Ergene *et al*., 2006; John *et al*., 2007). As part of our on-going studies on the genus *Heracleum* from Western Ghats of India, we now report on the composition of the volatile oils of the fruits of *H. aquilegifolium* for the first time, together with their antioxidant properties.

Material and Methods

Plant material

The fruits of *H. aquilegifolium* were collected during December 2009 from the High Wavy Mountains of Western Ghats, India. The identification of the specimens was authenticated by comparison of herbarium sheets deposited in Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. The voucher specimens have been preserved in the Department of Botany, The Madura College, Madurai, Tamil Nadu, India.

Isolation procedure

The essential oils of mature fruits of *H. aquilegifolium* were isolated from fresh mature fruits (250 g) by hydrodistillation, for 4 h, using a Clevenger-type apparatus.

Gas chromatography (GC)

The GC analysis was carried out with a Hewlett-Packard HP6890 instrument, equipped with a HP-Innowax silica capillary column (60 m × 0.25 mm, film thickness 0.25 μm) and a flame ionization detector. Nitrogen was used as carrier gas with a flow rate of 0.8 ml/min. Injector and detector temperatures were both set at 250 °C. The Column temperature was programmed to 60 °C for 10 min, gradually increased to 220 °C at 4 °C/min, held for 10 min, and then increased to 240 °C at 1 °C/min. Split ratio was 50:1 and 1 μl of sample (dissolved in hexane as 20% v/v) was injected into the system.

Gas chromatography-mass spectrometry (GC-MS)

The GC-MS analysis of the oil was carried out on an Agilent 6890N Network GC system combined with an Agilent 5973 Network mass selective detector. The capillary column used was an Agilent 19091N-136 column (HP-Innowax capillary; 60.0 m × 0.25 mm × 0.25 μm). Helium was used as carrier gas at a flow rate of 1.0 ml/min with 1 μl injection volume. Samples were analysed with the column held initially at 60 °C for 10 min after injection, then increased to 220 °C at 4 °C/min and kept at 220 °C for 10 min. The final temperature was increased to 240 °C at 1 °C/min. The injection was performed in the split mode (50:1). Detector and injector temperatures were 230 °C and 280 °C, respectively. Run time was 80 min. MS scan range was (m/z): 35–450 atomic mass units (amu) under electron impact (EI) ionization (70 eV).

Identification of the volatile oil components

The components were identified by comparing their relative retention times with those of authentic samples and their mass spectra with the data from the library of essential oil constituents as well as Wiley and Nist Libraries. Percentage contents of components were determined as area under peaks using Agilent software. The results were expressed as average of three determinations in all cases. GC and GC-MS analyses were both conducted at the Sophisticated Analytical Instrument Facility (SAIF), Central Drug Research Institute, Lucknow, India.

Reducing power assay

The reducing power ability was measured by mixing 1.0 ml sample oils of various concentrations prepared with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide and incubating at 50 °C for 30 min. After that 2.5 ml of trichloroacetic acid (10%) were added, and the mixture was centrifuged for 10 min at 3000 × *g*. 2.5 ml from the upper layer were diluted with 2.5 ml water and shaken with 0.5 ml fresh 0.1% ferric chloride. The absorbance was measured at 700 nm using a UV spectrophotometer. All experiments were done in triplicate using the standard BHT as positive control (Yildrim *et al*., 2001).

DPPH radical scavenging activity

The hydrogen donating ability of extracts was examined in the presence of stable DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals (Mensor *et al*., 2001). 1 ml of 0.3 mM ethanolic DPPH solution was added to 2.5 ml sample of different concentrations of essential oils from *H. aquilegifolium* and allowed to react at room temperature. After 30 min, the absorbance was measured at 517 nm.

Nitric oxide radical scavenging activity

Various concentrations of the test samples and sodium nitroprusside (10 mM) in phosphate-buffered saline (0.025 M, pH 7.4) in a final volume of 3 ml were incubated at 25 °C for 150 min. Thereafter, 0.5 ml of incubation solution was removed and diluted with 0.5 ml Griess' reagent (1% sulfanilamide, 2% orthophosphoric acid, and 0.1% naphthylethylene diamine dihydrochloride) and allowed to react for 30 min. The absorbance of the chromophore formed during diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylene diamine dihydrochloride was read at 546 nm. The experiment was done in triplicate using test samples (25–400 μg/ml) as positive control (Sreejayan and Rao, 1996).

Hydrogen peroxide scavenging activity

Hydrogen peroxide solution (2 mM) was prepared with standard phosphate buffer (pH 7.4). Test oil samples (25–400 μg/ml) in distilled water were added to the hydrogen peroxide solution (0.6 ml). The absorbance of hydrogen peroxide at 230 nm was determined spectrophotometrically after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging of hydrogen peroxide of both test samples and standard compound (α-tocopherol) were determined (Gulcin *et al*., 2003).

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and test samples for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. The reaction mixture contained deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM), EDTA (ethylenediaminetetraacetic acid; 0.1 mM), H_2O_2 (1 mM), ascorbic acid (0.1 mM), KH_2PO_4-KOH buffer (20 mM, pH 7.4), and various concentrations of essential oils of *H. aquilegifolium* fruits in a final volume of 1 ml. The reaction mixture was incubated for 1 h at 37 °C, and the intensity of the yellow colour formed was measured spectrophotometrically at 412 nm (Gulcin *et al*., 2003).

Superoxide anion scavenging activity

The superoxide anion scavenging activity of *H. aquilegifolium* was determined by the nitroblue tetrazolium (NBT) photoreduction method (McCord and Fridovich, 1969). The reaction mixture contained EDTA (0.1 M) supplemented with 0.00015% NaCN, riboflavin (0.12 mM), NBT (1.5 mM), various concentrations of essential oils of *H. aquilegifolium* fruits, and phosphate buffer (pH 7.8) in a final volume of 3 ml. The tubes were uniformly illuminated under an incandescent lamp for 15 min, and the optical density was measured at 530 nm before and after illumination.

Table I. Chemical composition of the fruit oil of *Heracleum aquilegifolium.*

Compound	RI	Composition (%)
1-Hexanol	867	0.02
α-Thujene	929	0.18
α-Pinene	952	1.27
1-Heptanol	965	1.22
β-Pinene	976	22.26
Myrcene	985	0.15
α-Phellandrene	995	1.05
n-Octanal	1003	1.00
1,8-Cineole	1006	20.32
Limonene	1026	2.12
β-Phellandrene	1028	12.38
cis-Ocimene	1034	0.09
trans-Sabinene hydrate	1039	0.21
1-Octanol	1067	1.85
α-Terpinolene	1086	1.25
Camphor	1095	0.15
Linalool	1098	0.52
trans-Pinocarvol	1121	1.21
Nerol oxide	1128	0.18
Verbenol	1141	0.51
p-Cymen-8-ol	1183	4.40
trans-Carveol	1187	0.10
α-Terpineol	1189	1.20
Nerol	1206	1.38
1-Ethyl-2,4-dimethyl benzene	1222	0.22
Geraniol	1238	1.42
trans-Decanol	1258	0.24
Thymol	1290	0.76
Geranyl acetate	1370	1.39
δ-Selinene	1381	0.29
β-Caryophyllene	1429	7.20
Myristicin	1514	0.14
Unknown	1520	1.25
Hedycariol	1537	0.14
Nerolidol	1552	0.14
Spathulenol	1564	1.52
Calarene	1571	1.39
Viridiflorol	1583	2.83
Identified compounds		93.95
Monoterpene hydrocarbons		87.7
Oxygenated monoterpenes		2.1
Sesquiterpene hydrocarbons		1.8
Oxygenated sesquiterpenes		1.3
Total identified compounds		93.9

Metal chelating activity

The reaction mixture containing different concentrations of essential oils (1.0 ml) was added to 2 mM ferrous chloride (0.1 ml) and 5 mM ferrozine (0.2 ml) to initiate the reaction, and the mixture was shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. The positive control was ascorbic acid. All tests and analyses were run in triplicate (Huang *et al*., 2000).

Statistical analysis

Data are reported as the mean ± S.E.M. of three measurements. Statistical analysis was performed by the student t-test and by ANOVA. IC_{50} values for all the above experiments were determined by the linear regression method. A *p*-value less than 0.05 was considered as indicative of significance.

Results and Discussion

Essential oil composition

The oil constituents obtained from the fruits of *H. aquilegifolium* are listed in Table I. The oil yield obtained from fruits was 1.3% (v/w). This yield was relatively lower than the average oil yields reported in related species for example of *H. candolleanum* from Western Ghats of India (George *et al*., 2001). The different harvesting period of the samples could partly be responsible for these differences because both the oil yields and the portions of several constituents of essential oil may vary greatly according to the developmental phase of the plant (Charma *et al*., 1963).

Thirty eight components could be identified, representing 93.95% of the total oils (Table I). Although monoterpenes were dominant in fruit oils (87.7%), the importance of the oxygen-containing or monoterpene hydrocarbons varied. β-Pinene was the dominant component in fruit oils (22.26%) of *H. aquilegifolium*, which is in accordance with the report by Saraswathy and Sasikala (1999) for the *Heracleum* spp. oils, although different from most of the Indian species of *Heracleum* chemotypes. β-Pinene (22.26), 1,8-cineole (20.32%), and β-phellandrene (12.38%) were the dominant components in fruits of *H. aquilegifolium*, which constituted 55% of total oil constituents. The results are showing some similarities to some previously studied populations of related species, *H. candollenaum* and *H. concanense* (Berenbaum, 1981; Chacko *et al*., 2000). 1,8-Cineole and β-pinene were the main compounds in all parts of *Heracleum* oils, which is also in agreement with previous reports for other species (Mojab and Nickavar, 2003).

According to the essential oil composition of *H. aquilegifolium*, the main compounds are impor-

Table II. Effect of *Heracleum aquilegifolium* fruit oils on different antioxidant methods.

Test material	Percentage of inhibition (%)						
	Reducing power assay	DPPH radical scavenging	Nitric oxide radical scavenging	H_2O_2 scavenging	Hydroxyl radical scavenging	Superoxide anion scavenging	Metal chelating assay
H. aquilegifolium							
50 μg/ml	31.20 ± 0.03	47.90 ± 0.02	24.80 ± 0.46	27.19 ± 0.07	4.65 ± 0.06	27.97 ± 0.41	39.62 ± 0.78
100 μg/ml	35.46 ± 0.08	61.07 ± 0.30	35.20 ± 0.46	46.15 ± 0.05	20.67 ± 0.51	31.46 ± 1.21	50.13 ± 1.64
200 μg/ml	46.72 ± 0.05	76.83 ± 0.011	49.37 ±1.75	57.12 ± 0.03	40.87 ± 0.05	50.54 ± 1.28	59.21 ± 0.53
300 μg/ml	57.81 ± 0.03	91.90 ± 0.32	67.46 ± 0.26	69.17 ± 0.09	60.14 ± 0.06	72.53 ± 0.28	69.62 ± 0.73
400 μg/ml	89.10 ± 0.10	98.62 ± 0.03	85.33 ± 1.20	81.15 ± 0.09	81.40 ± 0.05	81.71 ± 0.21	75.33 ± 1.43
IC_{50} (μg/ml)	51.2 ± 0.53	4.3 ± 0.88	83.93 ± 0.384	80.30 ± 0.43	21.0 ± 0.32	107.7 ± 1.081	41.6 ± 0.44
Standard drugs (control, 100 μg/ml)							
BHT	0.713 ± 0.05	NT	NT	NT	NT	NT	NT
Ascorbic acid	NT	42.98 ± 0.03	NT	NT	NT	43.58 ± 0.07	42.50 ± 0.71
α-Tocopherol	NT	NT	NT	25.57 ± 0.10	NT	NT	NT
Curcumin	NT	NT	37.37 ± 0.26	NT	NT	NT	NT
Quercetin	NT	NT	NT	NT	112.66 ± 0.06	NT	NT
IC_{50} (μg/ml)	48.4 ± 0.40	32.2 ± 0.15	76.50 ± 1.021	72.00 ± 0.57	18.0 ± 0.33	91.2 ± 0.568	35.13 ± 0.52

Values are means ± S.E.M. of 3 replicates. NT, not tested.

tant for the characteristic flavour and fragrance of this species. Flavour and fragrance are extremely important for the food, cosmetic, chemical, and pharmaceutical industries. Pinenes (α and β) are bicyclic monoterpenes of higher plants and have great value as precursors for flavour and fragrances (Mickibben, 1989).

Antioxidant activity

The results of the antioxidant studies indicated that the essential oils of *H. aquilegifolium* have reducing power and radical scavenging activities (Table II). Samples of essential oils exhibited dose-dependent increases in activity in all tests of ferricyanide reduction, DPPH radical scavenging, nitric oxide scavenging, hydrogen peroxide scavenging, hydroxyl radical scavenging, superoxide scavenging, and metal chelating. Based on the literature, the mechanism of reduction of DPPH radicals is correlated with the presence of hydroxy groups at the molecule. The present investigation can infer that the higher activity of essential oils of *H. aquilegifolium* is probably due to the presence of substances with available hydroxy groups. Similar results were obtained for the related species *Heracleum nepalense* that possesses strong DPPH radical scavenging activity (Dash *et al*., 2005). At 400 μg/ml concentration, the highest nitric oxide scavenging activity was shown by the fruit oil which was comparable with that of the standard curcumin.

The essential oil gave the lowest IC_{50} value (80.30 μg/ml) for hydrogen peroxide scavenging which is comparable with that of the standard α-tocopherol (72.00 μg/ml). The sample oil was the most efficient in hydroxyl radical scavenging (IC_{50} 21 μg/ml) that was comparable with the standard quercetin (IC_{50} 18.0 μg/ml). Among the essential oils studied, at 400 μg/ml concentration of fruit oil of *H. aquilegifolium* the highest superoxide anion scavenging activity was determined comperable with the standard ascorbic acid (IC_{50} 91.2 μg/ml). The maximum metal chelating activity was observed with the lowest IC_{50} value of 41.6 μg/ml. This value is close to the IC_{50} value of ascorbic acid standard which is 35.13 μg/ml (Table II).

The high antioxidant and radical scavenging activity is a attributed to the presence of some major components in the essential oils, β-pinene, 1,8-cineole, and β-phellandrene (Houghton, 2004).

Indeed, these compounds are known to possess a weak antioxidant activity (Tabe *et al*., 2005).

The present study indicates that essential oils of *H. aquilegifolium* might be potential antioxidants for application in food products. But it is very difficult to assign the antioxidant effect of total essential oil to one or more active principles, because there is always a mixture of different chemical compounds in essential oils.

The recent years have witnessed resurgence of interest in herbal drugs globally, as more and more people are turning to the use of herbal medicinal products in health care. About 80% of individuals from developing countries use traditional medicine, which incorporates compounds derived from medicinal plants. It is high time to revive the hidden wonders of plant compounds with the modern tolls of target-based screening to develop a new advanced generation of antioxidants with novel modes of action. However, further studies are urgently needed for screening of individual compounds in essential oils and determing their bioactivities and properties to understand the complete phenomena of its potential.

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