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Free Radical Scavenging Activities of Extracts of *Evolvulus alsinoides* Linn. Leaves and GC - MS Analysis of Methanol Extract

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Extracts of *Evolvulus alsinoides* L. leaves were investigated for their radical scavenging activity using two different *in vitro* assays, 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The methanolic extract possessed the highest radical scavenging activity in both assays, DPPH (IC_{50}-120 μg/ml) and ABTS (IC_{50}-100 μg/ml). Total phenolic content was also determined using Folin–Ciocalteu reagent. The highest total phenolic content was detected in the methanol and ethyl acetate extracts (16.8mg/g GAE and 15.6mg/g GAE, respectively). A correlation between radical scavenging capacities of samples with total phenolic compound content was observed. GC-MS analysis of methanolic extract showed that the Bicyclo 5.1.0 octane (7.02%), Hexadecanoic acid (5.75%), Heptadecanoic acid (5.58%) were the major constituents.

Key words: *Evolvulus alsinoides* L., DPPH, ABTS, Phenolics, GC-MS analysis.

Recent developments in biomedicals point to the involvement of the free radicals in many diseases[1]. Free radicals attack the unsaturated fatty acids in the biomembranes resulting in membrane lipid peroxidation, a decrease in membrane fluidity, loss of enzymes, receptor activity and damage to membrane proteins leading to cell inactivation[2,3]. For these reasons antioxidants are of interest for the treatment of many kinds of cellular degeneration[4]. Antioxidants are compounds that inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reactions[5].

It has long been recognized that naturally occurring substances in higher plants have antioxidant activity. In recent years there has been increasing interest in the presence and availability of compounds in plant materials that may possess bioactive properties, in particular, antioxidant activity. Most antioxidants isolated from higher plants are phenolic compounds (e.g. phenolic acids, tannins, coumarins, flavonoids)[6].The antioxidant activity of phenolic compounds is reported to be

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mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides[7,8]. One of the best approaches for discovering new antioxidants is the screening of plant extracts. The goal is to use phytochemicals in foods and pharmaceutical preparations to replace synthetic antioxidants, which are being restricted due to their carcinogenicity[9 10,11].

Evolvulus alsinoides L. (family : Convolvulaceae) is a Dwarf morning glory, a perennial herb with small woody and branched rootstock. The plant grows widely in open grassy places throughout India. Its branches are annual, numerous, more than 30 cm long, often prostrate, slender and wiry with long hairs. Leaves are small, entire, elliptic to oblong, obtuse, apiculate, base acute and densely hairy[12]. The plant bears numerous small sky-blue flowers. The plant contains an alkaloid shankapushpine.

The leaves are used in treating chronic bronchitis and asthma. Using the whole plant in the form of decoction with cumin and milk is used to treat fever. It is helpful in nervous exhaustion, memory loss, general weakness, loss of memory, urinary disorders and hypertension[13]. Due to uniqueness of leaves property in curing of different ailments this part was selected for the study. The aim of this study is to investigate total phenolic content and the antioxidant property of *Evolvulus alsinoides* L. as a potential source of natural antioxidants and to determine the possible chemical components from *Evolvulus alsinoides* L. leaves by GC-MS.

MATERIAL AND METHODS

Plant material

The plant *Evolvulus alsinoides* L. was collected from Aliyar field area, Pollachi, Coimbatore District, Tamil Nadu in the month of September 2008. It was identified by the Botanical Survey of India, Coimbatore and a voucher specimen (No.BSI/SC/5/23/09-10/Tech.133) was kept for future reference.

Plant sample extraction

The leaves were collected from the plant, shade dried and mechanically powdered to obtain a course powder, which was then subjected to successive extraction in a soxhlet apparatus using petroleum ether (60-80ºC), chloroform, ethyl acetate and methanol. All the extracts were concentrated to dryness under reduced pressure and used for antioxidant studies and the methanolic extract was subjected to GC-MS analysis.

DPPH radical scavenging assay

The effect of extracts on DPPH radical was determined using the method of Blois[14]. To 1 ml of each of the extracts, 5 ml of 0.1mM methanol solution of DPPH was added, vortexed, followed by incubation at 27ºC for 20 min. The control was prepared without any extract and absorbance of the sample was measured at 517 nm using UV/VIS Spectrophotometer. The ability to scavenge DPPH radical was calculated by the following equation : DPPH radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})]/(Abs_{control}) \times 100$. Butylated hydroxy toluene (BHT) was taken as reference standard. The experiment was performed in triplicate. The percentage of inhibition versus concentration was plotted and the concentration required for 50% inhibition of radicals was expressed as IC_{50} value.

ABTS radical cation scavenging assay

For ABTS assay, the method of Re *et al.*,[15] was adopted. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 hrs at room temperature in the dark. The solution was then diluted by mixing 1 ml $ABTS^{\cdot+}$ solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Fresh $ABTS^{\cdot+}$ solution was prepared for each assay. Plant extracts (1 ml) were allowed to react with 1 ml of the $ABTS^{\cdot+}$ solution and the absorbance was taken at 734 nm after 7 min using the spectrophotometer. The $ABTS^{\cdot+}$ scavenging capacity of the extract was compared with that of BHT and percentage inhibition calculated as ABTS radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})]/(Abs_{control})] \times 100$ where $Abs_{control}$ is the absorbance of ABTS radical + methanol; Abs_{sample} is the absorbance of ABTS radical + sample extract/ standard.

Determination of total phenolics

Total phenolic contents in the extracts were determined by Folin-Ciocalteu method[16]. An aliquot of the extracts was mixed with 5 ml of Folin-

Ciocalteu reagent (previously diluted with water 1:10v/v) and 4 ml (75g/l) of sodium carbonate. The tubes were allowed to stand for 15min and the total phenols were determined using UV/VIS spectrophotometer at 765nm. Total phenol values are expressed in terms of gallic acid equivalent (mg/g of dry mass), which is a common reference compound.

GC-MS analysis

GC-MS (gas chromatography coupled with mass spectrometry) analysis of methanolic extract of the sample were carried out with a Fisons Instrument GC 8000, equipped with mass selective detector with quadrupole analyser, MD 800. A fused silica capillary column (AB-35MS) of dimention 30m x 0.25 mm i.d. and 0.5mm film thickness was used. GC conditions : Injector temperature 250ºC, column temperature isothermal at 100ºC then programmed to rise upto 250ºC at 6ºC/min and held at this temperature for 10 min. Helium was used as carrier gas at a constant flow of 1ml/min and an injection volume of 1µl was employed. The electron ionisation energy was 70eV, ion-source temperature 200°C and the interface temperature 250°C.The constituents were identified after comparison with those available in the Computer Library (NIST ver.08) attached to the GC-MS instrument and published data[17].

RESULTS AND DISCUSSION

Effect of DPPH radical scavenging activity

DPPH is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Due to its odd electron, the methanolic solution of DPPH shows a strong absorbtion band at 517 nm. DPPH radicals react with suitable reducing agents and then electrons become paired off and the solution loses colour steoichometrically with the number of electrons taken up. Such reactivity has been widely used to test the ability of plant extracts to act as free radical scavengers[18].

Reduction of DPPH radicals can be observed by decrease in absorbance at 517 nm. Among all the extracts, methanol extract showed the highest scavenging activity with IC_{50} value of 120 µg/ml, which is comparable to that of BHT (Fig. 1).

Other extracts such as ethyl acetate, petroleum ether and chloroform showed weaker scavenging potency than that of methanol with IC_{50} values of 140, 200 and 245 µg/ml respectively. The results suggested that some components within methanol fraction were significantly strong radical-scavenging components.

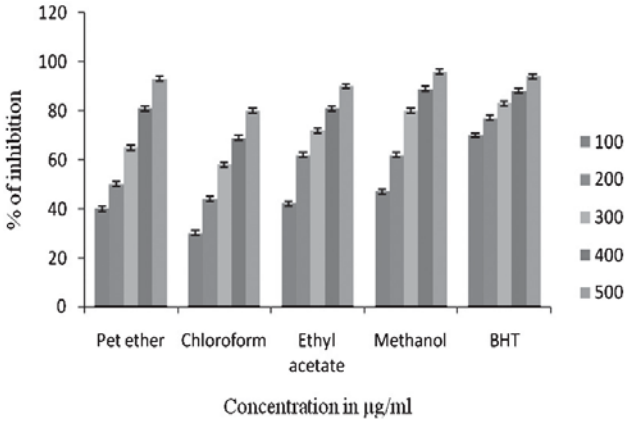

Fig. 1. DPPH radical scavenging activity

Effect of ABTS radical scavenging activity

Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maximum at 734 nm which decreases with the scavenging of proton radicals[19]. ABTS activities of petroleum ether, chloroform, ethyl acetate and methanol extract of the plant was measured (Fig. 2). The effect

of the investigated samples on ABTS radicals has been checked at various concentrations from 100 to 500 µg/ml. Higher concentration of the extracts were more effective in quenching free radicals. The highest scavenging potential (IC_{50} -100 µg/ml) was found in methanol extract and this activity was comparable to that of BHT. On the other hand, ethyl acetate and petroleum ether extracts exhibited less scavenging potency with IC_{50} -150 µg/ml and 200 µg/ml respectively. As can be seen from the results chloroform extract showed the weakest antioxidant activity (IC_{50} -260 µg/ml) similar to DPPH method.

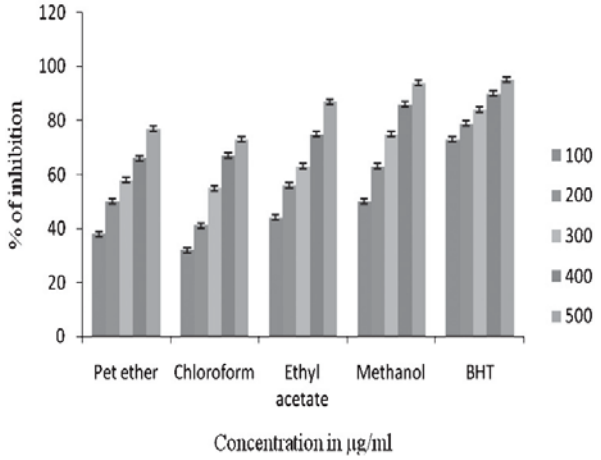

Fig. 2. ABTS radical scavenging activity

Total phenolic content

Polyphenols are the major compounds with antioxidant activity. It is well known that polyphenols are widely distributed in the plant kingdom and they are sometimes present in surprisingly higher concentrations[20]. The total phenol content of various extracts of the investigated plant material is presented in Table 1. Results obtained in the study revealed that the total phenolic content in methanol extract of *Evolvulus alsinoides* L. was highest compared to other extracts. This may be due to the presence of more bioactive compounds in methanol extract.

Table 1. Total phenol contents of the *Evolvulus alsinoides* L. extracts

S. No	Samples	Total Phenol content (mg /g of GAE)
1	Petroleum ether extract	13.4+ 0.025
2	Chloroform extract	12.8+ 0.039
3	Ethyl acetate extract	15.6+ 0.022
4	Methanol extract	16.8 +0.029

Data are represented as mean + SD (n=3)

The chloroform extract showed the lowest total phenolic content, which correspond to its weakest antioxidant potential. The results strongly suggest that phenolic compounds are likely to contribute to the antioxidant potential of the investigated plant extract.

GC-MS analysis

Ten compounds were identified in the methanolic extract of *Evolvulus alsinoides* L. by GC-MS analysis. The active compounds with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) are presented in Table 2 (Fig 3). The results revealed that Bicyclo 5.1.0 octane (7.02%) was found to be the major component followed by Hexadecanoic acid (5.75%) and Heptadecanoic acid (5.58%). Other components identified were 1-Pentatriacontanol (1.60%), Caryophyllene oxide (1.01%), Aromadendrene 2 (0.69%), Germacrene-D (0.33%), Beta - elemene (0.07%), (Z,Z)-Alpha-farnesene (0.06%) and Beta -D- glucopyranose (0.02%). Many of the identified phytochemicals including Hexadecanoic acid, Heptadecanoic acid have the property of antioxidant activities[21].

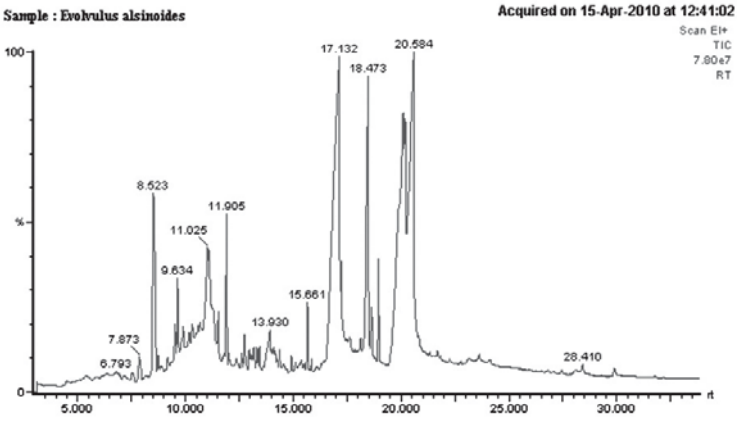

Fig. 3. GC-MS analysis of *Evolvulus alsinoides* L.

Table 2. Components identified in methanol extract of *Evolvulus alsinoides* L

S.No	RT	Name of the component	Molecular formula	MW	Peak area%
1	6.793	Beta -D- glucopyranose	$C_{12}H_{23}O_6NS_2$	341	0.02
2	7.873	Beta - elemene	$C_{15}H_{24}$	204	0.07
3	8.523	Aromadendrene 2	$C_{15}H_{24}$	204	0.69
4	9.634	Germacrene-D	$C_{15}H_{24}$	204	0.33
5	11.525	(Z,Z)-Alpha-farnesene	$C_{15}H_{24}$	204	0.06
6	11.905	Caryophyllene oxide	$C_{15}H_{24}O$	220	1.01
7	17.132	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	5.75
8	18.473	1-Pentatriacontanol	$C_{35}H_{72}O$	508	1.60
9	20.094	Heptadecanoic acid	$C_{17}H_{34}O_2$	270	5.58
10	20.584	Bicyclo 5.1.0 octane	C_9H_{14}	122	7.02

CONCLUSIONS

The results indicate that different solvent extracts exhibit varying degrees of free radical scavenging capacities. The present study elucidated the highest free radical scavenging potential in the methanol extract of *Evolvulus alsinoides* L. using two different *in vitro* assays DPPH and ABTS.

The total phenolic content correlates well with the radical scavenging activity. The antioxidant activity of the *Evolvulus alsinoides* L. found in the present study may be due to the presence of active components such as Bicyclo 5.1.0 octane, Hexadecanoic acid and Heptadecanoic acid. Antioxidative properties of various extracts from many plants are of great interest in both academia and the food industry, since their possible use as natural additives emerged from a growing tendency to replace synthetic antioxidants by natural ones. Thus, the *Evolvulus alsinoides* L. can be used as an easily accessible source of natural antioxidants and a preventing agent for many diseases which are caused by free radicals.

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