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Antioxidant Activity of *Talinum portulacifolium* (Forssk) Leaf Extracts

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SUMMARY

The aim of the study is to investigate the antioxidant activity through, reducing power, 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH), nitric oxide radical (NO), superoxide radical, hydrogen peroxide radical (H_2O_2) scavenging activity and the amount of total phenolic compounds of chloroform, ethyl acetate, methanol and aqueous extracts of the leaves of *Talinum portulacifolium.* Chloroform extract of leaves of *T. portulacifolium* showed highest antioxidant activity, with a direct relationship between activity and concentration of extracts (15 - 240 μg/mL). Among all the extracts, the highest amount of the total polyphenolic compounds was found in the chloroform extract. Chloroform extract of *T. portulacifolium* showed an important free radical scavenging activity towards the DPPH, NO, Superoxide and H_2O_2 radicals, with IC_{50} values of 133.26, 165.75, 156.34 and 135.29 μg/mL, respectively. In the lipid peroxidation assay, extracts of chloroform and ethyl acetate showed a remarkable inhibitory activity. The extracts showed significant activity in all the experiments but lower than the standard antioxidant, ascorbic acid.

Key words: Antioxidant activity, *Talinum portulacifolium,* Lipid peroxidation, Free radical scavenging

INTRODUCTION

Free radicals have aroused significant interest among scientists in the past decade. Their broad range of effects in biological systems has drawn the attention of many experimental works. Free radicals have been implicated in a variety of conditions including inflammation, atherosclerosis, diabetes, ageing and hepatotoxicity (Gokhale and Saraf, 2000). Free radicals attack membrane lipids there by generating lipid radicals, and this lipid radicals can combine with oxygen producing peroxy radicals, the peroxy radical can further cause peroxidation of cellular membrane lipids leading to cell necrosis.

Antioxidants are produced endogenously, to protect the body from damaging oxidation reactions by reacting with free radicals and other reactive oxygen species within the body, hence hindering the process of oxidation. During this reaction the antioxidant sacrifices itself by becoming oxidized. But, endogenous antioxidant supply is not unlimited as one antioxidant molecule can only react with a single free radical. Therefore, there is a constant need to replenish antioxidant resources, either endogenously or through supplementation

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(Ames *et al*., 1993). Although there are some synthetic antioxidant compounds, such as butylated hydroxyl toluene (BHT) and butylated hydroxyl anisole (BHA), which are commonly used in processed foods, it has been reported that these compounds have some side effects (Brainien, 1975; Ito *et al*., 1983). Several antioxidants of plant origin have been screened for their ability to scavenge free radicals and are used as effective protective agents against oxidative stress. For a long time the tribal people of the Rayalaseema region in Andhra Pradesh, India have used the leaves of the plant *Talinum portulacifolium* (Forssk: Portulacaceae) to keep away from Diabetes (Nagaraju and Rao, 1989). The scientific basis of such a beneficial effect of plant leaves is not clear. It is possible that the compounds present in the extracts might contribute to it's over all protective effect. Therefore, this study was designed to investigate the *in vitro* antioxidant activity of the different extracts of *T. portulacifolium* leaves to establish its potential therapeutic value.

Under physiological conditions, a wide range of antioxidant defences protects against the adverse effects of free radical production *in vivo* (Halliwell and Gutteridge, 1990). In diabetes mellitus, protein glycation and glucose auto oxidation may generate free radicals, which in turn catalyze lipid peroxidation **(**Baynes, 1991). Disturbances of antioxidant defense systems in diabetes mellitus such as alterations in antioxidant enzymes (Maritim *et al*., 2003; Seif and Youssef, 2004), lowered vitamin levels (West, 2000). It is well known that antioxidants can seize the free radical chain of oxidation and form stable free radicals, which would not initiate or propagate further oxidation.

The genus *Talinum* consists of approximately 500 species across the world. The family is cosmopolitan and it has 19 genera and more centered in South Africa and America (Heywood, 1978). It is Perennial, suffrutescent, shrubby plant distributed from Rajasthan, India south wards in to the peninsular region; also found in Nepal. It is cultivated in Africa and, like spinach, is used as a vegetable. It is also said to be used as an aphrodisiac (Anonymous, 1974). The leaf powder of this plant mixed with boiled milk is taken twice a day for 60 days to treat diabetes (Seetharami *et al*., 2004). This paper discusses the *in vitro* antioxidant activity of the water, chloroform, ethyl acetate and methanol extracts of the leaves of *T. portulacifolium* and investigates its possible antioxidant ability in comparison with commercially available antioxidant such as ascorbic acid.

MATERIALS AND METHODS

Chemicals

1,1-diphenyl-2-picryl-hydrazyl (DPPH), nicotinamide adenine dinucleotide (NADH) and trichloracetic acid, potassium ferricyanide, phenazine methosulphate, ferric chloride, tris-HCl buffer, L - ascorbic acid, hydrogen peroxide, folin - ciocalteu reagent, Gallic acid, sodium nitroprusside, sulphanilamide, naphthyl ethylene diamine dihydro chloride, phosphoric acid were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). All other solvents/ chemicals used were of analytical grade and obtained from MERCK (Mumbai, India). U.V – Visible spectra measurements were done using a Genesys-5 U.V-visible spectrophotometer (Shimadzu Corporation).

Plant material

The leaves of *T. portulacifolium* were collected from Tirupati (Andhra Pradesh, India) during October, identified and authenticated in Botanical Survey of India, Kolkata (No: CNH/I-I(72)/2006/Tech. II). The specimen was placed in the herbarium of Pharmacognosy and Phytotherapy Research Laboratory of Jadavpur University, Kolkata, India.

Extraction procedure

Plant material was exhaustively extracted successively with petroleum ether (40 - 60°C), chloroform, ethyl acetate, methanol, and distilled water, using a soxhlet extractor. The extracts were concentrated to

dryness in vacuum. The yield of petroleum ether, chloroform, ethyl acetate, methanol, and water extracts, were 1.5%, 2.4%, 3.2%, 12.3% and 9.2%, respectively. Petroleum ether extract didn't used in the experiment because it shows very less activity.

Preliminary phytochemical analysis

The different extracts obtained were thoroughly analyzed for the presence of different chemical groups using standard methods (Brain and Turner, 1975).

Determination of total phenolic content

Total phenolic content of chloroform, ethyl acetate, methanol and water extracts were determined using the Folin-Ciocalteau reagent (Singleton and Rossi, 1965). Samples were homogenized in 80% aqueous ethanol at room temperature and centrifuged in cold at 10 000 × g for 15 min and the supernatant was collected. The residue was re-extracted twice with 80% ethanol and supernatants were pooled, put into evaporating dishes and evaporated to dryness at room temperature. Residue was dissolved in 5 mL of distilled water. One-hundred microlitres of this extract was diluted to 3 mL with water and 0.5 mL of Folin-Ciocalteau reagent was added. After 3 min, 2 mL of 20% of sodium carbonate was added and the contents were mixed thoroughly. The color was developed and absorbance measured at 650 nm after 60 min using gallic acid as a standard. The results were expressed as μg gallic acid/100 g of dry weight material.

DPPH radical scavenging activity

The antioxidant activity using the DPPH assay was assessed by the method of Tagashira and Ohtake (1998). A test sample solution (15 - 240 μg/mL) 200 μL were added to 4 mL of 100 mM ethanolic DPPH. After vortexing, the mixture was incubated for 10 min at room temperature and the absorbance at 517 nm was measured. The difference in absorbance between a test sample and a control (ethanol) was considered as activity. The activity was shown as IC_{50} value (50% of inhibitory concentration in μg/ml). Ascorbic acid was used as reference substance. All values are shown as the mean of three parallel measurements.

Reducing Power

The reducing power of *T. portulacifolium* leaf extracts was determined according to the reported method (Oyaizu, 1986). The five concentrations of *T. portulacifolium* leaf extracts (15, 30, 60,120, 240 μg/mL) in 1mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 1000 × g (MSE Mistral 2000, U.K.). The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. Higher absorbance of the reaction mixture indicated greater reducing power.

Superoxide radical scavenging activity

Measurement of superoxide anion scavenging activity of different extracts of *T. portulacifolium* leaves was based on the method described by Liu *et al*. (1997) with slight modification (Gulcin et al., 2003a). Superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments, the superoxide radicals were generated in 3 mL of Tris-HCl buffer (16 mM, pH 8.0) containing 1mL of NBT (50 μM) solution, 1mL NADH (78 μM) solution, and a sample solution of *T. portulacifolium* leaf extracts (from 15 to 240 μg/mL) in water. The reaction started by adding 1mL of phenazine methosulfate (PMS) solution (10 μM) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance at 560 nm was measured against blank samples, ascorbic acid was used as a standard. Decreased absorbance of the reaction

mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of Superoxide anion generation was calculated using the formula.

$$\% \text{ Inhibition} = [(A_0 - A_1)] / A_0 \times 100$$

Where, in the instance A_0 was the absorbance of the standard (L-ascorbic acid), and A_1 was the absorbance of *T. portulacifolium* leaf extracts and standards (Ye *et al*., 2000; Gulcin *et al*., 2003b).

Scavenging of hydrogen peroxide radical

The ability of the *T. portulacifolium* leaf extracts to scavenge hydrogen peroxide was determined according to the method of Ruch *et al*. (1989). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm. *T. portulacifolium* leaf Extracts (15 - 240 µg/mL) in distilled water were added to a hydrogen peroxide solution (0.6 mL, 40 mM). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of both *T. portulacifolium* leaf extracts and standard compounds was calculated using the following equation:

$$\% \text{ Scavenged } [H_2O_2] = [(A_o - A_1) / A_o] \times 100$$

Where A_o was the absorbance of the control and A_1 was the absorbance in the presence of the sample of *T. portulacifolium* leaf extracts and standards (Gulcin *et al*., 2003c).

Nitric oxide radical scavenging activity

Nitric oxide scavenging activity was measured by using a spectrophotometer. Sodium nitroprusside (5 mM) in phosphate buffered saline was mixed with different concentrations of extract (15 – 240 µg/mL) dissolved in methanol and incubated at 25°C for 30 min. A control without test compound but with equivalent amount of methanol was taken. After 30 min 1.5 ml of the incubation solution were removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide and 0.1% napthyl ethylene diamine dihydrochloride in 2% H_3PO_4). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthyl ethylene diamine was measured at 546 nm (Sreejayan and Rao, 1997).

Effect on lipid peroxidation:

Rat brain homogenates

Rats were handled according to international regulations, and maintained under steady conditions of humidity, food, circadian cycles, and temperature. Brain homogenates were obtained from 3-month-old male Wister rats weighing 200 - 250 g. For the ferrous ion oxidation with xylenol orange (FOX) method, 40% (w/v) homogenates were prepared in HPLC - grade methanol (Elizabeth *et al*. 2006).

FOX method

Lipid peroxidation was conducted for a 60 min interval at 37 °C. The mixture for lipid hydroperoxide generation contained 10 µL of Fenton's reagent 1 (5 µL of 5 mM manganese Chloride plus 5 µL of 50 mM hydrogen peroxide), 10 µL of each extract, and 80 µL of brain homogenate. 900 µL of FOX reagent (49 mg of ferrous ammonium sulfate in 50 mL of 250 mM H_2SO_4, 0.397 g of BHT, and 0.038 g of xylenol orange in 950 mL of HPLC- grade methanol) was added to each sample and left to react for 30 min at room temperature. Absorbance was read at 560 nm. Hydrogen peroxide was used as a standard (Jiang *et al*., 1992).

Statistical analysis

Experimental results were mean ± S.E.M. of three parallel measurements. Analysis of variance was performed by one way ANOVA. Significant differences between means were determined by Duncan's multiple range tests. $P < 0.05$ were considered as significant.

RESULTS

Preliminary phytochemical analysis gave positive results for steroids, terpenoids in petroleum ether extract; alkaloids, glycosides, steroids, flavanoids, terpenoids in chloroform extract; Flavanoids, saponin, alkaloids in ethyl acetate extract; saponin, flavonoids, alkaloids in methanol extract; saponin, flavonoids, alkaloids in water extract. The total phenolic content of different extracts of *T. portulacifolium* is given in Table 1. The total phenolic compounds were expressed as μg of gallic acid (R^2 value is 0.9543) equivalent per 100 g of dry weight of leaves of *T. portulacifolium*. A varietal difference was noted for content of total phenolics among the extracts. The antilipid peroxidation, reducing power and free radical scavenging activities of various fractions of *T. portulacifolium* were evaluated and the results were summarized in Table 3 and 2. Results showed that all tested

Table 3. Effect of different extracts of *T. portulacifolium* on lipid peroxidation

Extract	Inhibitory Concentration (IC_{50}) (μg/ml)
Chloroform extract	10.98
Ethyl acetate extract	15.14
Methanol extract	22.45
Aqueous extract	41.66
Ascorbic acid	8.75

Data are mean ± S.E.M. values (n=3)

Table 1. Total phenolic contents of different extracts of *T. portulacifolium*

Extract	Total Phenolic content (μg gallic acid /100 g of dry weight material)
Chloroform extract	39
Ethyl acetate extract	30
Methanol extract	24.5
Aqueous extract	20

Table 2. Inhibitory concentration (IC_{50}) of *T. portulacifolium* leaves and ascorbic acid on various *in vitro* free radical systems

Method	Inhibitors (μg/ml)	Inhibitory Concentration (IC_{50}) (μg/ml)
DPPH Radical	Chloroform	133.26
	Ethyl acetate	171.22
	Methanolic	190.86
	Aqueous	209.19
	Ascorbic acid	101.071
Superoxide radical	Chloroform	156.34
	Ethyl acetate	187.28
	Methanolic	204.48
	Aqueous	217.73
	Ascorbic acid	105.304
Hydrogen peroxide Radical	Chloroform	135.29
	Ethyl acetate	157.79
	Methanolic	173.48
	Aqueous	189.86
	Ascorbic acid	107.07
Nitric oxide radical	Chloroform	165.75
	Ethyl acetate	176.92
	Methanolic	189.88
	Aqueous	218.55
	Ascorbic acid	146.60

Each value represents the mean ± S.E.M. of three replicates

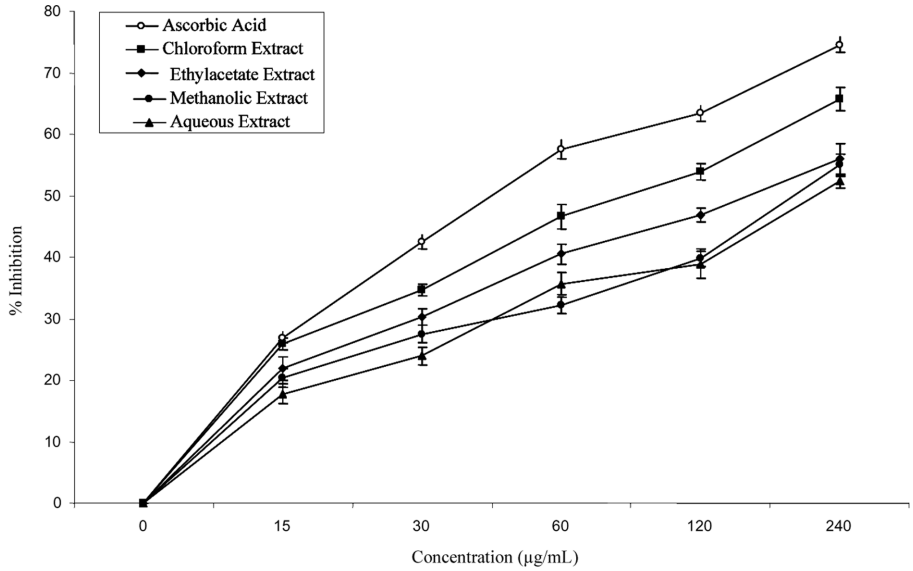

Fig. 1. Effect of different extracts of *T. portulacifolium* leaves and ascorbic acid on DPPH radical scavenging activity. The values are expressed as mean of three experiments.

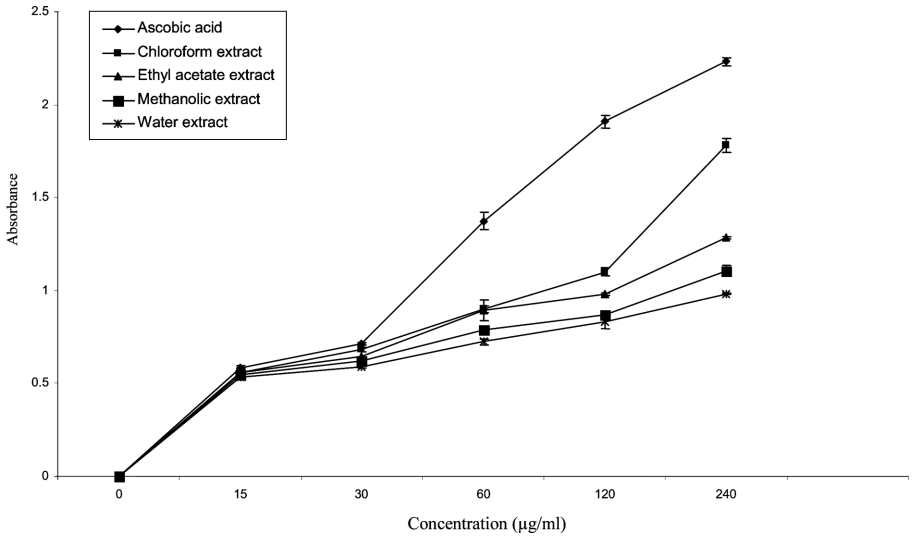

Fig. 2. Effect of different extracts of *T. portulacifolium* leaves and ascorbic acid on reducing power. Values are expressed as mean of three experiments.

several levels.

Further extracts were screened for their possible antiradical activity by four complimentary systems namely DPPH, nitric oxide, super oxide and hydrogen peroxide systems. Free radical scavenging capacities of the corresponding extracts are shown in Figs. 1-5. For the measurement of reducing ability the Fe^{3+}-Fe^{2+} transformation was investigated in the presence of various extracts of *T. portulacifolium*. Fig. 2 depicts the reductive effects of *T. portulacifolium* extracts compared with ascorbic acid. According to the findings presented in the Table 2 and 3 the most free radical scavenging extract of the plant was chloroform followed by ethyl acetate, methanol and water extracts. Water extract exhibited weakest antiradical activity in various *in vitro* radical

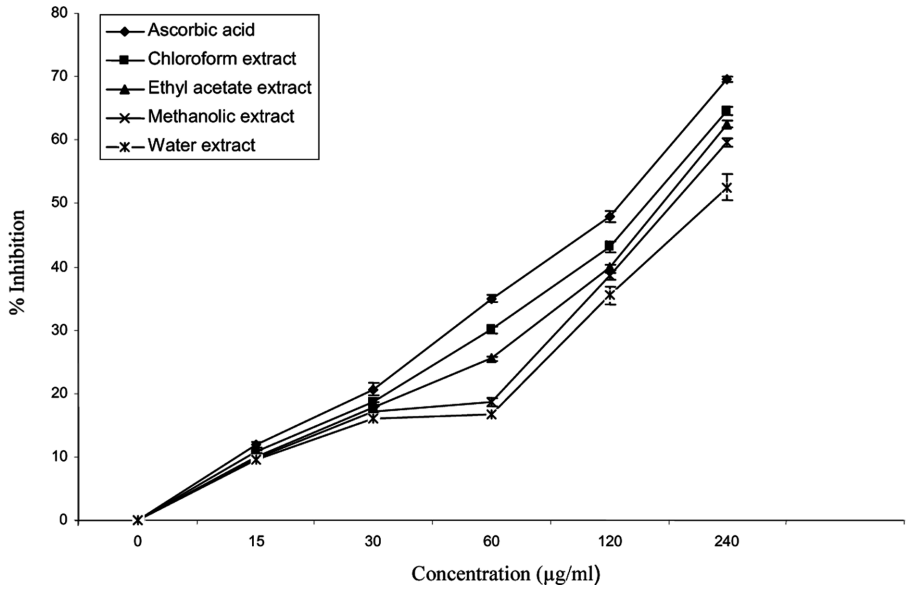

Fig. 3. Nitric oxide scavenging activity of different extracts of *T. portulacifolium* leaf extracts and ascorbic acid. The values are expressed as mean of three experiments.

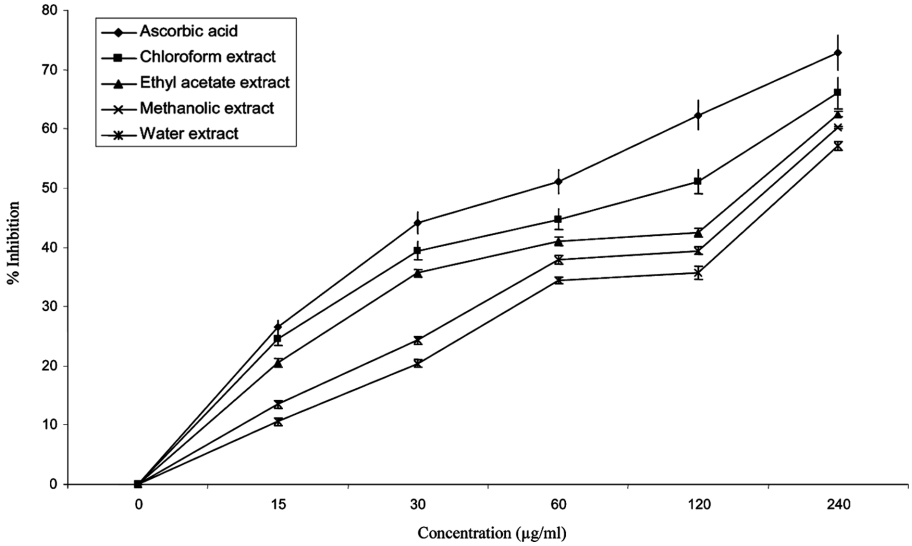

Fig. 4. Hydrogen peroxide scavenging activity of different extracts of *T. portulacifolium* leaves and ascobic acid. The values are expressed as mean of three experiments.

systems. But these antiradical activities are lower when compared with commercial antioxidants tested.

DISCUSSION

Reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals, iron-oxygen complexes, hydrogen peroxide and lipid peroxides are generated by several oxidative reactions (Vuillaume, 1987). Although ROS can help the immune system clean out extrusive microorganisms, excessive ROS can also react with biological molecules such as DNA, proteins and phospholipids, and eventually cause

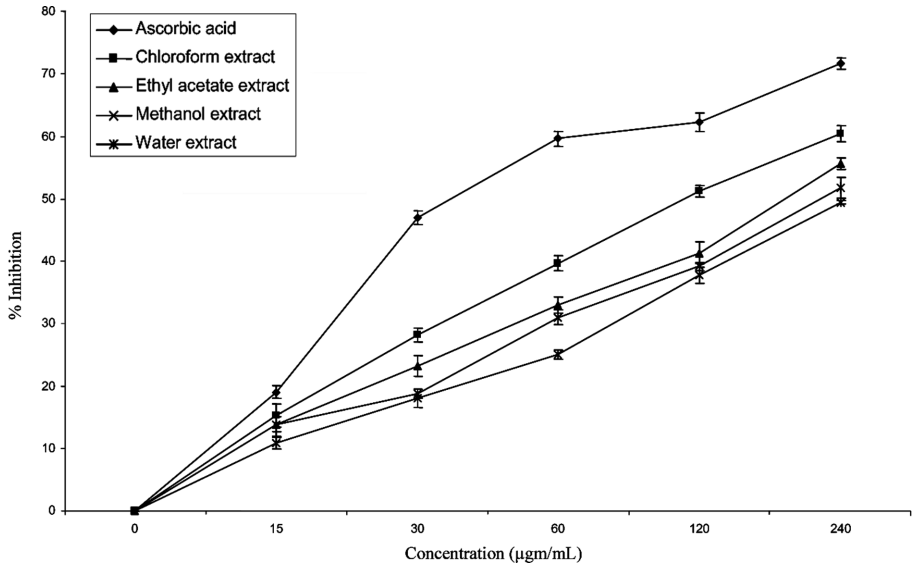

Fig. 5. Super oxide anion radical scavenging activity of different extracts of *T. portulacifolium* leaves and ascorbic acid. Values are expressed as mean of three experiments.

oxidative damage in tissue and free radical-related diseases such as inflammation, heart disease, diabetes, gout, cancer, etc (Slater, 1984) For aerobic organisms, the major system of defense against oxidative damage is the use of "antioxidant" enzymes to convert excessive ROS into non-toxic compounds. An imbalance between the amount of ROS and 'antioxidant' enzymes is a problem for our health. This is why the daily intake of foods with antioxidant activity is necessary (Halliwell and Gutteridge, 1984; Hochstein and Atallah, 1988). Many plants exhibit efficient antioxidant properties owing to their phenolic constituents. Most of tannins and flavonoids are phenolic compounds and may be responsible for antioxidant properties of many plants (Larson, 1988). The role of free radicals in many disease conditions has been well established. Several biochemical reactions in our body generate reactive oxygen species and these are capable of damaging crucial bio-molecules. If they are not effectively scavenged by cellular constituents, they lead to disease conditions (Halliwell and Gutteridge, 1985; Halliwell, 1994).

Antioxidant reacts with DPPH, which is a stable free radical, and converts it to 1, 1 – diphenyl -2-picryl hydrazyl. DPPH radical is scavenged by antioxidants through the donation of hydrogen, forming the reduced DPPH – H (Huang *et al*., 2004). The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm (Dong *et al*., 2004). The reducing power of bioactive compounds had been reported to be associated with their antioxidant effect. Our data on the reducing power of extracts suggested that it was likely to contribute significantly towards the observed antioxidant effect.

A significant correlation was obtained between the total phenolic content in the extracts and the inhibition of lipid peroxidation by the extracts (Table 3), we believe that the antioxidant activity shown by the extracts probably due to the presence of phenolic compounds. Our results are in agreement with those that reported the ability of phenolic compounds to scavenge free radicals and active oxygen species (Hatano *et al*. 1989, Duh *et al*. 1999) Hydrogen peroxide itself is not very reactive, but it can be toxic some time to cells because of it may give rise to a hydroxyl radical (Halliwell, 1991). Thus, removing H_2O_2 is very important for

protection of biological systems. Scavenging of H_2O_2 by *T. portulacifolium* extracts may be attributed to their phenolics, which could donate electrons to H_2O_2, thus neutralizing it to water (Halliwell and Gutteridge, 1985).

Super oxide radical is known to be very harmful to cellular components as a precursor of more reactive oxidative species, such as single oxygen and hydroxyl radicals (Aurand *et al*., 1977). Further more, Superoxide radical is considered to play an important role in the peroxidation of lipids (Dahl and Richardson, 1978). There fore studying the scavenging effects of extracts of *T. portulacifolium* on superoxide radicals is one of the most important ways of clarifying the mechanism of anti oxidant activity.

The results clearly indicate that the chloroform extract of *T. portulacifolium* has highest antioxidant activity against various radicals *in vitro*. It can be used as easily accessible source of natural antioxidants in food as a vegetable. However, the compounds responsible for the antioxidant activity have not been identified. Further study is currently under way aimed towards the isolation and characterization of the compound which is responsible for antioxidant activity. These facts can be further exploited, for instance, in obtaining a bioactive graded fraction, which will have improved antioxidant activity as compared to crude extract.

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