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ANTIOXIDANT STUDIES OF METHANOLIC EXTRACT AND ACTIVE FRACTION OBTAINED FROM *Ichnocarpus frutescens*

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ABSTRACT

In India, tribes used *Ichnocarpus frutescens* (L.) W.T. Aiton, as a substitute of Indian Sarsaparilla (*Hemidesmus indicus* (L.) R.Br.) to treat several illness including fever, dyspepsia, skin troubles and headache. The aim of this study was to investigate antioxidant activities of both methanolic extract and active fraction obtained from *I. frutescens*. Total Phenolic content of different solvent extracts was determined spectrophotometrically according to the Folin-Ciocalteu procedure and the total flavonoid content was determined by Aluminium chloride colorimetric assay. In this study, we assessed antioxidant potential of methanolic extract and active fraction, obtained from methanolic extract of *I. frutescens,* using DPPH, superoxide radical scavenging assay, ABTS, reducing power assay and ferric reducing antioxidant potential (FRAP). Total Phenolic content of different solvent extracts decreases in the following order: methanol > Ethyl acetate >chloroform > petroleum ether > hexane and it was found to be 169.50±0.294 mg GA/g, 146.63±0.306mgGA/g, 42.12±0.20mgGA/g, 11.89±0.34 mgGA/g and 9.99±0.27 mgGA/g extract respectively. Total flavonoid content (TFC) of methanol extract (149.28±0.6 mg QE/g extract) was found significantly higher as compared to other solvent extracts. Active fraction shows maximum inhibition (%) of 92.37%,70 %, 74.96 % and 85.7% in 1,1-Diphenyl-2-picryl-hydrazyl DPPH, superoxide radical scavenging assay, ABTS, and ferric reducing antioxidant potential (FRAP) assays at 100 µg/ml concentration respectively. In reducing power assay methanolic extract, active fraction and gallicacid showed absorbance of 0.62, 0.87, and 0.95 respectively at 100 µg/ml concentration. The results obtained in the present study indicate that *I. frutescens* can be used as an easily accessible source of natural antioxidants and could be harnessed as drug formulation.

KEYWORDS: **Antioxidant, Phenols, DPPH, Superoxide anion, ABTS, FRAP.**

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INTRODUCTION

Since ancient times, medicinal plants are widely used as alternative therapeutic tools for the prevention or treatment of many diseases due to their potent antioxidant activities. Phytochemical and dietary antioxidants are known to reduce the risk of many diseases.Each human cell receives a remarkable number of attacks per day from free radicals, so dealing with free radicals is very important in attaining and maintaining effervescent health. The potentially reactive derivatives of oxygen and nitrogen, attributed as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are continuously generated inside the human body during cell metabolism. However, overproduction of free radicals can easily affect and persuade oxidative damage to functional macromolecules such as DNA, proteins, and lipids if not eliminated quickly[1]. Free radical induced oxidative damage results in many chronic degenerative diseases like cancer, diabetes, neurodegenerative disease, atherosclerosis, cirrhosis, malaria and AIDS[2-5]. Recently, much attention has been dedicated on the therapeutic potential of natural antioxidants in reducing tissue injury caused by free radicals [6].*Ichnocarpus frutescens* (L.) W.T. Aiton, (Apocynaceae) is a climbing plant found throughout India and in the Himalayas under 5,000 feet. Leaves are elliptical, obviate or oblong and the small, greenish flowers grow in axillary umbels. Decoction of the shoots is used in fevers. Leaves are boiled in oil and applied in headaches and fevers[7, 8]. In the current study, efforts were made to evaluate antioxidant activities of methanol extract and active fraction obtained from *I. frutescens*.

MATERIAL AND METHODS

Plant material

The fresh leaves, stems and flowers of *I. frutescens* were collected from areas surrounding the Mysore (Karnataka, India) in the month of November 2014 Plant specimen was preserved at JSS College of Pharmacy, Mysore, India (accession number: Jsscp-Pcog-18). The leaves, stems and flowers were dried under shade and powdered by the help of mechanical process. The coarse powder of leaves stems and flowers have stored in airtight container for further studies.

Extract preparation

10g of sample was extracted by the method of crude extraction using 50mL of Methanol. Methanolic extract was filtered through Whatmann No. 1 filter paper and the solvent containing the phytochemical constituents was concentrated using a rotary flash evaporator[9]. Thus obtained extract was stored until further use.

Preparation of active fraction from methanolic extract

To the methanolic extract, obtained from the above process, methanol and hexane were added in 2:1 ratio and centrifuged at 2000 rpm for 10 minutes. Thus obtained brown coloured precipitate was used for antioxidant studies.

Determination of total phenols

The amount of total soluble phenolic content was estimated according to the Folin-ciocalteu procedure described by Singleton and Rossi [10]. Samples (200µl) were introduced into test tubes. To each tube 1 ml of Folin ciocalteu reagent and 0.8 ml of sodium carbonate (7.5%) were added. After 30 min of incubation, absorbance was measured at 765 nm against control using a spectrophotometer. The total phenolic content was calculated using standard gallic acid calibration curve. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram tissue**.**

Determination of total Flavonoids

The amount of flavonoid content was determined according to a modified colorimetric method of Bao *et al.*[11]. 1 ml of sample was mixed with 1ml of distilled water and 75µl of a 5% NaNOsolution. After 5 min of incubation 75µl of 10% $AlCl_3.H_2O$ solution was added. After 5 min, 0.5 ml of 1M Sodium hydroxide was added. The solution was mixed well and kept for 15 min. The increase in absorbance was measured at 510 nm using a UV-Visible spectrophotometer. The total flavonoid content was expressed as quercetin equivalents (QE) in milligrams per gram tissue as calculated from standard quercetin graph.

DPPH Radical scavenging assay

DPPH stable free radical scavenging activity was determined by the method of Blois [12]. 1.5 ml of each sample was added to 0.5 ml of 0.1mM solution of DPPH in methanol. The tubes were mixed and allowed to stand for 30 min at 37°C. Absorption was measured at 517 nm using a spectrophotometer. Gallic acid was used as the reference material. The percentage of DPPH scavenging was calculated by comparing the absorbance values of the test samples with those of the controls (not treated with extract). The percentage of DPPH radical scavenging was measured by using the following formula

I= (Abs control – Abs sample) / Abs control X 100

Superoxide radical scavenging activity

Superoxide radical scavenging activity was determined according to the method of Fontana *et al*[13]. All the reagents were prepared in 10mM phosphate buffer (pH 7.4). 0.5ml of Nitroblue tetrazolium (156µM), 0.5ml of NADH (468µM), 1.5ml of extracts (to produce final concentrations of 3.12-100µg/ml) was mixed. The reaction was initiated by adding 50 µl of phenazine-methosulfate (60µM) and then the mixture was incubated at 25℃ for 5 min followed by measurement of absorbance at 560nm. Ascorbic acid was used as positive control. The inhibition percentage (I) was calculated as radical scavenging activity as follows.

I= (Abs control – Abs sample) / Abs control X 100.

ABTS Radical scavenging assay

ABTS Radical scavenging activity was determined according to Auddy[14]. ABTS radical cations were generated by reacting ABTS and APS on incubating the

mixture in dark for 16 hours at room temperature. Thus obtained solution was further diluted with PBS to give an absorbance of 1.000. Different concentrations of the test sample and the reference standard (highest volume taken was 50µl) were added to 950 µl of ABTS working solution to give a final volume of 1ml, made up by adding PBS. The absorbance was recorded immediately at 734nm. The inhibition percentage (I) was calculated as radical scavenging activity as follows.

I= (Abs control – Abs sample) / Abs control X 100

Ferric-reducing antioxidant power (FRAP) assay of extracts

The determination of ferrous reducing antioxidant power was according to the Benzie and Strain method with some modifications[15]. 0.5 ml of each sample was added to 1.25 ml of 0.2 M potassium phosphate buffer (pH 6.6) and 1.25 ml of 1% (w/v) potassium ferricyanide. The mixture was incubated for 30 min at 50℃, after which 1.25 ml of 10% trichloroacetic acid was added. The mixture was then separated into aliquots of 1.5 ml and mixed with 1.5 ml of ddH2O. Then, 0.25 ml of 0.1% (w/v) FeCl3 was added to each tube and allowed to stand for 30 min. The absorbance of each tube was read at 700 nm.

Total reducing power

Reducing power assay was performed as described by Yen and Duh [16]. 0.5 ml of each test sample (3.12-100 µg/ml) was mixed with 250µl of 20mM phosphate buffer (pH 6.6) and 250µl of 1% potassium ferricyanide. The mixture was incubated at 50℃ for 20 min; then 250µl of 10% trichloro acetic acid was added, and the mixture was centrifuged at 2500 rpm for 10 min. The supernatant was mixed with 750µl of distilled water and 150µl of 0.1% ferric chloride and the absorbance of each tube were measured at 700nm using UV-VIS Spectrophotometer. The experiment was repeated thrice. Increase in the absorbance of the reaction mixture indicated increase in the reducing power.

STATISTICAL ANALYSIS

Statistical software (5th version) was used for data analysis using one way anova (analysis of variance). Mean separation was accomplished by Duncan's multiple range test. The value p≤0.05 was considered statistically significant.

RESULTS

Determination of total phenols

Total phenolic content of the different extracts of *I. frutescens* was determined by using the Folin-Ciocalteureagent and is expressed as mg of GA/g of extract using the following equation based on the calibration curve: Y = 0.006x + 0.0842, R^2= 0.9888 where x was the absorbance and Y was the mg GA/g. Among the studied plant extracts, methanolic extract contains the highest phenolic content with values of 169.5±0.294 mgGA/g and the lowest TPC values was observed in hexane extract with 9.99±0.271 mgGA/g dry weight.Total phenolic content of the extracts were found to decrease in the following order: methanol extract > ethyl acetate extract >chloroform extract >petroleum ether extract > n-hexane extract.

Table 1
Total phenol content in various extracts of I. frutescens

Solvent extracts	Total phenols in mg GA/g extract
Methanol	169.50±0.294[e]
Ethyl acetate	146.63±0.306[d]
Chloroform	42.12±0.20[c]
Petroleum ether	11.89±0.341[b]
Hexane	9.99±0.271[a]

values are mean ± Standard deviation (n=3). Mean values within the same series with different lowercase letter are significantly different (p<0.05).

Determination of total flavonoids

From the results obtained, total flavonoid content of the extracts (the standard curve equation: y = 0.0014x+0.1102, R2 = 0.9698) in terms of quercetin equivalent were between 10.07±0.9 mg QE/g and 149.28±0.6 mg QE/g d w. Methanolic extract contained the high value of total flavonoids (149.28±0.6 mg QE/g dw), followed by ethyl acetate (33.67±0.55 mg QE/g dw) and methanol extracts (24.6 mg QE/g dw). The lowest values were 10.07±0.9 mg QE/g d w and 11.42±0.6 mg QE/g d w in petroleum ether and hexane extracts, respectively Therefore, polar extracts had more total flavonoids content than non-polar extracts.

Table2
Total flavonoid content in various extracts of I. frutescens

Solvent extracts	Total flavonoid content in mg QE/g dry weight
Methanol	149.28±0.6[b]
Ethyl acetate	33.67±0.55[c]
Chloroform	24.60±0.85[d]
Petroleum ether	10.07±0.9[a]
Hexane	11.42±0.6[a]

Values are mean ± Standard deviation (n=3). Mean values within the same series with different lowercase letter are significantly different (p<0.05).

DPPH Radical scavenging activity

The results of the present study were shown in figure 1. Active fraction showed the higher scavenging activity against DPPH radicals, in a dose dependent manner ranging from 3.12 to 100 µg/ml when compared to methanolic extract. Active fraction exhibited the higher DPPH radical scavenging activity with an inhibition of 92.31% at 100µg/ml, while methanolic extract showed an inhibition of 69.57% at the same concentration.The IC_{50} of methanolic extract, active fraction and gallicacid were found to be 44µg/ml, 30µg/ml and 8µg/ml respectively. Active fraction showed 1.5 fold higher activities when compared with methanolic extract.

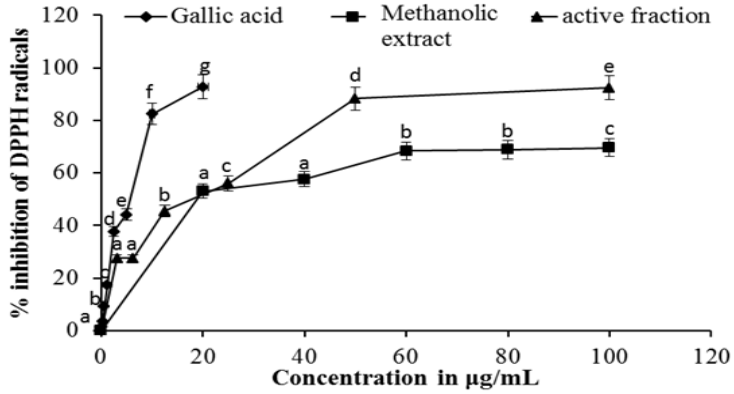

Figure 1

DPPH Scavenging activity. Values are mean ± Standard deviation (n=3). Mean values within the same series with different lowercase letter are significantly different (p<0.05).

Superoxide anion radical scavenging activity

The superoxide radical scavenging activities of methanolic extract, active fraction and the reference compound are increased markedly with increasing concentrations. Active fraction showed 70.36 % of inhibition at 100 µg/ ml concentration, while methanolic extract showed an inhibition of 65.96% at the same concentration. Ascorbic acid showed an inhibition of 25.73% at 100µg/ml. This data shows that the percentage inhibition of methanolic extract and active fraction are significantly higher than the percentage inhibition of ascorbic acid at 100 µg/ml concentration.

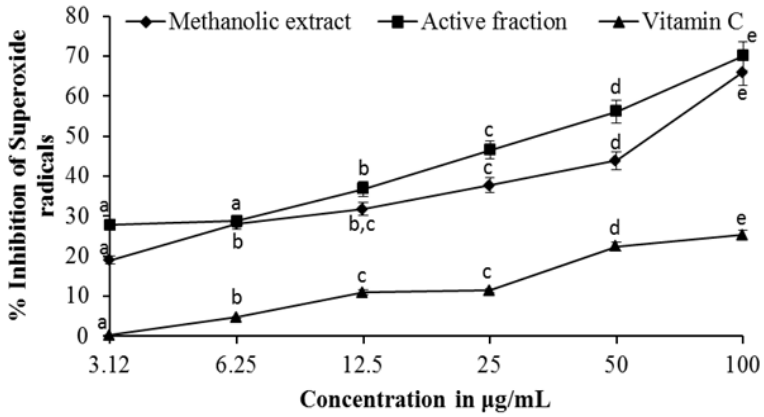

Figure 2

Superoxide anion radical Scavenging activity. Values are mean ± Standard deviation (n=3). Mean values within the same series with different lowercase letter are significantly different (p<0.05)

ABTS Radical scavenging activity

Differences for the $ABTS^{\bullet+}$ (2, 2 azobis-(3-ethylbenzothiozoline-6-sulphonic acid) radical cation scavenging capacities of methanolic extract, active fraction and gallicacid were recorded in this study (figure 3). As can be seen, the scavenging effect of methanolic extract and active fraction increased with increasing concentration. Active fraction exhibited the higher ABTS antiradical properties with an inhibition of 75% at 100µg/ml, while methanolic extract showed an inhibition of 56% at the same concentration.Gallic acid showed an inhibition of 94% at 100µg/ml.

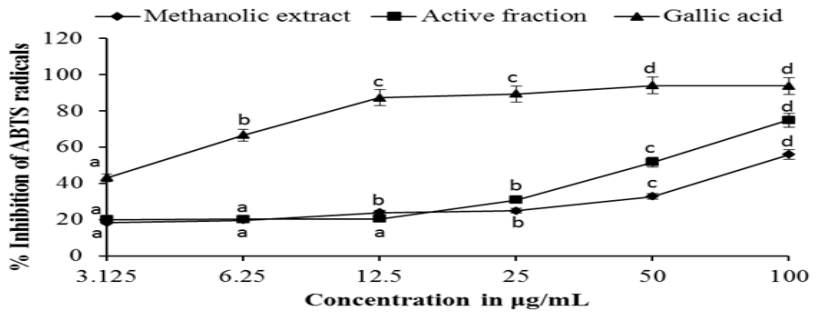

Figure 3
ABTS radical scavenging activity. Values are mean ± Standard deviation (n=3). Mean values within the same series with different lowercase letter are significantly different (p<0.05)

Ferrous reducing antioxidant power assay (Total antioxidant activity assay)
Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Figure 4shows the FRAP assay results of methanolic extract and active fraction compared with gallic acid. Methanolic extract and active fraction showed dose-dependent FRAPs and active fraction showed the higher reducing power than methanolic extract. Ferrous reducing antioxidant power of methanolic extract and active fraction were observed to be 80.96 % and 85.77 % at a concentration of 100µg/ml, whereas the reducing power of the standard gallic acid was found to be 87.75 %.

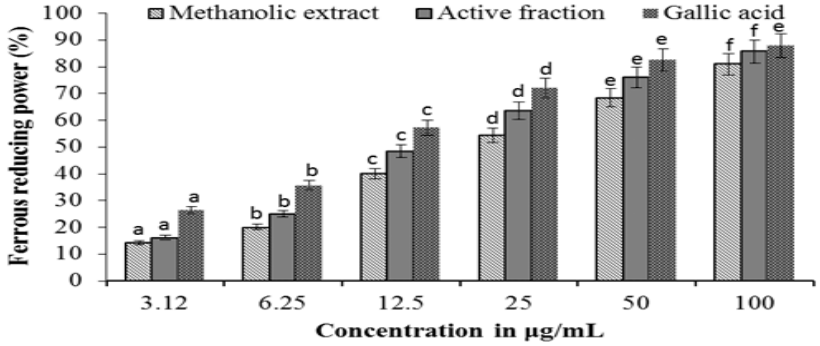

Figure 4
FRAP assay. Values are mean ± Standard deviation (n=3). Mean values within the same series with different lowercase letter are significantly different (p<0.05).

Reducing power assay
The presence of antioxidants in the extracts resulted into reduction of the ferric cyanide complex (Fe^{3+}) to the ferrous cyanide form (Fe^{2+}). Figure 5 showed the reducing activities of methanolic extract and active fraction in comparison with gallic acid as standard. The higher the absorbance of the reaction mixture, the higher would be the reducing power. At 100µg/ml concentration methanolic extract, active fraction and gallic acid showed absorbance of 0.62, 0.87 and 0.95 respectively.

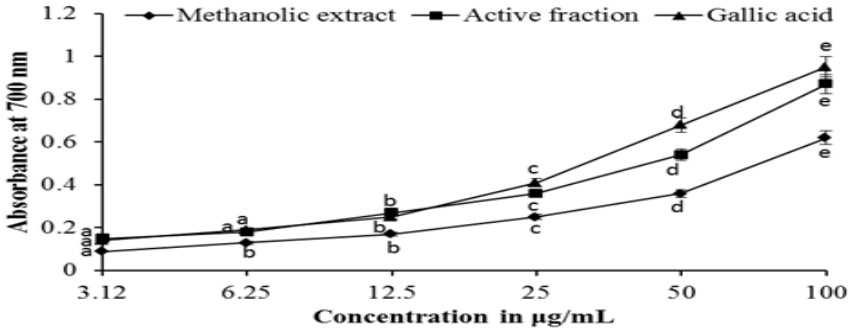

Figure 5
Reducing power assay. Values are mean ± Standard deviation (n=3). Mean values within the same series with different lowercase letter are significantly different (p<0.05).

DISCUSSION

Determination of total phenols

Medicinal plants rich in phenolics are of great interest since these compounds serve in plant defense mechanisms to counteract reactive oxygen species (ROS) in order to prevent damage by microorganisms, insects, and herbivores[17]. Plant materials that have a significant amount of phenolics are increasingly being used in the food industry because they exhibit antioxidant activity by inactivating lipid free radicals and thereby improve the quality and nutritional value of food.[18]. From the results obtained, the methanolic extract of *I. frutescens* showed most potency in phenolic content determination assay.

Determination of total flavonoids

Flavonoids are considered as one of the most widespread groups of natural constituents, which occur in different plant parts both in freestate and as glycosides. Scavenging of injurious free radicals such as super oxide and hydroxyl radicals by the flavonoids may be due to their polyphenolic nature [19]. It has been recognized that flavonoids are particularly beneficial, acting as antioxidants and having considerable effects on human nutrition and health. Antioxidant activity of Flavonoids depends on the structure and position of hydroxyl group [20.] Total amount of flavonoids present in plant extracts depends on the polarity of solvents used in the preparation of extract[21].

DPPH radical scavenging activity

The stable radical DPPH has been widely used to determine the primary antioxidant activity of pure antioxidant compounds, plant and fruit extracts and food materials. DPPH radical scavenging assay is a widely used, quick and reliable method to test the free radical-scavenging ability of various dietary antioxidants [22]. DPPH method is based on the reduction of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) to 2, 2-diphenyl-1-picryl hydrazine. In visible spectroscopy, DPPH gives a strong absorption band at 517 nm because of its odd electron. As this odd electron of the radical becomes paired off in the presence of a free radical scavenger, the absorption reduces and the DPPH solution isdecolourised as the colour changes from deep violet to light yellow and the resulting decolorization is stoichiometric with respect to the number of electrons captured[12]. In the current study IC50 value of methanolic extract is high when compared to the active fraction which may be due to its poor tendancy to donate a proton. Active fraction exhibited a good potential to act as a free radical scavenger with IC50 value of 30µg/ml comparable to gallic acid (8µg/ml).

Superoxide radical scavenging activity

Methanolic extract and active fraction were tested for their scavenging effect on the *in vitro* generation of superoxide radical. In living systems, Superoxide anion plays a vital role in the production of other reactive oxygen species such as hydrogen peroxide, hydroxyl radicals, or singlet oxygen which induce oxidative damage[23]. Superoxide anion is one of the strongest reactive oxygen species among the free radicals that are generated using a nonenzymatic reaction of phenazine methosulphate in the presence of NADH and molecular oxygen [24, 25]. Thus generated superoxide radicals reduce NBT to a blue colored formazan at pH 7.8 that is measured at 560 nm. Consumption of superoxide anions in the reaction mixture by the antioxidants is indicated by the decrease of absorbance at 560 nm. In the present study active fraction showed highest superoxide radical scavenging activity with an inhibition of 70.48% at 100 µg/ ml concentration. Ascorbic acid showed an inhibition of 22.25% at the same concentration. The superoxide anion radical-scavenging activity of the extract may be due to the presence of phenolic compounds and flavonoids.

ABTS radical scavenging assay

The ABTS assay is based on the antioxidant ability to scavenge the long life radical cation ABTS+ generated in the system. ABTS radical assay can be used in both organic and aqueous solvent system to assess either radical scavenging or antioxidant activity. Therefore it is often used in evaluating total antioxidant power of single compounds and complex mixtures of various plants[26]. High molecular weight phenolics such as catechin and rutin derivatives have more ability to quench free radicals ($ABTS^{\bullet}$ + by forming a stable ABTS-H[27]. From the results obtained, ABTS scavenging ability of *I. frutescens* has significant values and corresponds to the presence of high quantity of phenolic compounds.

Ferrous reducing antioxidant power assay

FRAP assay is based on electron transfer reaction and generally reducing properties are associated with compounds that can donate hydrogen atoms to free radicals to convert them into stable non-reactive molecules and then terminate the free radical chain reactions[28]. Initially this method was employed to assess plasma antioxidant capacity, now it can be used to measure the antioxidant capacity of a wide range of biological samples and pure compounds to fruits, wines, and animal tissues[29]. According to recent studies, a highly positive linear correlation between total phenols and total antioxidant activity appears to be the trend in many plant species [30]. In the present study, methanolic extract and active fraction showed dose-dependent FRAPs and active fraction showed the higher total antioxidant activity than methanolic extract. Similarly, antioxidant activity of the ethanol extracts increases with increasing concentration of the extracts in 200 µl to 1000 µl in all the samples according to Saikiya and Upadhyaya[31].

Reducing power assay

The reducing power assay is often used to assess the ability of an antioxidant to donate an electron which is an essential mechanism of phenolic antioxidant action [32]. In the reducing power assay, Presence of reducers causes the conversion of the oxidation form of iron (Fe+3) in ferric chloride to ferrous

(Fe+2) form as determined sectrophotometrically by measuring the formation of Pearl's Prussian blue at 700nm[33]. Many reports have revealed that antioxidant activity is concomitant with the development of reducing power [34]. Generally, reducing properties are associated with the presence of reductones which have been shown to exert antioxidant action by terminating the free radical chain reactions by donating a hydrogen atom [28]. Active fraction contains high amount of reductones than methanolic extract and hence active fraction showed higher reducing power than methanolic extract. In the present Study, an increase in reducing power with an increase in the concentration of the extract have been observed which is in agreement with the reports of Kumar *et al.* and Kalaivani *et al.*[35, 36].

CONCLUSION

This work describes invitro antioxidant activity of the methanolic extract and active fraction of *I. frutescens* for the first time.Both methanolic extract and active fraction *of I. frutescens* showed excellent free radical scavenging activity. Thus this plant may be a valuable source of natural antioxidants or nutraceuticals with potential application to scavenge free radicals via a chain terminating reaction.

CONFLICT OF INTEREST

Conflict of interest declared none.

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