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Active Mechanism Against Free Radical using *Tragia involucrata* L. Leaves and Root Extract by *In vitro* Antioxidant Models

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ABSTRACT

Tragia involucrata L. is a medicinal plant from the family Euphorbiaceae that has been selected for this study due to its widespread usage in the traditional, folklore and ethnobotanical importance. In this study, the antioxidant potential of methanolic extract of leaves and root of *Tragia involucrata* L. was clearly demonstrated. The total phenol, flavonoid content, DPPH$^{\bullet}$, ABTS$^{\bullet+}$ free radical scavenging activity and reducing power assay of the extracts were performed. *Tragia involucrata* L. leaves and roots contain 19.21±2.30, 2.90±0.75 mg/g^{-1} dry weight of total phenolic content as gallic acid equivalent and 17.56±1.80, 1.45±0.50 mg/g^{-1} dry weight of total flavonoid content as quercetin equivalent respectively. IC_{50} value of *Tragia involucrata* L. leaves and roots were found to be 10±1.20 and 156±3.50 µg/mL^{-1} in DPPH$^{\bullet}$ radical scavenging activity and 170±7.00 and >250.00 µg/mL^{-1} in ABTS$^{\bullet+}$ radical scavenging decolorization assay respectively. The ferrous reducing power found to be higher in leaves extract when compared to the root extract. This shows the methanolic extract of *T. involucrata* L. leaves and root posses the maximum antioxidant capacity.

Keywords: *Tragia involucrata* L. , Euphorbiaceae, radical scavenging, L-ascorbic acid, Quercetin

INTRODUCTION

Free radicals rotate on the peripheral layer around the nucleus as unpaired electron and it act as chemical substances causing oxidative stress. ROS are ions, atoms or molecules that have the ability to oxidize reduced molecules and it various forms of activated oxygen such as superoxide anion radicals (.O_2) and hydroxyl radicals (OH·), as well as non-free radicals (H_2O_2) and singlet oxygen[1]. Reactive Oxygen species are the major sources of primary catalysts that create oxidative stress cause oxidative damage leading to numerous diseases and disorders[2],[3] such as cancer[4], cardiovascular disease[5], neural disorders[6], Alzheimer's disease[7], DNA damage[8] and various lung disorders like asthma, chronic obstructive, pulmonary disorders, acute lung injury and lung cancer[9]. The ROS directly stimulate histamine release from mast cells and mucus secretion from airway epithelial cells resulting in asthma.

Plants are important source of natural antioxidants such as vitamin C and E, carotenoids, flavonoids and Tannins[10] and potent free radical scavengers[11]. At present synthetic antioxidants are used but causes many side effects[12] such as, risk of liver damage and carcinogenesis[13]-[15]. Hence, there is a need to produce natural, more effective, less toxic and cost effective antioxidants.

T. involucrata L. is commonly found in tropical regions of India as a weed and it belongs to the family Euphorbiaceae. The parts of this plant are used for many disorders such as scabies, cut wounds, inflammation and skin infection, headache, fever, skin, venereal complaints and baldness among the tribal communities[16],[17]. The fresh leaves causes burning pain and inflammation because of the leaf hairs that contain acid. This acid effect disappears, when the leaves are made to powder form, which has been proved using laboratory animals[18]. Hence, we attempted to find out the antioxidant capabilities of methanolic extract of *T. involucrata* L. leaves and root using different *in vitro* models.

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MATERIAL AND METHODS

Collection and authentication of the plant samples

T. involucrata L. was collected in and around Kozhinjampara, Kerala, India during winter season and the specimen was identified and authenticated by Dr. G. V. S. Murthy, Scientist 'F', Botanical Survey of India, Coimbatore-641 003, Tamilnadu, India. (Vide No. BSI/SRC/5/23/2011-12/Tech-1344 dated 30th November 2011) and the voucher specimen was deposited at the same institute for future reference.

Preparation of the extract

The collected plant materials (leaves and root) were washed thoroughly in tap water, chopped, air dried for 2-3 weeks at 35-40^{0}C and pulverized in electric grinder. The 150 g dry powder successively extracted with methanol (64-66^{0}C), finally 24.0 g and 16.0 g of extracts were obtained.

Phytochemical screening of T. involucrata L.

Phytochemical screening was carried out to identify the secondary metabolites present in methanolic extract of *T. involucrata* L.[19] [20].

Determination of total phenolic content

The total phenolic content of the *T. involucrata* L. methanolic extracts from leaves and root were determined using Gallic acid equivalence (GAE)[21]. The dry extracts were diluted in methanol to get the known concentration (mg/ml), from which 50µl of the sample was transferred to a 10 ml volumetric flask and 0.5 ml undiluted Folin's-Ciocalteu reagent was added. After one minute, 1.5 ml of 20% (w/v) Na_2CO_3 was added and the volume made up to 10 ml with distilled water. The reaction mixture incubated at 25^0 C for one-hour and the absorbance was measured at 760 nm and compared with a pre-prepared Gallic acid calibration curve. The blue colour formation is the end point of a reaction mixture.

Estimation of total flavonoid content

The total flavonoid content in methanolic extract of *T. involucrata* L. leaves and roots were estimated using singleton *et al.*, method[22] with modification. The 1ml extract (mg/ml) mixed with 75 µl of 5% sodium nitrate solution. After 10-minute incubation at room temperature, added 150 µl of 10% aluminium chloride and 0.5ml sodium hydroxide. Finally the reaction mixture made up to 2.5ml with deionized water and if found turbid the reaction mixture is centrifuged at 5000 rpm, the absorbance of the supernatant of the reaction mixture was recorded at 510 nm. A yellow color indicated the presence of flavonoid. The total flavonoid content was calculated as quercetin equivalent (mg/g^{-1} dry weight basis).

DPPH$^{\bullet}$ radical scavenging activity

The ability of *T. involucrata* L. extracts to scavenge the DPPH$^{\bullet}$ radical was assessed using Susanta *et al.*, method[23] with modification. Briefly, aliquots of the extract 10, 20, 40, 80, 160µg /ml was mixed with 3.0 ml DPPH$^{\bullet}$ (0.5 mmol/l, in methanol), the reduction of the absorbance was recorded at 517 nm after 30 min incubation at 37^0C. The percentage of scavenging ability was estimated using the following formula,

Percentage of inhibition (%) =[($A_{control} - A_{sample}$) / $A_{control}$] X 100

Where $A_{control}$ - absorbance of DPPH

A_{sample} - absorbance of reaction mixture (DPPH with Sample)

ABTS$^{\bullet+}$ radical cation decolourisation assay

The methanolic extract of *T. involucrata* L. leaves and roots were evaluated for their ABTS$^{\bullet+}$ radical scavenging ability by following[24] method with modification. The experiments were carried out using an improved ABTS$^{\bullet+}$ decolourisation method. ABTS$^{\bullet+}$ was generated by oxidation of ABTS$^{\bullet+}$ with potassium persulfate. Various concentration (50-250 µg /ml) of the sample mixed with 0.6 ml of generated ABTS$^{\bullet+}$ solution prepared and the reaction mixture make up to 2.5 ml with ethanol. The dark blue colored reduction reaction is the indication of the scavenging ability of the extract. The decreasing absorbance of the reaction mixture was measured at 734 nm by using UV-Visible spectrophotometer (Shimadzu-2450, Japan). The inhibition of the ABTS$^{\bullet+}$ radical scavenging assay was calculated using the above formula,

Percentage of inhibition (%) =[($A_{control} - A_{sample}$) / $A_{control}$] X 100

Where $A_{control}$ - absorbance of ABTS

A_{sample} - absorbance reaction mixture (ABTS with Sample).

Ferrous reducing power assay

The reducing ability of methanolic extract of *T. involucrata* L. leaves and root was assed according to the method of Oyaizu (1986)[25]. Various concentrations (50-250 µg /ml) of the methanolic extracts (0.250ml) was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (1%), after incubation at 50°C for 20 min. add 2.5 ml TCA (10%). Then the reaction mixture was centrifuged at 3000 rpm for 10 min. after centrifugation, 2.5ml of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride (1%), the increasing absorbance was measured at 700 nm. The increasing absorbance of the increasing concentration of the reaction mixture indicated greater reducing power. The reducing power of *T. involucrata* L. methanolic extract was compared with standard antioxidant L-ascorbic acid (mg/ml).

RESULTS AND DISCUSSION

In most developing countries 80% of people utilize medicinal plants for the maintenance of good health as they have powerful natural antioxidants properties[26]. Nowadays, most people use natural antioxidants as nutraceuticals or as food additives[27]. Many medicinal plants contain higher phenolic compounds such as flavonoids, monophenols and polyphenols[28], by keeping this in mind we have screened the major secondary metabolites, free radical scavenging ability and reducing power of the *T. involucrata* L. leaves and root extracts.

***Phytochemical screening of T. involucrata* L.**

The methanolic extract of *T. involucrata* L. leaves and root revealed the presence of Alkaloids, Terpenoids, Flavonoids, Steroids, Cardioglycosides, Tannins, Aminoacids and Proteins (Table 1), which shows the most of the major phytoconstituents are present in the leaves and root extract.

Table: 1 Phytochemical screening of *Tragia involucrata* L.

Extracts	AL	S	TER	FLA	STE	CG	T	AA	P
Leaves	+	-	+	+	+	+	+	+	+
Roots	-	-	-	+	+	-	-	+	+

AL-Alkaloids; S-Saponins; TER- Terpenoids; FLA- Flavonoids, STE-Steroids, CG-Cardioglycoside, T-Tannins, AA-Aminoacids, P-Proteins.

This lead to screen the antioxidant capacity of the crude extract by *in vitro* models like DPPH$^{\bullet}$ radical scavenging activity, ABTS$^{\bullet+}$ radical scavenging activity and Reducing power assay.

Total phenolics and flavanoid contents

The total content of flavonoids and phenolics are the ones active against oxidative stress in the cell as it acts as the free radical scavenger and antioxidant agent. Earlier studies have shown that some phenolic and flavanoid components such as quercetin[29], L-ascorbic acid and gallic acid[30] have active mechanism against oxidative damage and other active mechanism such as prevention of cancer, malignant transformation, apoptosis in leukemia, lung cancer[31].

Polyphenolic compounds in plants have strong antioxidant activity and it help to protecting cell against oxidative damage caused by free radicals. Due to this, the total phenolic content in *T. involucrata* L. methanolic extract of leaves and root were determined with the Gallic acid linear curve ($y = 0.1165x - 0.1045$, $R^2 = 0.981$). Using this linear curve, *T. involucrata* L. leaves and roots showed 19.21±2.30 and 2.90±0.75 mg/g dry weight and estimated the total flavonoid content with the regression calibration curve $y = 0.1205x - 0.1336$ ($R^2 = 0.9963$) of quercetin and extracts showed 17.56±1.80 and 1.45±0.50 mg/g dry weight respectively (Table-2). In both, the total flavonoid and phenolic contents were found to be high in leaves compared to the root (Table-2). The phenolic compounds were directly correlated with its antioxidant ability[32] and it is found to be abundant in some fruits, vegetables and flowers[33]. Flavonoids are one of the most diverse and widespread groups of natural compounds. The plant derived antioxidants especially polyphenols and flavonoids have been used to treat various disease such as cancer, diabetic, aging and prevention of cardiovascular disease[34].

Table: 2 Quantification of total phenolics (TPC) and flavanoids (TFC)

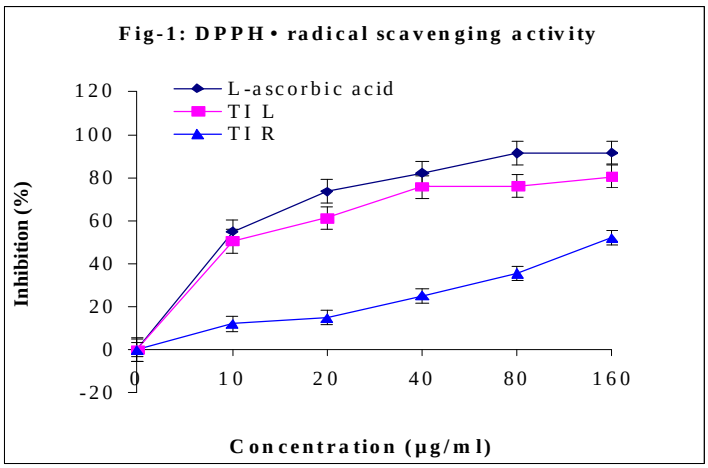
Substance	Method	Equivalent	mg/ g^{-1} of dry weight
TI-Leaves	TPC	Gallic acid	19.21±2.30
TI-Roots	TPC	Gallic acid	2.90±0.75
TI-Leaves	TFC	Quercetin	17.56±1.80
TI-Roots	TFC	Quercetin	1.45±0.50

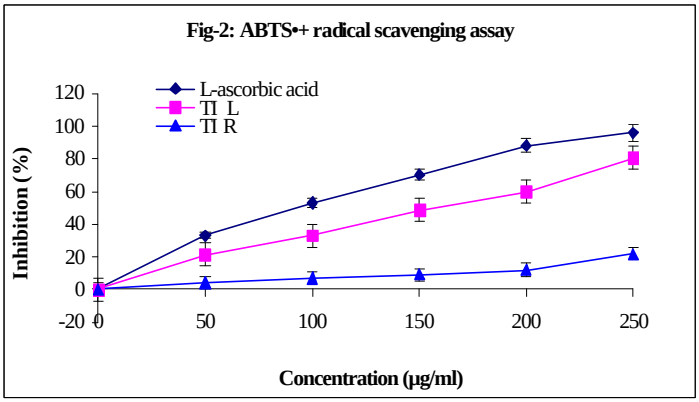
All analyses are the mean of triplicate measurements ± standard deviation

DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ free radical scavenging activity

The photometric evaluation of free radical scavenging ability of the methanolic extract of *T. involucrata* L. leaves and root showed good antioxidant capacity (Figure 1 & 2). More reduction of absorbance were observed in the

DPPH• radical scavenging activity due to the scavenging ability of the extracts. The IC_{50} value calculated for the leaves and root extracts and compared to the standard antioxidant L. ascorbic acid such as 10±1.20, 156±3.50 and 8±0.30 µg/mL^{-1} in DPPH• and 170±7.00, >250.00 and 90±5.50 µg/mL^{-1} in ABTS•+ respectively (Table-3). A lower IC_{50} value indicates a higher free radical scavenging activity. The ability of DPPH• radical scavenging is higher in leaves extract when compared to the root extract. DPPH• is a stable free radical that accepts an electron of hydrogen radical to become stable diamagnetic molecule. It produced hydrazine by converting the unpaired electrons to paired electron due to the hydrogen donating ability of the extract.





The reduction capacity of DPPH• radicals was determined by the decrease in the absorbance at 517nm. Hence DPPH• is usually used as a substrate to evaluate antioxidative capacity of antioxidants. In this study two different radical scavenging models were used such as DPPH• and ABTS•+, these are the synthetic nitrogen centered radicals and also it not found to be biologically relevant, but are often used as "indicator compounds" in testing hydrogen donating capability[35].

Table: 3 *In vitro* antioxidant activity

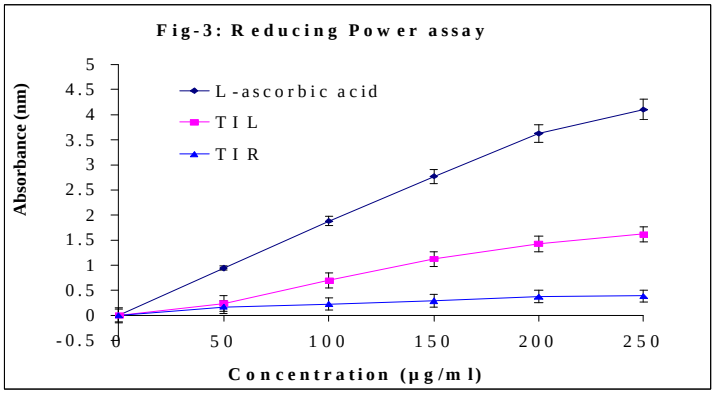
Compound	Method	IC_{50} value (µg/mL^{-1})
L-Ascorbic acid	DPPH•	8±0.30
TI-L	"	10±1.20
TI-R	"	156±3.50
L-Ascorbic acid	ABTS•+	90±5.50
TI-L	"	170±7.00
TI-R	"	>250.00

All analyses are the mean of triplicate measurements ± standard deviation

DPPH radical scavenging activity involves H atom transfer and ABTS•+ radical involve an electron transfer process[36] and reduction were recorded at 517 nm. In ABTS•+ blue chromophore produced by the reaction between ABTS•+ and potassium persulfate in the presence of the plant extract or L-ascorbic acid, where cation radicals reduced and the remaining cation concentration in the reaction were recorded at 734nm.

Ferric reducing power assay

The reducting ability were evaluated using the Fe^{3+}- Fe^{2+} transformation using *T. involucrata* L. Leaves and Root extracts. Increased absorbance were showed with increasing concentration of the sample[37] The higher absorbance value indicated that high antioxidant capacity of the extracts. The result showed significant reduction in leaves extract when compared to the root. The result showed the extract posses ferric ions (Fe^{3+}) reducing ability (Fig-3).



The reduction capability of the extracts indicated them as potent antioxidant. The reducing reaction terminates the radicals that may be very damaging to the tissues. The yellow color of the reaction mixture changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing ability of the extract can be measured by the direct reduction of Fe[(CN6)]3 to Fe[(CN6)], the addition of free Fe^{3+} to the reduced product leads to the formation of the intense prussian blue complex, Fe4[Fe(CN^{-})6]3, which show strong absorbance at 700 nm. It indicates the electron donating capacity of the extracts. The increasing absorbance of the reaction mixture indicates the reducing capacity due to the increasing blue colour complex formation.

5. CONCLUSION

This study clearly showed, *T. involucrata* L. leaves and root extracts posses antioxidant potentials. The methanolic extract of *T. involucrata* L. leaves and root revealed the presence of secondary metabolites such as Alkaloids, Terpenoids, Flavonoids, Steroids, Cardioglycosides, Tannins, Aminoacids and Proteins. *T. involucrata* L. shown to have effective antioxidant capacity in several *In vitro* assays including: DPPH• free radical scavenging, ABTS•+ radical scavenging and reducing power activity, may be due to the high amount of phenolic and flavonoid contents. Our results also showed that the free radical scavenging activity is high in leaves extract compared to the root extracts.

Statistical Analysis

All experiments were repeated at least thrice. The results were expressed as mean ± standard deviation.

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