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PHYTOCHEMICAL ANALYSES AND ANTIOXIDANT ACTIVITY OF THE METHANOLIC EXTRACTS OF *TRIDAX PROCUMBENS* LINN. (ASTERACEAE)

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ABSTRACT

The present study evaluates the phytochemical constituents and *in vitro* antioxidant activity of *Tridax procumbens*. The antioxidant potential of the methanol extracts of leaf, stem and flower of *Tridax procumbens* were tested using different assays such as Reducing power assay, DPPH, hydroxyl, superoxide, nitric oxide, and hydrogen peroxide scavenging assays. The extracts gave positive results for all the assays and the radical scavenging ability was detected to be comparable to those of the corresponding standards which was more prominent in the flower extract. Quantitative estimation of antioxidant phytochemicals such as phenols, flavonoids and proanthocyanidins were also carried out in the methanolic extracts of *Tridax procumbens*. The flower extract was found to have higher levels of flavonoids and proanthocyanidins than leaf and stem extracts. Thus, the plant would be considered as a potential source of antioxidant phytochemicals.

K**eywords:** *Tridax procumbens*, phytochemical screening, antioxidant assays, phenols, flavonoids, proanthocyanidins.

INTRODUCTION

Oxidative stress plays a crucial role in the development of various ailments such as arthritis, asthma, dementia, mongolism, carcinoma and Parkinson's disease. The chain reaction leading to such a damage is known to be initiated by reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, nitric oxide, hydroxyl radical, hypochlorite radical, singlet oxygen and various lipid peroxides [1, 2, 3].

Antioxidants are substances that help organisms to deal with oxidative stress caused by free radicals. The amount of protection provided by any antioxidant depends on its concentration and its reactivity towards the particular ROS [4]. Antioxidants are produced within the body besides being derived from certain food substances. Plants as a rich source of natural antioxidants have a potential role in preventing human diseases [5, 6].

Many studies have reported that several plant chemicals such as phenols and flavonoids inhibit the chain propagation reactions initiated by free radical reactions and hence possess antioxidant property [7]. Polyphenols are reported to have anticarcinogenic, antimutagenic and cardioprotective effects, owing to their free radical scavenging activity [8]. Flavonoids can protect the human body through their antitumor, anti-inflammatory, antimicrobial, antiallergic and antioxidant activites [9, 10].

The present study deals with the analysis of antioxidant potential of the methanolic extracts of leaf, stem and flower of *Tridax procumbens* (Asteraceae). *Tridax procumbens* is a weed widely found in India [11]. The species has been extensively documented in the literature for its variety of medicinal properties such as antiseptic, antiviral, anti-inflammatory, antifungal, anticoagulant and wound healing activities [12, 13]. However, there has been no extensive reports on the antioxidant properties of various parts of this plant. Therefore, the present study focuses on the free radical scavenging potential of *Tridax procumbens* along with a quantitative analysis of the antioxidant phytochemicals.

MATERIALS AND METHOD

Plant Collection:

The plant sample, *Tridax procumbens*, an invasive weed was collected from Nalanchira (Thiruvananthapuram, Kerala) in the month of January, 2015. The plant was washed thoroughly after the root portion was cut off. The leaves, flowers and stem portion were shade dried separately for 25 days. The dried plant parts were powdered and stored for further use.

Chemicals:

Methanol, Gallic acid, Ascorbic acid, Folin-ciocalteau reagent, Sodium carbonate, Vanillin, Aluminium chloride, Potassium acetate, Phosphate buffer, Butylated hydroxyl toluene (BHT), 1, 1- Diphenyl-2-picryl hydrazyl (DPPH), Potassium persulphate, Potassium ferricyanide, Trichloro acetic acid, Sodium nitroprusside, Hydrogen peroxide, Hydrochloric acid, Sulfanilic acid, Glacial acetic acid, Naphthyl ethylenediamine dichloride (NEDD), ferric chloride, Potassium metabisulphite, EDTA, DMSO, Ammonium acetate, Acetone, NADH. All chemicals were of analytical grade.

METHODS:

Preparation of Extract:

About 20g of the powdered plant material was subjected to extraction by soxhlet apparatus using 300 ml of methanol. The extracts were then concentrated under reduced pressure and preserved in refrigerator until further use [14].

Preliminary phytochemical screening:

Phytochemical screening of the methanolic extract of the plant material was carried out to investigate the presence or absence of the different phytochemical constituents like phenols, flavonoids, saponins, tannins, steroids, terpenoids, coumarins and cardiac glycosides[15].

Quantitative estimation of antioxidant phytochemicals:

Determination of total phenol-

2.5ml of 10% Folin-Ciocalteu reagent and 2ml of Na_2CO_3 (2% w/v) was added to 0.5ml of each sample of plant extract solution (1mg/ml). The resulting mixture was incubated at 45ºC with shaking for 15 min. The absorbance of the samples was measured at 765nm using UV/Visible light. Results were expressed relative to Gallic acid (0-0.5 mg/ml) dissolved in distilled water.[16]

Determination of total flavonoids-

Aluminium chloride colorimetric method was used for flavonoid determination. One ml of sample (1mg/ml) was mixed with 3ml of methanol, 0.2ml of 10% aluminium chloride, 0.2ml of 1M potassium acetate and 5.6ml of distilled water and allowed to remain at room temperature for 30 min. The absorbance of the reaction mixture was measured at 420nm with UV-visible spectro-photometer. The content was determined from extrapolation of calibration curve which was made by preparing gallic acid solution (0-0.8 mg/ml) in distilled water. The concentration of flavonoids was expressed in terms of mg/ml.[15]

Determination of total proanthocyanidins

The mixture of 3ml vanillin-methanol (4% v/v), 1.5ml of hydrochloric acid was added to 0.5 ml (1mg/ml) of methanol extracts and vortexed. The resulting mixture was allowed to stand for 15 min at room temperature followed by measurement of the absorbance at 500nm. Total proanthocyanidin content was expressed as gallic acid equivalent (mg/ml) from the standard curve.[17]

Antioxidant assays:

Reducing power assay

Different concentrations of the plant extract (25 - 100 µg/ml) was combined with 2.5 ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The combinations were incubated for 20 min at 50°C. After incubation, 2.5 ml of 10% trichloroacetic acid were added to the mixtures, followed by centrifugation at 2000 rpm for 10 min. The upper layer (5 ml) was mixed with 5 ml of distilled water and 1 ml of 0.1% ferric chloride. The absorbance of the obtained solution was measured at 700 nm.[18]

DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) assay

One ml of 0.135mM DPPH prepared in methanol was mixed with 1.0 ml of methanol extract ranging from 0.2-0.8 mg/ml. The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30 min. The absorbance was measured spectrophotometrically at 517nm.[19] The scavenging ability of the plant extract was calculated using the equation:

DPPH scavenging activity (%) = [(*Abs control-Abs sample)] / (Abs control)] x 100

*Abs control is the absorbance of DPPH + methanol; Abs sample is the absorbance of DPPH radical + sample.

Hydroxyl Radical Scavenging Activity

Various concentrations (20, 40, 60, 80 and 100 µg/ml in methanol) of extracts were taken in different test tubes and evaporated to dryness. One ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5ml of EDTA (0.018%) and 1ml of DMSO (0.85% v/v in 0.1M phosphate buffer, pH 7.4) were added to these tubes, and the reaction was initiated by adding 0.5ml of 0.22% ascorbic acid. Test tubes were capped tightly and heated on a water bath at 80-90^{o}C for 15 min., the reaction was terminated by the addition of 1ml of ice-cold TCA (17.5% w/v). Three ml of Nash reagent (75.0 g of ammonium acetate, 3 ml of glacial acetic acid and 2ml of acetyl acetone were mixed and made up to 1L with distilled water) was added to all of the tubes and left at room temperature for 15min for color development. The intensity of the yellow color formed was measured spectrophotometrically at 412nm against the reagent blank.[20]

Nitric oxide Scavenging Activity

Sodium nitoprusside in aqueous solution at physiological pH spontaneously generate nitric oxide, which interacts with oxygen to produce nitrite ions determined by the use of Griess reagent. Two ml of 10mM Sodium nitroprusside dissolved in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant extract at various concentrations (0.2-0.8 mg/ml). The mixture was incubated at 25^{o}C. After 150 min, 0.5 ml of incubation solution was withdrawn and mixed with 0.5ml of Griess reagent [(1.0ml sulfanilic acid reagent (0.33%) in 20% glacial acetic acid at room temperature for 5 min with 1ml of naphthylethyl enediamine dichloride (0.1% w/v)]. The mixture was incubated at room temperature for 30 min. The absorbance was measured at 540nm.[21] The amount of nitric oxide radical was calculated following this equation:

$$\% \text{ of inhibition of NO} = [*Ao - A_1] / Ao \times 100.$$

*Ao is the absorbance before reaction and A_1 is the absorbance after reaction has taken place.

Superoxide anion Scavenging Activity

The reaction mixture consisting of 1ml of plant extract (0.2-0.8 mg/ml), 1 ml of PMS (60µM) prepared in phosphate buffer (0.1 M pH7.4) and 1 ml of NADH (phosphate buffer) was incubated at 25^{o}C for 5 min. The absorbance was read at 560nm against blank samples.[22]

Hydrogen peroxide Scavenging Activity

The plant extracts (4ml) prepared in distilled water at various concentrations (0.2-0.8 mg/ml) were mixed with 0.6 ml of 4mM H_2O_2 solution prepared in phosphate buffer (0.1M pH 7.4) and incubated for 10

min. The absorbance of the solution was taken at 230 nm against blank solution containing the plant extract without H_2O_2.[23]

RESULTS:

Preliminary phytochemical screening of the plant extract: Table 1 shows the phytochemicals detected in the methanolic extract of various parts of *Tridax procumbens*.

Table 1: Phytochemicals detected in the methanolic extracts of *Tridax procumbens*

Sl. No.	Tests	Observation		
		Leaf extract	Stem extract	Flower extract
1.	Test for phenols	+	+	+
2.	Test for flavonoids	+	+	+
3.	Test for saponins	+	+	-
4.	Test for tannins	+	+	+
5.	Test for steroids	+	+	+
6.	Test for terpenoids	+	+	+
7.	Test for coumarins	+	+	+
8.	Test for cardiac glycosides	+	+	-

Quantitative estimation of antioxidant phytochemicals:

Quantitative estimation showed that the content of flavonoids and proanthocyanidins were greater in flower extract with decreasing quantity in the leaf and stem extracts. Phenols formed a predominant group in the leaf extract (Table 2).

Table 2: Antioxidant Phytochemicals in the Methanolic Extracts of *Tridax procumbens*

Plant Extract	Phytochemicals (extract equivalent of gallic acid (mg/g)		
	Phenols	**Flavonoids**	**Proanthocyanidins**
Leaf extract	4.48	3.59	2.00
Stem extract	3.83	2.98	1.01
Flower extract	4.00	4.32	2.93

Antioxidant Assays:

The results of the antioxidant assays are presented in tables 3-8.

Reducing Power Assay:

The reducing capacity of the methanol extracts of *Tridax procumbens* was found to be appreciable. The results showed that there was an increase in absorbance value and hence, an increase in reducing power of the plant extracts as the extract concentration increased which was more prominent in flower extract (Table 3).

Table 3: Reducing Power Assay using the Methanolic Extracts of *Tridax procumbens*

Concentration of the extract µg/ml	Absorbance value in leaf extract	Absorbance value in stem extract	Absorbance value in flower extract	Absorbance value by *BHT
20	0.05	0.04	0.072	0.091
40	0.145	0.139	0.153	0.167
60	0.231	0.215	0.254	0.283
80	0.282	0.256	0.298	0.358
100	0.359	0.334	0.364	0.387

*BHT- Butylated Hydroxyl Toluene

DPPH Assay:

The DPPH scavenging effect of the extracts and the standard follow the following trend: Ascorbic acid > flower extract > leaf extract > stem extract. (Table 4)

Table 4: DPPH Assay using the Methanolic Extracts of *Tridax procumbens*

Concentration of the extract µg/ml	% of inhibition in leaf extract	% of inhibition in stem extract	% of inhibition in flower extract	% of inhibition by ascorbic acid
20	28.32	25.92	30.22	41.67
40	41.02	36.23	45.31	53.01
60	59.83	54.28	60.78	75.24
80	76.69	63.55	78.72	81.54

Hydroxyl Radical Scavenging Assay:

In Hydroxyl radical scavenging assay, comparable inhibition percentages were noticed in the leaf and stem extracts with the flower extract showing higher percentage of inhibition values almost nearing ascorbic acid (Table 5).

Table 5: Hydroxyl Radical Scavenging Assay using the Methanolic Extracts of *Tridax procumbens*

Concentration of the extract µg/ml	% of inhibition in leaf extract	% of inhibition in stem extract	% of inhibition in flower extract	% of inhibition by ascorbic acid
20	22.41	23.61	26.02	31.00
40	37.15	35.74	38.29	40.24
60	45.96	46.03	51.58	59.97
80	59.41	57.27	63.94	67.72
100	66.24	66.05	70.00	75.32

Nitric Oxide Scavenging Assay:

All the extracts were found to be potent scavengers of nitric oxide radical but the value was lesser than the standard (Table 6).

Table 6: Nitric Oxide Scavenging Assay using the Methanolic Extracts of *Tridax procumbens*

Concentration of the extract. mg/ml	% of inhibition in leaf extract	% of inhibition in stem extract	% of inhibition in flower extract	% of inhibition by BHT(*)
0.2	29.02	27.29	34.98	40.00
0.4	39.80	35.82	40.03	46.38
0.6	52.95	50.45	54.91	60.02
0.8	69.76	63.37	72.00	81.09

Superoxide Scavenging Assay:

Superoxide scavenging assay showed that the superoxide scavenging activity of all the extracts was evidently appreciable in comparison to BHT (Table 7).

Table 7: Superoxide Scavenging Assay using the Methanolic Extracts of *Tridax procumbens*

Concentration of the extract mg/ml	% of inhibition in leaf extract	% of inhibition in stem extract	% of inhibition in flower extract	% of inhibition by BHT(*)
0.2	56.65	53.17	59.01	59.18
0.4	63.32	60.65	69.93	72.46
0.6	72.86	71.40	76.45	77.11
0.8	76.72	74.91	77.89	79.60

Hydrogen Peroxide Scavenging Assay:

In hydrogen peroxide scavenging assay, the ability to quench peroxyl radicals was higher in the flower extract and in BHT standard in comparison to leaf and stem extracts (Table 8).

Table 8: Hydrogen Peroxide Scavenging Assay using the Methanolic Extracts of *Tridax procumbens*

Concentration of the extract mg/ml	% of inhibition in leaf extract	% of inhibition in stem extract	% of inhibition in flower extract	% of inhibition by BHT(*)
0.2	51.73	46.98	54.33	68.50
0.4	56.28	52.17	59.62	72.22
0.6	61.10	59.96	65.74	76.53
0.8	69.95	65.72	77.49	79.01

DISCUSSION

The free radicals generated constantly in the living systems cause considerable damage to biomolecules, ultimately leading to tissue damage and associated degenerative disease conditions. Such a tissue damage could be prevented through the mediation of cell signalling modulations by antioxidants. [14, 24] Many synthetic antioxidants like butyl hydroxyl anisole (BHA) and butyl hydroxyl toluene (BHT) are reported to have specific toxic effects to lungs, foremost stomach and liver.[25] Hence, combating free radicals using natural antioxidant phytochemicals is an area of recent research interest.

Preliminary phytochemical studies in the methanolic extracts of *Tridax procumbens* revealed the presence of phenols, flavonoids, saponins, tannins, steroids, terpenoids, coumarins and cardiac

glycosides in the leaf and stem extracts. While saponins and cardiac glycosides were found to be absent in the flower extract (Table 1). Jude *et al*.[26] reported six phytochemicals from the leaves of *T. procumbens*. Sawant *et al*. [27] detected the presence of steroid, tannin, saponin, anthocyanin, coumarins, phenol and flavonoids in the acetone-water extract of leaves of *T. procumbens*.

Antioxidant activity in plants is mainly due to the presence of phytochemicals such as phenols, flavonoids and proanthocyanidins.[28] The results indicated that the leaf, stem and flower extracts contain substantial amount of phenols and flavonoids. Phenolic content was higher compared to flavonoids and proanthocyanidins in leaf extracts while flower extract was found to have a higher proportion of flavonoids and proanthocynadins (Table 2). These compounds have good antioxidant potential and significant effects on human health and nutrition. [28, 29]

The antioxidant activity of phenolics is basically due to their free radical scavenging activity owing to the presence of hydroxyl groups in relation to carboxyl groups in the aromatic ring system.[28] Flavonoids act through direct scavenging or chelating process. The iron chelating property of flavonoids prevent ROS- iron complexation and subsequent lipid peroxidation [30]. The highly reactive hydroxyl group of the flavonoids inactivates the free radicals through their hydrogen donating ability.[31] Ethanolic extracts of *T. procumbens* is reported to have the flavonoid, quercetin through HPLC and HPTLC studies [32].

Proanthocyanidins also have antioxidant activity and play a role in the stabilization of collagen and elastin besides having antitumor and anticancer activity.[29, 33] Thus the results indicate that the antioxidant activities exhibited may be due to the presence of phenols, flavonoids and proanthocyanidins in the extracts of *T. procumbens*.

The antioxidant potential of the extracts is usually tested by different antioxidant assays. The principle of reducing power assay is based on the reduction of Fe (III) to Fe (II) in the presence of antioxidants. The reducing ability depends on the electron donating ability of the antioxidant phytochemical in the extract which may reduce the intermediates in the chain reactions involved in the peroxidation of lipids, thereby act as both primary and secondary antioxidants.[34] All the three extracts showed some amount of electron donating capacity (Table 3). Nevertheless, the flower extract showed maximum reduction of the ferric to ferrous form as evident through the higher absorbance value observed at 700nm, but the capacities were lower than that BHT.

DPPH is a stable free radical with a characteristic absorption peak at 517nm. The absorption value decreases in the presence of a free radical scavenger as DPPH accepts an electron and get decolourised from deep violet to yellow. The degree of reduction in absorbance measurement is indicative of radical scavenging potential of the extract.[19] Table 4 highlights the DPPH radical scavenging ability of various extracts. The results of DPPH scavenging activity in this study indicated that both the flower and leaf extracts were potentially active than stem extract of *T. procumbens*. The phytochemicals present in the plant extract could be capable of donating hydrogen to a free radical so as to eliminate the unpaired electron, accountable for its reactivity.

Hydroxyl radicals are extremely reactive species formed in living systems and are capable of destroying almost all kinds of biomolecules present in living cells.[35] This highly damaging radical has the capacity to break DNA strands leading to cytotoxic and carcinogenic effects. The radical is known to be a potential source of active oxygen species that cause lipid peroxidation and subsequent damage to tissues 36. The results of the study indicated that the flower extract of *T. procumbens* was much stronger hydroxyl radical scavenger when compared to its leaf and stem extracts (Table 5).

Nitric oxide radicals are produced in the body during inflammatory reactions. Such inflammatory reactions are associated with various chronic disease conditions such as multiple sclerosis, arthritis, juvenile diabetes and ulcerative colitis.[37] Moreover, the radical interfere with the cell cycle regulatory processes owing to their mutagenic potential. The nitric oxide generated from sodium nitroprusside initiates the chain reaction by reacting with oxygen to form nitrite anion.[38, 39] The nitrite formation is inhibited by the extract by competitive elimination of the oxygen molecules involved in the reaction. The results indicated that all the extracts of *T. procumbens* are potent scavengers of nitric oxide. (Table 6).

Superoxide anion being a strong reactive free radical has damaging effects on cells and DNA leading to associated disorders.[40] The result suggested that all the extracts of *T. procumbens* possess potent superoxide scavenging ability compared appreciably with the standard BHT (Table 7).

Hydrogen peroxide is a weak oxidising agent and as such it is not very reactive. They are capable of directly inactivating certain enzymes involved in cellular signalling by the oxidative modification of critical thiol groups[24]. It can be toxic once they penetrate the cell membrane as it possibly gets converted to hydroxyl radicals through their probable reaction with ferrous and cuprous ions. [39]. Scavenging of H_2O_2 by the extracts of *T. procumbens* may be ascribed to their phenolics which provide electron to H_2O_2, thereby reducing it to water. In the present study, all the extracts were capable of scavenging H_2O_2 in a concentration dependant manner with flower extract showing a significant scavenging ability, comparable to the standard BHT. (Table 8).

On the whole, it appears that *T. procumbens* has an appreciable radical scavenging ability and may be utilized effectively.

CONCLUSION

The present work shows the antioxidant efficacy of the methanolic extracts of *Tridax procumbens*. From the results, it is concluded that the methanolic extracts of various aerial parts of the plant exhibits high antioxidant and free radical scavenging activities comparable to those of standards such as gallic acid, BHT and ascorbic acid. The activity is due to the presence of large amounts of phenolic compounds and flavonoids. These in vitro assays suggest that the plant extract especially the flower extract is an excellent source of natural antioxidant that might help prevent the progress of numerous oxidative stress related disorders. However, the chemical compounds

responsible for the antioxidant activity are at present not clear. Therefore, further research is required to isolate and identify the antioxidant compounds present in the plant extract. Moreover, the in vivo antioxidant activity of the extracts need to be conducted prior to clinical use.

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