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PJBS

ISSN 1028-8880

**Pakistan
Journal of Biological Sciences**

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Phytochemical Screening and *In-vitro* Antioxidant Activity Isolated Bioactive Compounds from *Tridax procumbens* Linn

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Abstract: *Tridax procumbens* L., Asteraceae, has been extensively used for various ailments in the Ayurvedic system of medicine. Previous studies have revealed remarkable phytoconstituents from *Tridax procumbens* L. with significant antioxidant activity. The aim of the present study is to measure the anti-DPPH activity of the purified isolated compounds from n butanol soluble part and ethyl acetate soluble part of successive methanolic extract of *Tridax procumbens* L. We thus quantified the total phenolic and total flavonoids in different purified isolated compounds, the whole of the tests were evaluated with a sample conc. of 100 µg mL⁻¹ and were determined spectrophotometrically using Folin-ciocaltue and AlCl₃ reagents, respectively. DPPH (1, 1-diphenyl, 2-picryl hydrazyl) assay was used to determine the *in vitro* antioxidant activity of different isolated compounds. Isolated compounds, one from ethyl acetate soluble part (EF-I) and one from n butanol soluble part (BF-II) were reported to possess a significant anti DPPH activity with lowest IC₅₀ values 67.26 and 80.90 µg mL⁻¹, respectively while comparable to standard ascorbic acid with IC₅₀ value of 59.62 µg mL⁻¹, due to the high concentration of phenols 146.4 µg mL⁻¹ from EF-I and 142.2 µg mL⁻¹ from BF-II and flavonoids 48 and 42.5 µg mL⁻¹ found in EF-I and BF-II isolated compounds, respectively.

Key words: *Tridax procumbens* L., phytochemical screening, antioxidant activity, DPPH

INTRODUCTION

The health promoting benefits of antioxidants of plants are thought to be resulted from their potential effects against the reactive oxygen/nitrogen species. In the present scenario, focus on natural antioxidants has restricted the use of synthetic antioxidants, due to their evil effects on human health (Wang *et al.*, 2011). More than 80% of world's depends for their primary health care wholly and solely on the traditional herbal treatment, a conventional and alternative treatment with less or no side effects. As such, plants possess a wide range of primary and secondary metabolites that can be used to treat chronic as well as infectious diseases (Alshawsh *et al.*, 2012). The use of herbal medicine for the treatment of diseases and infections is as old as mankind. The world Health Organization supports the use of traditional medicine provided they are proven to be efficacious and safe. A vast part of population in the developing and underdeveloped countries like our India, are living in extreme conditions of poverty and some are

suffering and dying for want of safe water and medicine, because of unavailability of alternatives for primary healthcare (GOI, 2001; AYUSH, 2012). Present research as plant drugs and their traditional use has honored herbal medicine as an integral part of standard healthcare. Body resistance to infections has been found to be enhanced due to the use of medicinal plants (Kunle *et al.*, 2012). The family Asteraceae consists of tropical trees and shrubs, a few herbs with about a total of 1400 species and 674 species in India (NISCAIR, 2004).

A noxious wide spread weed, *Tridax procumbens* Linn. commonly known as coat button is found in the West African sub region and other Tropical Zones of the world. It is native to tropical America but it has been introduced to tropical, subtropical and mid temperate regions worldwide. Being a noxious weed and pest plant has been valued for its pharmaceutical properties (Sahoo and Chand, 1998). Investigations into the health maintaining properties of *Tridax procumbens* L. have resulted in the identification of a wide array of bioactive compounds like alkaloids, carotenoids, flavonoids,

fumaric acid, β -sitosterol, saponins and tannins. A rich content of Carotenoids, Saponins, Oleanolic acid and ions like sodium, potassium and calcium has also been reported. Flowers of the plant has been found to contain a wide range of bioactive compounds like Luteolin, glucoluteolin, quercetin and isoquercetin (Jude *et al.*, 2009; Verma and Gupta, 1988). The plant as a procumbent herb has known for its number of pharmacological activities like hepatoprotective activity (Ravikumar *et al.*, 2005), anti-inflammatory (Prabhu *et al.*, 2011), wound healing (Nia *et al.*, 2003; Bhat *et al.*, 2007), antidiabetic activity (Bhagwat *et al.*, 2008), hypotensive effect, immunomodulating property (Tiwari *et al.*, 2004; Oladunmoye, 2006), anticancer activity (Vishnu *et al.*, 2011), antioxidant activity (Chander *et al.*, 2005) etc. Antioxidants are means for the substances or group of the substances that delay or inhibit oxidative damage to a molecule. This defense system is having many modes of classification such as based on their mechanism of action (chain breaking, preventive) (Racek *et al.*, 2005; Yildirim *et al.*, 2001). The literature survey reveals that *Tridax procumbens* L. plant possesses good antioxidant activity (Harborne, 1984). Hence efforts were taken to evaluate the phytochemical screening of *Tridax procumbens* L. and an antioxidant activity of isolated compounds from the methanolic extract using DPPH Assay.

MATERIALS AND METHODS

Plant material: The plant sample was collected by uprooting the whole plant from the fields and along road sides of a local village "Shamsabad" Somehow 85 kms from Bhopal. The authentication of plant was done by a Taxonomist Dr. Shail Bala Sanghi, Professor, Department of Botany, M.L.B. Girls College Bhopal M.P., Voucher specimen was deposited in College Herbarium (V.No. TPR/25/2010). The whole plant was washed with water and shade dried for one week.

Extraction of plant material: Dried material was coarsely pulverized to powdered form 1.5 kg of powdered plant material was defatted with petroleum ether exhaustively extracted with 700 mL of methanol by maceration process. MeOH extract was concentrated using Vacuum Rotary Evaporator and residue was dried in Petri-dish till crystalline deep green mass (102.37 g) was available.

Fractionation of MeOH extract: Methanol extract of *Tridax procumbens* L. (SMETP) was then suspended in water and fractionated with ethyl acetate (3 \times 300 mL) and then n butanol (3 \times 300 mL) in a separatory funnel to enrich methanol extract into flavonoids and saponins,

respectively. Methanolic extract and its fractions (ethyl acetate fraction and n-butanol fraction) were then tested phytochemically and thin layer chromatography was performed for both the fractions to confirm the presence of required bioactive compounds.

Phytochemical study: Biologically active Pet ether extract, Methanolic extracts and its bioactive fractions (ethyl acetate fraction EAF and n-butanol fraction nBF) were evaluated for preliminary phytochemical screening to know the presence of different primary and secondary metabolites present in them i.e., Alkaloids, Terpenoids, Glycosides, Steroids, Triterpenoids, Flavonoids, Carbohydrates, Saponins, Tannins and proteins etc. (Harborne, 1984; Evans and Trease, 1989).

Thin layer chromatography (silica gel G 60 F₂₅₄ TLC plates E. Merck, layer thickness 0.2 mm) was used to confirm the presence of bioactive compounds from the n-butanol soluble fraction. The solvent system used was chloroform: methanol (90:10 v/v). After spraying the plates with vanillin-sulphuric acid and Liebermann Buchard's reagent separately, they were heated at 100°C for 10 min to visualize the spots (Harborne, 1984).

Similarly, presence of bioactive compounds from ethyl acetate soluble fraction were confirmed using thin layer chromatography but the solvent system was ; n butanol: acetic acid: water (4:1:5), Ethyl acetate spotted plate was sprayed with anisaldehyde sulphuric acid and heated at 110°C for 5 min. Plate was also treated separately with 1% AlCl₃ in methanol and observed under UV light (Harborne, 1984).

Bioactivity guided isolation of compounds: Both the fractions were subjected to glass column 60 \times 4.5 cm) (having sintered glass disc at its bottom) packed with a slurry of adsorbent (silica gel; 60-120 mesh), prepared by mixing the adsorbent in the chloroform and used as stationary phase.

Fractions eluted from n-butanol soluble fraction, with 18-39 fraction of chloroform: methanol (95:5), designated as compound-I (BF-I) (12% w/w, 0.6 g). Fraction 40-58 eluted with chloroform: methanol (80:20) afforded a (5.5% w/w, 0.275 g) compound-II (BF-II). Which showed single spot on TLC and chromatogram was developed in chloroform: methanol (90:10 v/v). Those fractions which were eluted in considerable quantities and in pure form (after evaporating the respective mobile phase) were characterized after recrystallization from methanol.

The same procedure was followed for ethyl acetate soluble part using chloroform, chloroform: ethyl acetate (50:50), ethyl acetate, ethyl acetate: methanol (90:10) and methanol to give compound-III (EF-I) (27% w/w, 1.3 gm) and Compound-IV (EF-II) (19.6% w/w, 0.98 gm). These

compounds had shown single spot on TLC plate where the chromatograms were developed in n butanol: acetic acid: water (4:1:5) and the plates were sprayed with anisaldehyde sulphuric acid and heated for 5 min at 110°C. These compounds were purified by recrystallization from methanol. Each fraction was dried in oven at 40-45°C. (Imam *et al.*, 2007; Seebacher *et al.*, 2003).

Chemical characterization: Thin layer chromatography (silica gel G 60 F₂₅₄ TLC plates E. Merck, layer thickness 0.2 mm) was used to confirm the presence of bioactive compounds from the isolated fractions of n-butanol fraction. The solvent system used was chloroform: methanol (90:10 v/v). After spraying the plates with vanillin-sulphuric acid and Liebermann Buchard's reagent separately, they were heated at 100°C for 10 min to visualize the spots (Harborne, 1984).

Similarly presence of isolated bioactive compounds from ethyl acetate soluble fraction were confirmed using thin layer chromatography but the solvent system was ; n butanol: acetic acid: water (4:1:5), Ethyl acetate spotted plate was sprayed with anisaldehyde sulphuric acid and heated at 110°C for 5 min. Plate was also treated separately with 1% AlCl₃ in methanol and observed under UV light (Harborne, 1984).

Further these compounds are sent to IIT Indore for spectral analysis (IR, UV, ¹H NMR, ¹³C NMR, Mass Spectroscopy) for the elucidation of structure, their physical and chemical properties after interpretation of the data.

Total five bioactive compounds two of n-butanol soluble fractions (BF-I, BF-II), two of ethyl acetate soluble fraction (EF-I and EF-II) and ruminant water soluble fraction or Aqueous Fraction (AQF) were isolated in significant quantities and were evaluated for Total phenolic and Flavonoid content then after for antioxidant activity to check the significance of the fractions for their bioactivity as a potential antioxidant.

Determination of total phenolic content: The concentration of phenolic in fractions was determined using spectrophotometric method (Singleton *et al.*, 1999). Methanolic solution of the fractions in the concentration of 0.01 mg mL⁻¹ was used in the analysis. The reaction mixture was prepared by mixing 0.5 mL of methanolic solution of fraction, 2.5 mL of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 mL 7.5% NaHCO₃. Blank was concomitantly prepared, containing 0.5 mL methanol, 2.5 mL 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 mL of 7.5% of NaHCO₃. The samples were there after incubated at room temperature in dark for 45 min. The absorbance was determined using spectrophotometer at λ_{max} = 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was

repeated for the standard solution of gallic acid and a dilution series of gallic acid of concentration 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg mL⁻¹ was prepared and calibration line was construed. Based on the measured absorbance, the concentration of phenolic was read (µg mL⁻¹) from the calibration line; then the content of phenolic in different fractions was expressed in terms of Gallic acid equivalent (µg of GA mg⁻¹ of Fraction).

Determination of total flavonoids content: The content of flavonoids in the examined fractions was determined using spectrophotometric method (Quettier-Deleu *et al.*, 2000). The sample contained 1 mL of methanol solution of the fractions in the concentration of 0.01 mg mL⁻¹ and 1 mL of 2% AlCl₃ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at λ_{max} = 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of rutin and a dilution series of rutin of concentration 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg mL⁻¹ was prepared and the calibration line was constructed. Based on the measured absorbance, the concentration of flavonoids was read (µg mL⁻¹) on the calibration line; then, the content of flavonoids in examined fractions was expressed in terms of rutin equivalent (µg of RU mg⁻¹ of Fraction).

Evaluation of isolated compounds from methanolic extract of *Tridax procumbens* Linn. for antioxidant activity

DPPH free radical scavenging activity

Principal: The Capacity of biological reagents to scavenge the DPPH radical can be expressed as its magnitude of antioxidant ability. The DPPH alcohol solution is deep purple in colour with an absorbance peak of 517 nm. Which appears with the presence of the radical scavenger in the reactive system and when an odd electron of the Nitrogen in the DPPH is paired?

Preparation of stock solutions: An accurately quantity of fractions (10 mg) was dissolved in methanol and volume was brought up to 100 mL with methanol (100 µg mL⁻¹).

Preparation of test solution: The portion of stock solutions of different fractions were diluted appropriately with methanol to obtain a dilution series of concentration range of 25, 50, 75, 100, 125 µg mL⁻¹.

Method: The free radical scavenging activity of the *Tridax procumbens* L. fractions and Ascorbic acid was measured in terms of hydrogen donating or radical scavenging ability using the stable radical 1,1-

diphenyl-2-picrylhydrazyl (DPPH, Sigma-Aldrich) (Ravishankara *et al.*, 2002; Taddei and Rosas, 2000). 0.1 mM solution of DPPH in methanol was prepared and 1.0 mL of this solution was added to 3 mL of test solution in water at different concentrations (25-125 µg mL⁻¹). After 30 min incubation in darkness at room temperature (23°C), the absorbance was recorded at 517 nm. Control sample contains all the reagents except the extract. Percentage inhibition was calculated using Eq. 1, while IC₅₀ values were estimated from the percentage inhibition versus concentration plot, using a non-linear regression algorithm. The data were presented as mean values±standard deviation (n = 3).

$$\text{Percentage inhibition} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \quad (1)$$

where, “A” stands for Absorbance.

RESULTS AND DISCUSSION

The Phytochemical screening of petroleum ether and methanolic extract of *Tridax procumbens* L. showed the presence of Flavonoids, Carbohydrates, Saponins,

Tanins, amino acids, steroids, purines as Shown in Table 1. However Phytochemical analysis of ethyl acetate soluble fraction showed the presence of only flavonoids in significant quantity, further confirmed by using TLC with R_f value (x100) as 34, 58, 64, 69, 89, an n-butanol soluble fraction showed the presence of Carbohydrates, Saponins, Tannins, Steroids and terpenoids, also confirmed using TLC with R_f value (x100) 28, 33, 46, 64. On Column chromatography performed to the bioactive fractions of methanolic extract of *Tridax procumbens* L., a total of 5 compounds with single spots were obtained confirmed after thin layer chromatography as per the procedure discussed above. All the fraction were further studied for the estimation of phenols and flavonoids, that resulted the phenolic concentration of 146.4±2.83, 81.6±2.11, 57.4±1.47, 142.2±2.26 and 6.4±0.2 µg mg⁻¹ equivalent to Gallic acid in EF-I, EF-II, BF-I, BF-II and AQE, respectively, calculated from the calibration plot of Gallic acid concentration versus absorbance as shown in Fig. 1 (Standard Curve equation y = 0.005x + 0.081, R² = 0.988) and as shown in Table 2. and Flavonoid concentration as 48±4.58, 19.5±3.22, 18±2.0, 42.5±1.36, 2.1±1.12 µg mg⁻¹ equivalent to Rutin in EF-I, EF-II, BF-I, BF-II and AQE, respectively, calculated from the

Table 1: Phytochemical composition of various crude extracts of *Tridax procumbens* L.

Phytochemical constituent	Tests	Observation			
		Pet. Ether Extract	MeOH extract	EAF	nBF
Alkaloids	Mayer's reagent test	-	-	-	-
	Wagner's reagent test	-	-	-	-
	Hager's reagent test	-	-	-	-
	Tannic acid test	-	-	-	-
Flavonoids	Alkaline reagent test	-	+	+	-
	Zinc HCl test	-	-	+	-
	Shinod's test	-	+	+	-
Carbohydrates	Molish's tests	+	+	-	+
	Pentose test	-	-	-	+
Saponins	Froth formation test	-	+	-	+
	Hemolytic test	-	+	-	+
Tanins	FeCl ₃ test	-	+	-	+
	Lead acetate test	-	+	-	+
Fats	Saponification test	-	-	-	-
Aminoacids	Ninhydrine test	+	+	-	+
Anthraquinoneglycoside	Borutrage's test	-	-	-	-
Sterols/Steroids	Salkowaski test	-	-	-	+
Terpenoids	Liebermann burchard test	-	-	-	+
Proteins	Biuret test	-	+	-	-
	Xanthoproteic test	-	-	-	-

+: Present; -: Absent; (EAF) Ethyl acetate Fraction, (nBF) n-Butanol Fraction

Table 2: Total phenolic content in fractions expressed in µg mg⁻¹ equivalent to gallic acid

Isolated compounds	Absorbance (Mean±SD)	Conc. of Fraction (µg mL ⁻¹)	Total phenolic content µg mg ⁻¹ equivalent to Gallic acid (Mean±SD)
EF-I	0.813±0.002	100	146.4±2.83
EF-II	0.489±0.006	100	81.6±2.11
BF-I	0.792±0.003	100	57.4±1.47
BF-II	0.367±0.008	100	142.2±2.26
AQE	0.113±0.003	100	6.4±0.2

Table 3: Total Flavonoid content in fractions expressed in $\mu\text{g mg}^{-1}$ equivalent to Rutin

Isolated compounds	Absorbance (Mean \pm SD)	Conc. of fraction $\mu\text{g mg}^{-1}$	Total flavonoid content $\mu\text{g mg}^{-1}$ equivalent to rutin (Mean \pm SD)
EF-I	0.193 \pm 0.002	100	48 \pm 4.58
EF-II	0.136 \pm 0.005	100	19.5 \pm 3.22
BF-I	0.182 \pm 0.003	100	18 \pm 2.0
BF-II	0.133 \pm 0.003	100	42.5 \pm 1.36
AQE	0.083 \pm 0.001	100	2.1 \pm 1.12

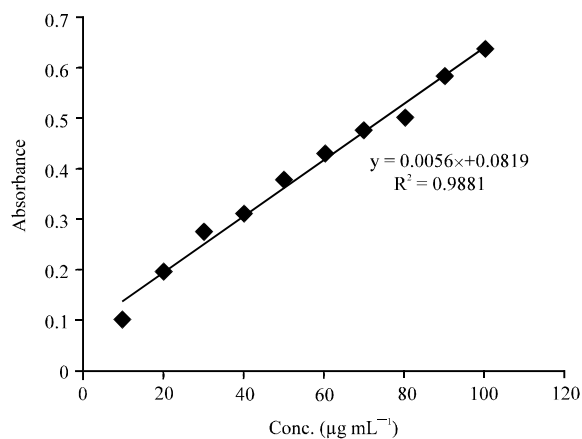


Fig. 1: Calibration plot for the determination of Phenols

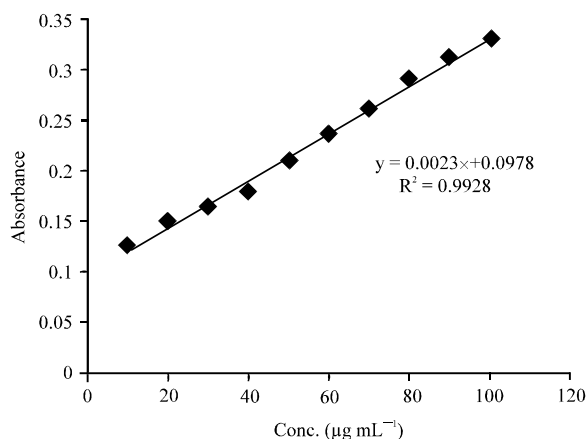


Fig. 2: Calibration plot for the determination of Flavonoids

calibration plot of rutin, concentration versus absorbance as shown in Fig. 2 (Standard Curve equation $y = 0.002x + 0.097$, $R^2 = 0.992$) and as depicted in Table 3 accordingly.

An electron uptake on pairing with a suitable reducing agent resulted the solution to lose colour, as the electron or hydrogen is easily accepted by nitrogen centered free radical; DPPH (Blois, 2001). DPPH assay provides a good assessment for evaluation of *in vitro* antioxidant activity. The results showed that the ethyl

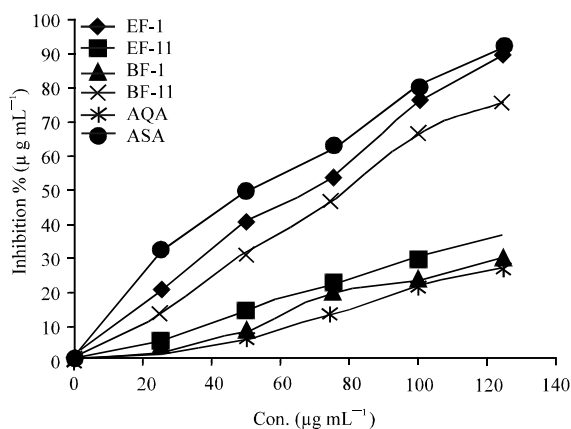


Fig. 3: Effect of isolated bioactive fractions from methanolic extract of *Tridax procumbens* L. on accumulation of DPPH

acetate fractions (EF-I) and n butanol fraction (BF-II) showed significant antioxidant activity with IC_{50} value 67.26 and 80.90 $\mu\text{g mL}^{-1}$ which is comparable to the activity of ascorbic acid with IC_{50} value of 59.62 $\mu\text{g mL}^{-1}$. While as the other fractions EF-I, BF-I and AQA shows less or no significant antioxidant activity, when compared to the standard ascorbic acid, as shown in Fig. 3. Fractionation of the parent extract reduced the complexity of material and provided more accurate idea related to the phytochemicals, responsible for antioxidant activity of *Tridax procumbens* L. Earlier studies reported that antioxidant activity of plants may be due to the plant metabolites like flavonoids, tannins, catechins and other phenolic compounds (Rice-Evans *et al.*, 1995). Further Studies like spectral analysis are in progress to confirm the isolated fractions for their structure elucidation and properties.

CONCLUSION

From the studies it is concluded that compounds isolated from the ethyl acetate fraction possess potent antioxidant activity because of the presence of high concentration of Phenolic and Flavonoids. Which may be either in itself or in combination with other may be of interest in pharmaceutical industry as a new drug as potent antioxidant. Further clinical studies are yet to be needed.

ACKNOWLEDGMENT

We are highly thankful to M.S. Karchule, Amit Nayak, of Pinnacle Biomedical Research Institute, Shymla Hills Bhopal M.P. for providing the necessary laboratory facilities for the conduct of this research work.

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