

A comparative analysis of *in vitro* antioxidant potential of crude extracts of *Tridax procumbens* L. in different solvents and *in vitro* hypoglycemic potential of its hydro-alcoholic extract

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Abstract

The therapeutic potential of crude extracts of aerial parts (stem, leaves and flowers) of *Tridax procumbens* was screened for *in vitro* antioxidant potential and alpha amylase inhibitory action. The crude hydro-methanolic, aqueous and petroleum ether extracts were obtained by percolation-maceration method using 50% methanol, double distilled water and petroleum ether as solvents. Phytochemical screening of these extracts revealed the presence of flavonoids, tannins, alkaloids, glycosides, saponins, phenols, steroids and carbohydrate. Antioxidant activities of the extracts were evaluated for free radical scavenging by hydrogen peroxide scavenging and superoxide scavenging potential. The *in vitro* alpha amylase inhibition action of hydro-methanolic extract of aerial parts of the plant (HMETP) was evaluated for hypoglycemic properties using starch as substrate. Mode of inhibition of the enzyme was also determined. The results indicated that hydro-methanolic extract showed significant antioxidant potency at concentration of 25-80 µg as compared to aqueous and petroleum ether extracts and also possess alpha amylase inhibitory property. Hence it can be suggested that hydroalcoholic extract of aerial parts of *Tridax procumbens* has potential as an antioxidant and probably in biological systems as a nutraceutical for hypoglycemia.

Keywords: *Tridax procumbens*, antioxidant activity, phenolic content, α -amylase inhibition, competitive inhibition

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Introduction

Diabetes mellitus (DM) is a chronic disease with increasing worldwide prevalence. Type 2 DM (T2DM) accounts for majority (90-95%) of diabetes and poses a huge burden on healthcare systems, especially in developing countries (1). Moreover, conventional therapies, as of yet, have been unable to achieve a cure. Hence, systematic and intensive search in medicinal plants for new drugs to treat Type 2 diabetes mellitus seem to be of great utility. Plants have been the major source of drugs in Indian system of medicine and other ancient systems in the world (2,3). The present study aims to study the comparative efficacy of hydro-alcoholic, aqueous and petroleum ether extracts of *Tridax procumbens* (Compositae) as antioxidants for the utilization in the treatment of diabetes. *Tridax procumbens*, a plant belonging to the daisy family, is found perennially in various tropical and subtropical

regions as well as mildly temperate regions worldwide (4). It inhabits waste places, road sides and hedges throughout India (2). Listed as a weed and a pest plant, it has been known by several names including 'Tridax daisy' in English, 'Jayanti veda' in Sanskrit, 'Ghamra' in Hindi, 'Dagadi pala' in Marathi, 'Herbe caille' in French and 'Thata poodu' in Tamil. Some reports from tribal areas in India state that the leaf juice can be used to cure fresh wounds, stop bleeding and also as a hair tonic(4), (5,6,9). Many reports have focused on the immense potential of this plant which has antimicrobial, wound healing, anti-inflammatory and Immunomodulatory properties (6,7,8,12,14). Antioxidant properties have also been demonstrated in various researches (13,15). Antioxidants can scavenge free radicals and play important role in prevention of diabetes (16,18,20). The role of oxidative stress in

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diabetes and diabetic complications has been reported (7,10,11). Although antihyperglycemic effects of leaf extracts of the plant have been reported (16,18) but a comprehensive study of effect of hydro-alcoholic extract as against other pure solvents like water or petroleum ether needed attention to identify a better anti-diabetic agent. Hence in present study, comparative antioxidant effects of aerial parts of *T. Procumbens* extracts in three solvents, its alpha amylase inhibition activity as well as its mode of inhibition of amylase were evaluated.

Materials and methods

Collection of the material Whole plant of *Tridax procumbens* (Compositae) was collected from the premises of Jawaharlal Nehru Cancer Hospital and Research Centre, Bhopal, Madhya Pradesh, India. The material was identified and authenticated. The material was identified and authenticated by Dr. Madhuri Modak, Professor, Department of Botany, Shaheed Bhagat Singh Govt. Degree College, Ashta, Sehore, Madhya Pradesh, India with voucher number 1212-88.01-408.

Preparation of extracts

Fresh and healthy leaves were collected and allowed to shade dry for 2-3 weeks. The shade dried parts were pulverized and weighed and the powdered leaves were divided into three portions. Each portion was macerated in a separating funnel with 50% methanol, petroleum ether or water. The mixtures were vigorously shaken intermittently for 72 hours. The extracts were collected in separate beakers and concentrated in water bath at 45 °C. This process was repeated 3 times at least till colorless marc was obtained for each solvent. It was dried at 45 °C in oven, powder of crude extract collected and weighed. After this, the crude extracts was used for the study of phytochemicals, antioxidant study and enzyme inhibition assay.

Phytochemical screening of extract

The crude hydro-methanolic extract, aqueous extract and petroleum ether extract were subjected to qualitative chemical tests to identify various classes of bioactive chemical constituents present in the plant using standard procedures (11).

Antioxidant assay

Hydrogen peroxide (H₂O₂) scavenging activity

The estimation of H₂O₂ scavenging activity was done by according to the reported method of Nabavi *et al.* (21). The concentration of H₂O₂ solution (40 mM/L) prepared in 50 mmol/L phosphate buffer (pH 7.4) was determined by measuring absorption at 230 nm. Absorbance of extract at different concentrations (200 , 400 , 600 , 800 and 1000 µg/ml) or standard BHT solution with 2mL of H₂O₂ was determined after 10 min against a blank solution of phosphate buffer without H₂O₂. The percentage of H₂O₂ scavenged was determined as:

$$\% \text{H}_2\text{O}_2 \text{ scavenging activity} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Where A_{control} is the absorbance of the BHT and A_{sample} the absorbance of *Tridax procumbens* or standards.

Measurement of superoxide radical (O₂⁻) scavenging activity

The estimation of superoxide radical scavenging activity of the extracts was done based on the modified method described by Liu *et al.* (23). The superoxide is generated by reacting 0.1 mL of NBT (50 µM) solution with 0.3 ml sample solution of different extracts in Dimethyl Sulphoxide (DMSO) in a concentration of 1-10 µg/ml. The reaction was started by adding 1mL of alkaline DMSO 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 the blank samples. Butylated Hydroxy Toluene (BHT) was used as positive control.

$$\% \text{Inhibition} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Where A_{control} is the absorbance of the BHT and A_{sample} the absorbance of *Tridax procumbens* or standards. The data at each point is the average of three measurements.

Determination of total phenolic content (TPC)

The concentration of phenolics in the extract was determined using Folin-Ciocalteu assay (24). In brief, 1 mL of extract (10 mg/ml in DDW) or a series of standard solution of Gallic acid (1 mg/ml) were taken in test tubes and volumes were made up to 10 mL using DDW. 1.0 mL of Folin-Ciocalteu reagent was added to the tubes and shaken. After 5 min, 1 mL of saturated Na₂CO₃ was added to the tubes and the volume made upto 10mL using DDW. The reaction was kept in the dark for 90 minutes at room temperature and the

absorbance noted against blank at 760 nm using UV-1660, Shimadzu Spectrophotometer. All samples were analyzed in triplicate.

Determination of total flavonoid content (TFC)

The determination of flavonoids was carried out according to aluminium chloride colorimetric method (25). In brief, to 1ml of different concentrations of the extract (1 mg/ml hydro-methanolic) or standard solution of Rutin (20, 40, 60, 80 and 100 mg/l), 5 mL of 2% AlCl_3 in methanol was added. After a 60 minute incubation at room temperature (23 ± 2 C) the absorbance against blank consisting of water instead of extract was determined at 510 nm using UV-1660, Shimadzu Spectrophotometer and the flavonoids content was calculated with (\pm) rutin and the concentration was expressed as (\pm) rutin equivalents. All samples were analyzed in triplicate.

Alpha amylase inhibitory assay

This assay was carried out according to modified procedure of McCue and Shetty (26). A series of HMETP solutions were prepared in varying concentrations (1.25-10 mg/ml). To the tubes, 250 μL of 0.02 M sodium phosphate buffer (pH 6.9) containing 0.5 mg/mL of α -amylase was added. The solution was incubated at 25 °C for 10 min, after which 250 μL of 1% starch solution in 0.02 M sodium phosphate buffer was added at timed intervals and then further incubated in boiling water bath for 5min and cooled to room temperature. The reaction was terminated by adding 500 μL of dinitrosalicylic acid (DNS). The tubes are again incubated in boiling water for 5mins and cooled to room temperature. The reaction mixture was diluted with 5 mL distilled water and the absorbance was measured at 540 nm. A control was prepared by replacing the extracts with distilled water. Each experiment was performed in triplicates, along with appropriate blanks. Acarabose at various concentrations (10-100 $\mu\text{g}/\text{ml}$) was included as a standard. The result is expressed as percentage inhibition, which was calculated as,

$$\text{Inhibition (\%)} = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100$$

The result is also expressed in terms of IC_{50} value.

Mode of α -amylase inhibition

The mode of inhibition of α -amylase by HMETP was performed according to the modified method of Ali *et al.* (27). Briefly, 250 μL of the extract (5 mg/ml) was preincubated with 250 μL of α -amylase solution for 10 min at 25°C in one set of tubes. A second set of tubes was used as control with α -amylase preincubated with 250 μL of phosphate buffer (pH 6.9). In order to initiate the reaction, 250 μL of starch solution at increasing concentrations (6.25-50.0 mg/ml) was added to both sets of reaction mixtures. The mixture was then incubated for 10 min at 25 °C and then boiled for 5 min after the addition of 500 μL of DNS to stop the reaction. The resultant reducing sugar produced during the reaction of each extract solution was spectrophotometrically analyzed at 540 nm for concentration determination using standard maltose curve and converted to reaction velocities. A Lineweaver-Burk plot ($1/V$ versus $1/[S]$) where V is reaction velocity and $[S]$ is concentration of substrate, was plotted. The mode of inhibition of α -amylase enzyme by the crude extract was determined by the help of double reciprocal plot or Lineweaver-Burk plot using Michaelis-Menten kinetics (27).

Statistical analysis

All the determinations were done in triplicate. The statistical processing of the data obtained from all studies is expressed as means \pm standard deviation (SD) of three separate experiments. IC_{50} values were calculated from linear regression analysis.

Results

The active components found in the extract include alkaloids, flavonoids, glycosides, saponins, polyphenols, tannins etc the results are shown in table 1.

Total phenolics and flavonoids

The total phenolic content was calculated using calibration curve for Gallic acid standard curve ($y = 0.002x + 0.004$; $R^2 = 0.956$). The results, as shown in table 2, were expressed as Gallic acid equivalent per gram dry weight of extract (mg of GAE/g of extract). The total phenolic content of hydro-methanolic extract of *T.procumbens* was 24.99 ± 3.1 mg gallic acid equivalent/g of extract which was highest in comparison to extracts of double distilled water (22.86 ± 2.4 mg

Table 1 Phytochemical study of *Tridax procumbens*

Sample Number.	Phytochemical analysis	Aqueous extract	Hydro-methanolic extract	Petroleum ether extract
1.	Flavonoids	+	+	+
2.	Tannins	+	+	+
3.	Alkaloids	+	+	-
4.	Glycosides	-	+	-
5.	Saponins	-	+	-
6.	Phenols	+	+	-
7.	Tepenoids	+	+	+
8.	Steroids	-	-	-
9.	Carbohydrates	+	+	+
10.	Proteins	+	+	+

Table 2 Total phenolic and flavonoid content of *Tridax procumbens*

Sample extracts in different solvents	Total polyphenols (mg GAE/g extract)	Flavonoids (mg Ru/g extract)
Hydro-methanolic	24.99 ± 3.1	8.331 ± 2.6
Water	22.86 ± 2.4	8.07 ± 1.8
Petroleum ether	16.12 ± 1.2	3.28 ± 2.5

of GAE/g of extract) and petroleum ether (16.12 ± 1.2 mg of GAE/g of extract). The total flavonoid content was calculated using calibration curve for Rutin standard curve ($y = 0.006x + 0.014$, $R^2 = 0.985$) and was found to be 8.331 ± 2.6 mg Rutin equivalent/g of hydro-methanolic extract, 8.07 ± 1.8 mg Rutin equivalent/g for aqueous extract and 3.28 ± 2.5 mg Rutin equivalent/g for petroleum ether extract.

Hydrogen peroxide scavenging

It was found that the radical scavenging activity of extract augmented with the increase in concentration. The extract exhibited good reducing power at 25 - 80

µg/ml but its activity was not comparable with that of BHT ($P > 0.05$). Extract was capable of scavenging hydrogen peroxide in a concentration dependent manner with maximum activity at 80 µg/ml as shown in Figure 1. The hydro-alcoholic extracts showed better scavenging as compared to aqueous and petroleum ether extracts. The effect of alcoholic extract eventually got stabilized above concentration of 80%. The IC_{50} value for BHT was found to be 24.97.0 µg/ml, 24.9 µg/ml for hydro-methanolic extract, 49.92 µg/ml for aqueous extract and 49.97 µg/ml for petroleum ether extract.

Superoxide radical (O₂⁻) scavenging activity

The scavenging activity of *T.procumbens* on superoxide radicals enhanced with increase in concentration as given in figure 2. The scavenging effect of the *T.procumbens* and standards on the superoxide radical showed that the hydro-alcoholic extracts showed better scavenging as compared to aqueous and petroleum ether extracts. IC₅₀ values of the *T.procumbens* were- hydro-methanolic extract (2.13 µg/ml), water extract (2.24 µg/ml) and petroleum ether extract (2.51 µg/ml).

Alpha amylase inhibition

As antioxidant potency of hydro-methanolic extract was found to be better in comparison to other solvent extracts studied, henceforth, the antihyperglycemic effects of the hydro-methanolic extract (HMETP) only was studied ($y = 0.57x + 39.13$ $R^2 = 0.989$) and it was found that the hydro-alcoholic extract of *T.procumbens* has IC₅₀ value of 19.07 µg/ml. Results are shown in figure 3. Acarbose showed α- amylase inhibitory activity with IC₅₀ value of 0.33 µg/ml.

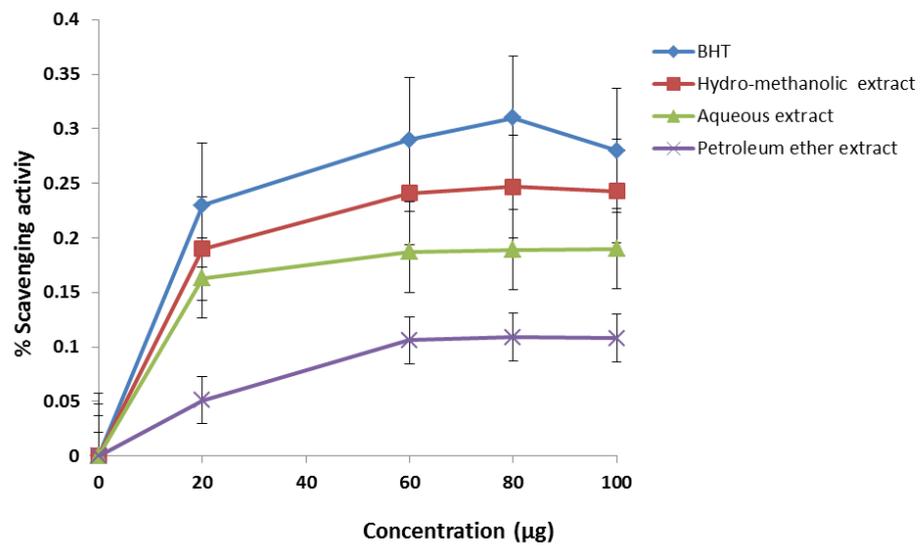


Figure 1 H₂O₂ Scavenging activity of *Tridax procumbens* in different solvents

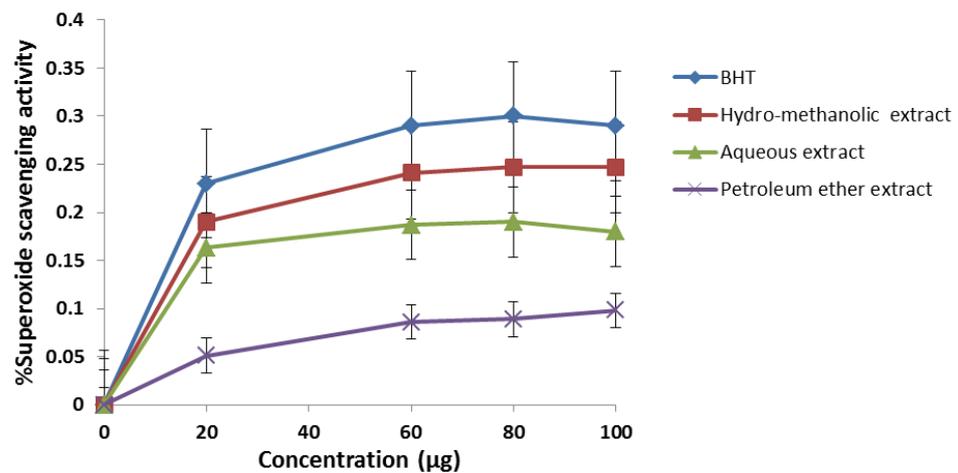


Figure 2 superoxide scavenging activity of *Tridax procumbens* in different solvents

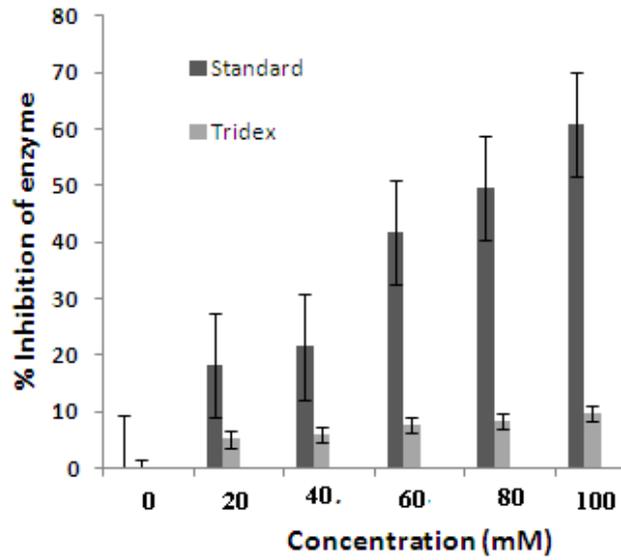


Figure 3 Alpha Amylase inhibition assay of hydro-methanolic extract of *T.procumbens*

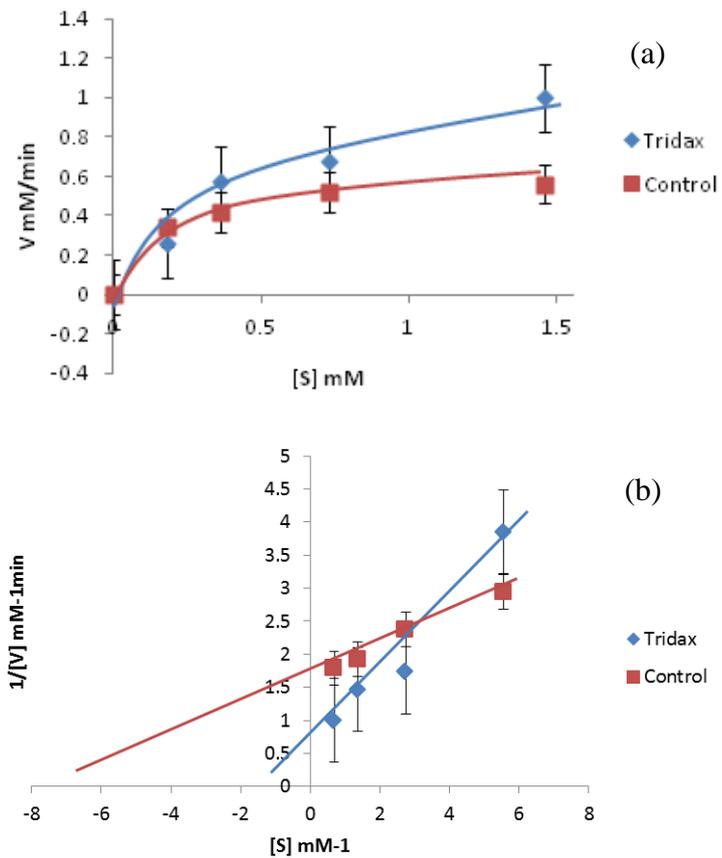


Figure 4 Mode of inhibition of α -amylase by HMETP (a) Michaelis-Menten plot and (b) Lineweaver-Burk plot.

Mode of inhibition

The mode of inhibition of the aqueous extract of HMETP leaf on α -amylase was determined using the Lineweaver-Burk plot which displayed competitive inhibition of the enzyme (Figure 4. A and B). As with increasing extract concentration, fewer active sites are available, so reaction velocity decreases. Michaelis-Menten plot shows lesser inhibition of the enzyme as compared to the standard used.

Discussion

Natural antioxidants not only protect lipids from oxidation, but also provide health benefits associated with preventing damage due to biological degeneration and it is well-known that plant phenolics are highly effective free radical scavengers and antioxidants due to their hydrogen donating ability (12,27). In general, antioxidant activities of plant are often explained with respect to their total phenols, flavonoids tannin content and antioxidant vitamins (28, 30). Table 1 gives the total polyphenols and flavonoids from different solvent extracts, which showed that hydro-methanolic extract contained high levels of polyphenols and flavonoids. Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive oxygen species (29). The superoxide radical is known to be produced *in vivo* and can result in the formation of H_2O_2 via dismutation reaction. Moreover, the conversion of superoxide and H_2O_2 into more reactive species, e.g., the hydroxyl radical, has been thought to be one of the unfavorable effects caused by superoxide radicals (30, 32). Hydro-methanolic extract was markedly a more potent scavenger of superoxide anion than other extracts. However, the reference compound, BHT exhibited higher superoxide scavenging activity than the extracts. Scavenging of H_2O_2 by *T.procumbens* extract may be attributed to its phenolics, which can donate electrons to H_2O_2 , thus neutralizing it to water. Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H_2O_2 is very important throughout food systems (31, 33). Extract was capable of scavenging hydrogen peroxide in a concentration dependent manner. It showed good activity.

Alpha Amylase enzyme is involved in a number of important biological processes, such as digestion of carbohydrate into glucose or processing of the

oligosaccharide moieties of glycoprotein. The search for amylase inhibitors has yielded a number of chemically distinct inhibitors from plants (25,26,33) because these are important biochemical tools for studying the mechanism of enzymes (32,34). The drugs that inhibit carbohydrate hydrolyzing enzymes have been demonstrated to increase postprandial hyperglycemia and improve impaired glucose metabolism without promoting insulin secretion in Non Insulin dependent Diabetes mellitus (NIDDM) patients (26,35). Thus the hydro-methanolic extract of *Tridax procumbens* HMETP is beneficial in the control of Diabetes and oxidative stress by activation of various antioxidants.

HMETP seems to inhibit alpha amylase as a competitive inhibitor as shown by the Michaelis-Menten and Lineweaver-Burk plots. It binds with the active sites of the enzyme thereby reducing its capacity to bind with its main substrate (starch) and hence prevents its conversion to oligosaccharides.

Conclusion

The results clearly showed that hydro-methanolic extract of *Tridax procumbens* (HMETP) has potential active principles which are responsible for reducing postprandial glucose levels via competitive α -amylase inhibitory action. These effects of *T. procumbens* are especially promising in the light of preventing diseases linked to oxidative stress and cellular damage like cardiovascular diseases, cancer and diabetes. The present study can be used to further investigate the mechanism of action related to these effects.

Conflict of interest statement

The authors declared no potential conflict of interest with respect to the authorship, and/or publication of this study.

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