Assessment of Antiulcer, Anthelmentic and Antioxidant Properties of *Tabernaemontana dichotoma* Leaf Extracts

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ABSTRACT

Introduction and Aim: The present study is the first report of phytochemical analysis, antioxidant capacity, antiulcer property, and in vitro anthelmintic capacity of methanol (TCME) and aqueous extract (TCAE) of *T. dichotoma* leaf.

Materials and Methods: The leaves of *T. dichotoma* were used to evaluate the antioxidant, antiulcer, and antihelmentic properties.

Results: The phytochemical analysis confirmed the presence of secondary metabolites, antioxidant activity was established in both the extracts by DPPH and FRAPS assays. TCME displayed a higher degree of H+ K+ ATPase activity on par with standard drug, thus confirming the antiulcer potential of the plant. TCME also exhibited better wormicidal activity compared to the other extracts in both paralysis and death of earthworms (*Pheretima posthuma*) and confirming its role as anthelmintic medicine.

Conclusion: The study confirms the antiulcer and anthelmintic properties of *T. dichotoma* seed extracts, with TCME showing higher efficacy.

Key Words: Antioxidant, anthelmintic, Antiulcer, H+ K+ ATPase, phytochemical

INTRODUCTION

Abundant medicinal and wild edible plants have been studied and reported for their pharmacological and therapeutic properties (1). Even though a large number of literatures discloses the study of ethnopharmacology in different geographical areas, hitherto numerous plants have the potential to be explored for their medicinal properties. Oxidative stress is the result of free radical generation. Free radicals are highly unstable, and also reactive molecules are generated during metabolism. Adding to this, environmental stress also is an important factor in the radical generation. The free radicals include superoxide, hydrogen peroxide, hydroxyl, singlet oxygen, hydroperoxyl, alkyl hydroperoxide, alkylperoxyl radical, alkoxyl radical, hypochlorite ion, ferryl ion, periferryl ion, nitric oxide, peroxynitrite (2). These free radicles are responsible for many degenerative disorders such as cancer, ulcer, diabetes mellitus, etc. Antioxidants are responsible for the protection mechanisms at different levels within cells in the human body by inhibiting the generation of free radical species, disturbing radical-chained reactions, reducing existing free radicals into less harmful molecules and repairing oxidative stress. Even though synthetic antioxidants, such as butylated hydroxyanisol, butylated hydroxytoluene, and tert-butylhydroquinone are commercially available but are accompanied by adverse side effects during

chronic usage (3).

Plants are known to produce a wide array of compounds called secondary metabolites with diverse physiological and biochemical and metabolic activities. Secondary metabolites and their derivatives have been reported to show significant medicinal and therapeutic activities such as being hepatoprotective, antiulcer, anti-allergic, anticancer effects, and antioxidant (4-6). Phytochemicals like tocopherols, carotenoids, alkaloids, ascorbates, and phenols are known to be strong antioxidants and have been extensively used in the health care sector. This is creating new paradigms to identify new natural antioxidants from the living system for application in the Medicinal, therapeutic, and pharmaceutical industries.

Apocynaceae, a family of flowering plants that comprise of herbs, shrubs, trees, and vines, commonly familiar as the dogbane family (7). Several of these plants are known to have medicinal applications. Genus Tabernaemontana belonging to Apocynaceae family is usually found and distributed in Asia, Africa, and American continents. Some members of Tabernaemontana have been traditionally used as medicines and are also scientifically reported for their therapeutic properties. T. divaricata is reported to have potential antioxidant properties and anticancer properties (8,9). Alkaloids isolated from leaves of T. corymbosa have shown anticancer properties (10). T. catharinensis have been reported to be anticholinesterasic activity (11). Stem and root barks of *Tabernaemontana dichotoma* Roxb. ex wall have been used as traditional medicine for snake and centipede bites, eye infections, and toothaches. However, the potential of other parts of the plants is yet to be uncovered.

In the present, the leaves of *T. dichotoma* were used to evaluate the antioxidant, antiulcer, and antihelmentic properties. Novel approaches were used to investigate the antiulcer properties of leaf extracts of *T. dichotoma* Ulcer is caused due to ulcerogens, namely H. pylori, NSAIDs, alcohol, and stress. This causes the formation of reactive oxygen species (ROS), which triggers up-regulation of ATPase, mucosal damage, H. pylori infection, and gastric damage. Oxidative stress to membrane proteins or hemoglobin may affect RBC survival. Oxidative injury to erythrocytes membrane (lipid and protein peroxidation) may be concerned with hemolysis associated with some human disorders (12).

MATERIALS AND METHODS

Collection of plant source

T. dichotoma is a known species of family Apocynaceae. Tabernaemontana is a genus of trees and shrubs of the family Apocynaceae. The leaves of *T. dichotoma* were collected from the rural forests of Western Ghats, Karnataka, India.

Preparation of aqueous and methanolic extracts

A gram of *T. dichotoma* leaves was weighed (100g) followed by homogenization using pestle and mortar with a small quantity of methanol and water for diverse extraction and 10ml of boiling water/ methanol was added to the relevant homogenized sample. The homogenate was later centrifuged for 5min at 1500g. The pellets were discarded, and the supernatant was stored. The extracts were labeled as TDAE (*T. dichotoma* aqueous Extract) and TDME (*T. dichotoma* methanol Extract) correspondingly.

Preliminary phytochemical analysis

Both the extracts were qualitatively screened for the presence of secondary phytochemicals namely alkaloids, glycosides, flavonoids, phenols, lignins, saponins, tannins, sterols, anthraquinone and reducing sugar by Ghagane et al. method (13).

Determination of total phenolic contents: The polyphenol content was determined by the Folin-Ciocalteau (FC) method. Different aliquots of standard tannic acid were used, and the total volume was made up to 1ml with distilled water. Extracts were also taken, and 1ml of FC reagent (1:2 v/v with water) and 2 ml of Na_2CO_3 (10%) was added and set aside for incubation at room temperature for 1 hr. Absorbance was reported against blank at 765nm.

Free radical scavenging activity (DPPH assay): TDAE and TDME were investigated for antioxidant activity (14). Different aliquots of standard BHA and samples were taken, and the final volume was made up to 250 µl with water and methanol correspondingly. To this 1ml of DPPH was added and incubated in the dark at room temperature, for 20 minutes. Absorbance was measured against blank at 517 nm. The percentage of free radical scavenging activity was calculated based on the extent of reduction in the color.

Reducing power assay: Reducing power assay was performed out by ferric reducing antioxidant power assay (FRAP) (15). Antioxidants neutralize pro-oxidants, precisely expressing reducing power. Consequently, the rationale of reducing the power capacity of the test extract indicates and estimates antioxidant potential. Different aliquots of standard tannic acid and samples were taken, and the volume was made up to 0.5 ml with buffer (pH 6.6). To this 0.5 ml, potassium ferricyanide was added and incubated for 20 minutes at 50°C in a water bath. 0.5 ml of 10% TCA was added, which was then centrifuged at 2000 g for 10 minutes. Later 1.5 ml of supernatant was secured, and 1.5 ml of distilled water, 0.3 ml of 0.1% ferric chloride was mixed. The color obtained was assessed spectrophotometrically at 700 nm.

H+ K+ ATPase assay

Fresh sheep stomach was collected from a local slaughterhouse. The mucosa of the gastric fundus was removed, and the inner layer was scraped for parietal cells, which were then homogenized in 0.2 M Tris buffer (pH 7.4) containing 10% Triton X-100 and centrifuged at 6000 g for 10 minutes. The supernatant (enzyme extract) was used for the assay.

The enzyme extract was incubated with different doses of *T. dichotoma* extracts, tannic acid, cinnamic acid and omeprazole in a reaction mixture of 0.2 M Tris buffer (pH 6.6) and the reaction was initiated by adding substrate (2 mM ATP, 2 mM MgCl2, 2 mM KCl). Following 30 minutes of incubation at 37°C, the reaction was terminated by the addition of assay mixture (containing 4.5% ammonium molybdate and 60% perchloric acid) (16). The color of supernatant was obtained by the addition of n-butyl acetate. The sample tubes were centrifuged, and the inorganic phosphate formed was read spectrophotometrically at 400nm. Results were compared with known antiulcer proton potassium ATPase inhibitor drug Omeprazole.

Anthelmintic Evaluation

Adult earthworms (*Pheretima posthuma*), were used to investigate the anthelmintic activity in vitro. Earthworms were collected from moist soil and washed with normal saline to eliminate all faecal matter and used for the anthelmintic study.

Assay

The earthworms were acclimatized to the laboratory condition before experimentation (17). The earthworms were divided into six groups of five earthworms in each and placed in eight Petri dishes containing the leaf extract solutions and the standard drugs as below

Group -1: Received 2% gum acacia which served as the control

Group-2: Received Piperazine citrate suspension at a dose of 10mg/ml which served as the standard

Group-3: Received Methanolic extract at a dose of 100mg/ml

Group -4: Received Methanolic extract at a dose of 200mg/ml

Group-5: Received Aqueous extract at a dose of 100mg/ml

Group-6: Received Aqueous extract at a dose of 200mg/ml

All Petri dishes were kept in room temperature. The viable worms were kept under close observation. The observation was made for the time taken to complete paralysis (PT) and death (DT) for individual worms. Each worm was frequently applied with external stimuli that stimulate and induce movement in earthworms, if alive. Paralysis was considered when the worms do not revive even in normal saline. Death was reported when the worms lose their motility followed by fading of the body colour. The motionless worms were then transferred at 40ºC for confirmation of mortality.

Statistical analysis

All data were statistically analyzed and represented as Mean ± SE. In all tests, the level of statistical significance was set for P=0.05. The significance was calculated by Student`s t-test using MS-Excel.

RESULTS

Table 1: Preliminary phytochemical analysis of *T. dichotoma* leaf extracts

Constituents	Test	Methanol	Aqueous
Alkaloids	Iodine	+	+
	Wagner`s	+	+
Flavonoids	Pew`s	-	-
	NaoH	+	+
Glycosides	Molisch`s	-	-
	Conc H_2SO_4	-	+
	Keller-Killani	+	+
Phenols	Ellagic acid	+	+
	Phenol	+	+
Sterols	Salkowski`s	+	-
Tannin`s	Gelatin	+	+
Lignin`s	Labat	+	+
Saponins	Foam test	+	+

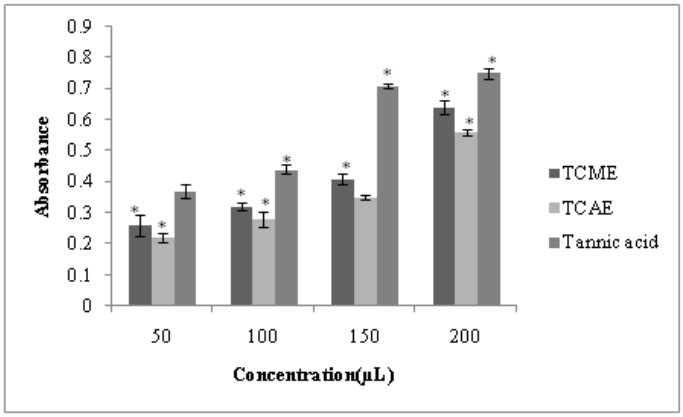
Table 2: Total Phenolic content of *T. dichotoma* methanol and aqueous leaf extracts

Concentration mg/mL	Methanol	Aqueous	BHA
100	54±0.73*	51±0.22	71±0.77
200	61±0.61*	55±0.56*	74±0.82

Table 3: Determination of percentage inhibition of 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity of *T. dichotoma* (%)

Leaf extracts	TPC	Units equivalents	R^2 values
Methanol extracts (TCME)	76.35±0.13	mg/g TAE	R^2=0.9877
Aqueous extracts (TCAE)	63.14±0.31	mg/g TAE	R^2=0.9841
Tannic acid	82.43±0.72	mg/g TAE	R^2=0.9972

Figure 1: FRAP analysis for leaf extracts of *T. dichotoma*

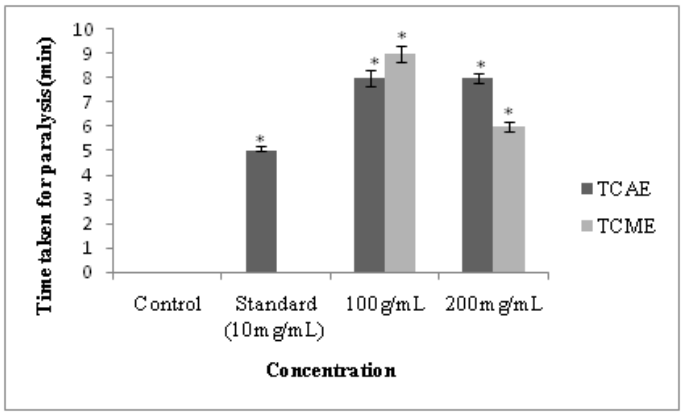


Data is presented as mean SEM (n = 3). *Significant value (*P*=0.05)

Table 4: $H^+ K^+$ ATPase assay expressed in IC_{50} in µg of phenols/ml

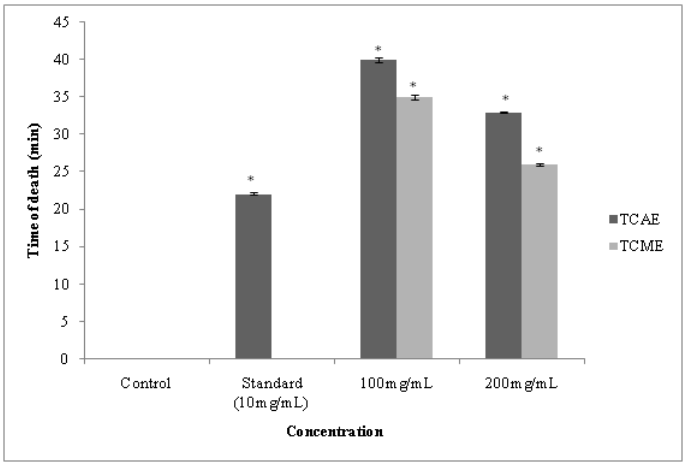
Sample	Concentration	IC_{50} in µg of phenols/ml
TDAE	100mg/mL	29.8
	200mg/mL	28.4
TDME	100mg/mL	26.3
	200mg/mL	22.7
Omeprazole	Standard proton blocker	17.2

Figure 2: Graphical representation for the anthelimintic activity of *T. dichotoma* methanolic and aqueous extract compared to standard drug (Paralysis)



Data is presented as mean SEM (n = 3). *Significant value (*P*=0.05)

Figure 3: Graphical representation for the anthelimintic activity of *T. dichotoma* methanolic and aqueous extract compared to standard drug (Death)



Data is presented as mean SEM (n = 3). *Significant value (*P*=0.05)

DISCUSSION

The present investigation is the first report to establish the traditional use of *T. dichotoma* plant, by evaluating the phytochemical constituents, antioxidant capacity, antiulcer properties and anti helmentic potentials in the methanol and aqueous extracts of *T. dichotoma* leaf extracts. Medicinal plants possess secondary metabolites that have potential antioxidant, which have radical scavenging agents that help in the prevention of diseases and disorders (18). An ulcer is a persistent disease, and free radicals have been associated with the pathogenesis of gastric damage. Various therapeutic formulations have been used to control and treat the disease. However, synthetic formulations are accompanied by side effects, giving sufficient reason for the identification of plant-based formulations (19). Helmentic infections of the gastrointestinal tract of animals and human beings are a major concern in infectious disease management. The development of drug resistance preparations to these pathogenic organisms has been a major cause of concern among the synthetic drugs. Consequently, plant-derived drugs have gained remarkable importance attributed to their decreased side effects,

given their compatibility with physiological flora (20).

The importance of plant-based therapeutics in the treatment of gastric problems and intestinal infections has paved the way for the identification of new plant sources with medicinal properties. *T. dichotoma* leaves were collected from the Western Ghats and identified according to their taxonomical characteristics. The methanol and aqueous leaf extracts were prepared labeled as TCAE and TCME respectively and primarily screened for the presence of secondary metabolites in both the extracts. Leaf powder of 100g of *T. dichotoma* yielded a total of 17.16g in methanol and 16.43g in the aqueous extract.

Preliminary phytochemical analysis: The phytochemical screening of the *T. dichotoma* leaf extracts revealed the presence of all major secondary metabolites. These secondary metabolites have been extensively reported to possess numerous medicinal and therapeutic properties (21).

Phytochemical screening (table 1) revealed the presence of alkaloids in both methanol and aqueous extract; flavonoids have also detected both extracts upon reaction with NaOH. Glycosides were found to be prominently present in aqueous extracts but were also confirmed in methanol extract. Phenols, saponins, lignin, and tannin were confirmed in both TCAE and TCME, whereas sterol was found to be in methanol extract preparations. Thus the confirmations of secondary metabolites further enrich and enhance the therapeutic potentials of *T.dichotoma*

Determination of total phenolic contents

The FCR method was adopted to estimate the amount of phenolic compound in both TCAE and TCME, the results showed (table 2) higher phenol content in methanol extract (76.35±0.13 mg/g TAE) when compared to aqueous extract (63.14±0.31 mg/g TAE) with standard tannic acid showing (82.43±0.72 mg/g TAE) phenol content. The presence of phenolic compounds is in agreement with the plants that are used as traditional medicines such as anti-inflammation, anti-oxidative, anti-diabetic, anti-tumor, and application on neuronal activity (22). Due to the presence of secondary metabolites, the extent of antioxidant capacity and their radical scavenging potential becomes very significant to consider these plant extracts as medicinal candidates.

Free radical scavenging activity (DPPH assay) and FRAP assay

The antioxidant activities of *T. dichotoma* leaf extracts with different concentrations of methanol and aqueous solvents were investigated using DPPH free radical scavenging activity. The results are presented in Table 3. The antioxidant capacity of the extract was compared with BHA as standard antioxidant. The antioxidant activity of *T.dichotoma* leaf extracts was investigated by its capacity to decolorize the DPPH radical and by FRAP assay. The results of percentage inhibition of 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity of *T. dichotoma* (%) are provided in table 3. Both TCAE and TCME showed an almost similar degree of scavenging potential when compared to the standard BHA. However, optimum activity was found at the highest concentration of 200mg/ml in both the extracts.

Similar to the DPPH radical scavenging results, FRAP assay also demonstrated (figure 1) higher scavenging activity in Methanol extract when compared to aqueous extract. Thus, the presence of a significant amount of antioxidants in plant extracts, have been reported to disturb free radical chain, thereby restoring metabolic equilibrium (23). This mechanism of donation of electron or hydrogen molecule for reduction by antioxidants is related to the presence of secondary metabolites like phenols, alkaloids, and flavonoids present in the plant extract (24).

H^+ K^+ ATPase assay

Given that H+K+ATPase is the principle enzyme in inducing acidity, we evaluated the ability of TCAE and TCME with different doses in inhibiting H+K+ATPase isolated from sheep stomach (Fig. 1). During our investigation, we evaluated the antiulcer potential of TCAE and TCME, thus asserting the possible upregulation of antioxidants. Gastric H+ K+ ATPase is situated in the apical membrane of parietal cells which pump protons to the gastric lumen using ATP and thus responsible for the secretion of gastric acid. The results showed (table 4) significant inhibition of sheep H+ K+ ATPase by both the extracts, however methanol extract at higher concentration (200mg/mL) exhibited inhibition with an IC50 of 22.7 µg/mL similar to the standard proton blocker omeprazole (IC50 of 17.2µg/mL). The results suggest the methanol extract (TCME) with remarkable H+ K+ ATPase inhibition potential might well replace the

synthetic proton pump inhibitors, which are reported to have adverse side effects (25). Negligent toxicity of TCME may also prove to be another important reason for the alternative therapeutic use of the extract. This investigation is the first report to establish TCME (leaf) as a potential antiulcer candidate, as the extract worked on similar lines of established drugs, by inhibiting the activity of H+ K+ ATPase.

Anthelmintic evaluation

Anthelmintic activity was observed in vitro, using adult earthworms (Pheretima posthuma), due to their availability and similarity to the intestinal worms. The standard used piperazine citrate acts by increasing the conductance of the chloride ions of the worm muscle membrane (26). This causes hyperpolarization and reduction in the excitability, which further results in muscle relaxation and flaccid paralysis (27). Different leaf extracts (TCAE and TCME) were investigated dose-dependently for paralysis and death of earthworms. Time of paralysis (figure 2) and death (figure 3) in TCME at 200mg/mL was on par with the standard drug piperazine citrate. This potential wormicidal mechanism of TCME against earthworms may support its efficacy against the parasitic infections in human beings.

CONCLUSION

The use of traditional plant *T. dichotoma* as an anti-ulcer and anthelmintic source has been reported for the first time, as the different extracts displayed potential to inhibit H+ K+ ATPase activity in the sheep stomach and enhancing chloride ions movement in the worm muscle. Based on these results obtained in the present investigation, it can be concluded that *T. dichotoma* leaf extracts can be viewed as a potential source of natural antioxidant, antiulcer and anthelmintic and compounds.

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