



Analyzing the Phytochemical, Anti-ulcer, Anthelmintic and Antioxidant Potentials of *Tabernaemontana dichotoma* Roxb. ex Wall Seed Extracts

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Abstract

In the current investigation, phytochemical analysis, antioxidant, anti-ulcer and *in vitro* anthelmintic studies of *Tabernaemontana dichotoma* Methanol Extract (TCME) and Aqueous Extract (TCAE) from seeds were evaluated. Phytochemical analysis confirmed the occurrence of various secondary metabolites. Biological evaluation showed a dose dependent response of different extracts of *T. dichotoma* seeds. Both the extracts showed antioxidant potential. TCME displayed higher degree of H⁺ K⁺ ATPase on par with omeprazole, thus confirming the anti-ulcer potential of the plant. TCME also exhibited better wormicidal activity compared to the other extract in both paralysis of earthworms (*Pheretima posthuma*), and confirming its role as anthelmintic medicine. The study confirms the anti-ulcer and anthelmintic properties of *T. dichotoma* seed extracts, with TCME showing higher efficacy.

Keywords: Antioxidant, Anti-ulcer, DPPH, Phytochemicals

1. Introduction

Numerous medicinal and wild edible plants have been studied and reported for their pharmacological and therapeutic properties¹. Even though a huge number of literatures disclose the study of ethno pharmacology in different geographical areas, yet numerous of 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 extremely reactive molecules generated during metabolism. In addition, environmental stress is also a major factor to the radical generation. The free radicals include superoxide, hydrogen peroxide, hydroxyl, singlet oxygen, hydroperoxyl, alkyl hydroperoxide, peroxy nitrite, alkylperoxyl radical, nitric oxide, alkoxy radical, hypochlorite ion, ferryl ion².

These free radicals are responsible for many degenerative disorders such as cancer, ulcer, diabetes mellitus etc. Antioxidants are responsible for the defense mechanisms at different levels inside the cells of human body by resisting the generation of free radicals and averting oxidative stress. Even though synthesized antioxidants, such as butylated hydroxyanisole etc are available but are accompanied with undesirable side effects during chronic usage³.

Plants generate a wide array of molecules known as secondary metabolites with varied physio- biochemical and metabolic activities. Secondary metabolites have been reported to show significant medicinal and therapeutic potentials such as hepatoprotective, antiulcer, anti-allergic, anti-proliferative and antioxidant effects^{4,5}. Chemical constituents such as tocopherols, carotenoids,

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alkaloids, ascorbates and phenols are known to be rich antioxidants and are extensively used in the health care sector. This is creating new paradigms to identify novel antioxidants from living organism for relevance in medicinal, therapeutic and pharmaceutical industries.

Apocynaceae is a family of flowering plants that comprise of herbs, shrubs, trees and vines, commonly familiar as the dogbane family⁶. 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^{7,8}. Alkaloids isolated from leaves of *T. corymbosa* have shown anticancer properties⁹. *T. catharinensis* have been reported to exert anticholinesterasic activity¹⁰. Stem and root barks of *Tabernaemontana dichotoma* Roxb. ex wall is being used as traditional medicine for snake and centipede bites, eye infections and toothaches. However potential of other parts of the plants are yet to be uncovered.

In the present study the seeds of *T. dichotoma* were evaluated for antioxidant, antiulcer, and antihelminthic properties. Novel approaches were used to investigate the antiulcer properties of seed extracts of *T. dichotoma*. Ulcer is caused due to ulcerogens namely *H. pylori*, NSAIDs, alcohol and stress. This causes the generation of reactive oxygen species (ROS) which triggers up regulation of ATPase, mucosal damage, *H. pylori* infection and gastric injury. Oxidative stress to membrane proteins or hemoglobin may affect RBC survival. Oxidative injury to erythrocytes membrane (lipid and protein peroxidation) may be concerned in haemolysis associated with some human disorders¹¹.

2. Materials and Methods

2.1 Collection of Plant Source

The seeds of *T. dichotoma* were collected from the rural forests of Western Ghats, Karnataka, India.

2.2 Preparation of Aqueous and Methanol Extracts

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

2.3 Preliminary Phytochemical Investigation

Both the methanol and aqueous extracts were investigated for the confirmation of secondary metabolites like tannins, flavonoids, phenols, glycosides, lignins, sterols, saponins, alkaloids, and reducing sugar¹⁰.

2.4 Determination of Total Phenolic Contents

The total polyphenols were determined by Folin-Ciocalteu (FC) method. Absorbance was reported against blank at 765 nm.

2.5 Free Radical Scavenging Assay (DPPH assay)

TDAE and TDME were investigated for antioxidant activity¹². Absorbance was read against the blank at 517 nm. Percentage of free radical scavenging potential was determined depending on the extent of reduction in the color.

2.6 H⁺ K⁺ ATPase Assay

Sheep stomach was bought from sheep slaughter house. The mucosa was detached followed by scraping of the inner layer of the parietal cells, which were later homogenized in 0.2 M Tris buffer (pH 7.2) with 10% Triton X-100 and centrifuged for 10 min at 6000 g. The 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. The sample

tubes were centrifuged and the inorganic phosphate obtained was measured at 400 nm. The observations were collated with established anti-ulcer drug omeprazole, a proton potassium ATPase inhibitor.

2.7 Anthelmintic Evaluation

Adult earthworms (*Pheretimaposthuma*), were used to investigate the *in vitro* anthelmintic activity. The earthworms were attuned to the laboratory environment before analysis. The earthworms were then segmented into six groups of five each and placed in seven petri dishes containing the seed extract solutions (150 mg/ml and 300 mg/ml) and the standard drug (10m/ml) while one group was treated with 2% gum acacia which served as the control.

All Petri dishes were incubated in lab temperature. The live worms were kept for careful observation. Observation was determined for the time taken to absolute paralysis (PT) for individual earthworms. Every earthworm was regularly treated with external stimuli which triggers and provoke movement in earthworms, if they are alive. Paralysis was reported if the earthworms did not revitalize even in regular saline.

2.8 Statistical Analysis

All data was statistically analyzed and represented as Mean \pm SE. In every experiments, the level of statistical significance was taken as $P < 0.05$. The significance was calculated by Student's *t*-test using MS-Excel.

3. Results

3.1 Total Yield of Extracts

Seed powder of 100 g of *T. dichotoma* yielded a total of 18.56 g and 15.3 g methanol and aqueous extract respectively.

3.2 Preliminary Phytochemical Analysis

The methanol and aqueous seed extracts of *T. dichotoma* were analyzed for preliminary phytochemical investigation, which confirmed a multiple secondary metabolites as shown in the Table 1. Alkaloids were found in both extract, flavonoids were reasonably confirmed in methanol extracts, phenols were

confirmed both in methanol and aqueous extracts, glycosides were present in aqueous solvents, lignin's were absent in both extracts, saponins were found to be present in both extracts, sterols confirmed its existence in both the samples, tannins were present in both extracts.

Table 1. Preliminary phytochemical analysis of *T. dichotoma* seed extracts.

Constituents	Test	Methanol	Aqueous
Flavonoids	Pew's NaOH	- +	- -
Alkaloids	Iodine Wagner	+ +	+ +
Sterols	Salkowski	+	+
Phenols	Ellagic acid Phenol	+ +	+ +
Glycosides	Molisch Conc H ₂ SO ₄ Keller-Killani	- - +	- + +
Tanin	Gelatin	+	+
Lignin	Labat	-	-
Saponin	Foam	+	+

3.3 Determination of Total Phenolic Concentration

Total phenolic contents of *T. dichotoma* methanol and aqueous extracts differed considerably, as given in the Table 2. The quantity of total phenolic levels was established by the standard curve of Tannic acid ($R^2 = 0.9962$) value and the results were reported as Tannic Acid Equivalent (TAE) mg per gram.

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

Seed extracts	TPC	Units equivalents	R ² values
Methanol extract (TCME)	75.82 \pm 0.66	mg/g TAE	R ² =0.9883
Aqueous extract (TCAE)	68.34 \pm 0.72	mg/g TAE	R ² =0.9853
Tannic acid	81.43 \pm 0.42	mg/g TAE	R ² =0.9962

3.4 Free Radical Scavenging Activity (DPPH Assay)

The antioxidant scavenging potentials of *T. dichotoma* seed extracts showed dose dependent response in DPPH method. The results are presented in Table 3. The antioxidant potential of the extracts was established with BHA as standard antioxidant.

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

Concentration mg/mL	Methanol	Aqueous	BHA
75	45±0.73*	47±0.52	71±0.77
150	52±0.23	53±0.22*	74±0.82
225	57±0.44*	58±0.75*	77±0.12
300	63±0.47*	67±0.46*	83±0.33

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

3.5 H⁺ K⁺ ATPase Assay

H⁺K⁺ATPase is the principle enzyme in generating acidity, the extracts were evaluated for its capability to inhibit H⁺K⁺ATPase obtained from sheep stomach (Table 4). Both TCAE and TCME repressed H⁺K⁺ATPase activity in a dose-dependent way with an IC₅₀ of 22.8 g/mL and 26.4 g/mL (TDAE) and 25.3 mg/mL and 21.8 mg/mL when juxtaposed to established anti-ulcer medicine-omeprazole (IC₅₀-16.6 g/mL) during comparable experimental environments.

Table 4. H⁺ K⁺ ATPase assay expressed in IC₅₀ in µg of phenols/ml

Sample	Concentration (mg/mL)	IC ₅₀ in µg of phenols/ml
TDAE	150	22.8
	300	26.4
TDME	150	25.3
	300	21.8
Omeprazole	Standard proton blocker	16.6

3.6 Anthelmintic Evaluation

Dose dependent anthelmintic activity of *T. dichotoma* methanol and aqueous extracts were evaluated for paralysis. The results for paralysis study showed

decrease in time to paralysis, comparable to standard albendazole suspension in the methanol extract (300 mg/mL).

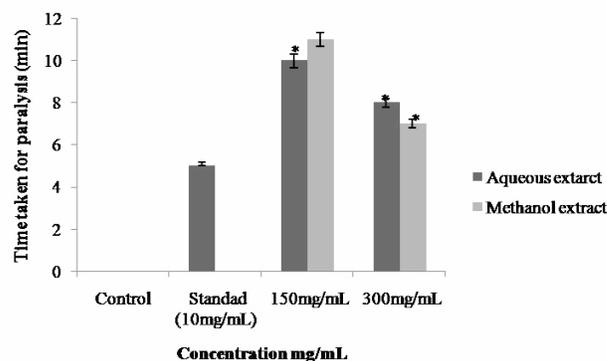


Figure 1. Anthelmintic activity of *T. dichotoma* methanol and aqueous seed extract compared to standard drug (Paralysis).

4. Discussion

The present study was undertaken to establish the traditional use of *T. dichotoma* plant, by evaluating the chemical constituents, antioxidant capacity, anti-ulcer properties and anti helmentic potentials in the aqueous and methanol extracts of *T. dichotoma* seeds. Herbal sources have significant secondary metabolites with antioxidant, radical scavenging properties that aid in alleviation of diseases and disorders¹⁴. Ulcer is a persistent disease and free radicals have been attributed to the pathogenesis of gastric damage. Emotional stress, burns, infections are among the known causes for gastritis. Different therapeutic formulations have been used to control and treat the disease. However synthetic formulations are accompanied by side effects, giving sufficient reason for identification of plant based formulations¹⁵. Helmentic infections in gastrointestinal tract of living organisms have been a major concern in infectious disease management. Development of drug resistance potentials of these pathogenic organisms have been a major cause of concern among the synthetic drugs. Consequently, plant derived drugs have gained remarkable importance attributed to their reduced side effects given their compatibility with physiological flora.

Importance of plant-based therapeutics in the treatment of gastric problems and intestinal infections

have paved way for the identification of new plant sources with potential medicinal properties. *T. dichotoma* seeds were collected from Western Ghats and recognized according to their taxonomical characteristics. The methanol and aqueous seed extracts were prepared labeled as TCAE and TCME respectively and primarily screened for presence of secondary metabolites in both the extracts. The phytochemical screening of the *T. dichotoma* seed extracts revealed presence of all major secondary metabolites. These secondary metabolites have been extensively reported to possess numerous medicinal and therapeutic properties¹⁶.

Phytochemical screening (Table 1) revealed the presence of alkaloids in both methanol and aqueous extract; flavonoids were detected only in methanol extracts upon reaction with NaOH. Glycosides was found to be prominently present in aqueous extracts but was also confirmed in methanol extract. Phenols, sterols, saponins and tannin were confirmed in both TCAE and TCME; whereas lignin was found to be absent in the both extract preparations. Thus the presence of major secondary metabolites may further enrich and enhance the therapeutic potentials of *T. dichotoma*. The FCR method was employed to estimate the amount of phenolic compound in both TCAE and TCME, the results showed (Table 2) higher phenol content in methanol extract (75.82 ± 0.66 mg/g TAE) when compared to aqueous extract (68.34 ± 0.72 mg/g TAE) with standard tannic acid showing (81.43 ± 0.42 mg/g TAE) phenol content. The presence of phenolic compounds is in agreement with their traditional use such as anti-inflammation, anti-oxidative, anti-analgesic, anti-tumor and significant application on neuronal activity¹⁷. Due to the presence of secondary metabolites, the extent of antioxidant activity and their radical scavenging potential becomes very significant to consider these plant extracts as medicinal candidates. Antioxidant activity of *T. dichotoma* seed extracts was investigated by its capacity to decolorize the DPPH radical and by FRAP assay. The results of percentage inhibition of DPPH radical scavenging activity of *T. dichotoma* (%) is provided in Table 3. Both TCAE and TCME showed almost similar degree of scavenging potential when compared to the standard BHA (83 ± 0.33); however optimum activity was found at the concentration of 300 mg/mL in both the extracts.

Further the anti-ulcer properties of TCAE and TCME were also studied, thus asserting the possible up-regulation of antioxidants. H^+K^+ ATPase are 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 (300 mg/mL) exhibited inhibition with an IC_{50} of 21.8 μ g/mL similar to the standard proton blocker omeprazole (IC_{50} of 16.6 μ g/mL). The result suggests that the methanol extract (TCME) has remarkable H^+K^+ ATPase inhibition potential, this extract might well be an alternative to replace the synthetic proton pump inhibitors which are reported to have adverse effects¹⁸. Negligent toxicity potential of TCME may also prove to be another important reason for alternative therapeutic use of the extract. This investigation is the first report to establish TCME as a potential anti-ulcer candidate, as the extract worked on similar lines of established drugs, by inhibiting the activity of H^+K^+ ATPase.

Anthelmintic activity was studied *in vitro* employing 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 membrane¹⁹. This leads to hyper-polarization and reduction in the excitability, which further results in muscle relaxation and flaccid paralysis²⁰. Different seed extracts (TCAE and TCME) were investigated dose dependently for paralysis of earthworms. Time of paralysis (Figure 1) in TCME at 300 mg/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 being.

5. 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 hamper H^+K^+ ATPase activity in the sheep stomach and enhancing chloride ions movement in the worm muscle. On the basis of these results obtained

in the present investigation, it can be concluded that *T. dichotoma* can be viewed as a potential candidate of natural antioxidant, anti-ulcer and anthelmintic properties.

6. Competence of Interest

The authors have no competence of interest.

7. References

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