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Evaluation of analgesic, antioxidant, antibacterial, and anti-inflammatory activities of *Trichosanthes dioica* leaf extract

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ABSTRACT

Background: *Trichosanthes dioica* is an indigenous medicine that is extensively utilized for its known pharmacological properties. The leaves are commonly used as folk medicine for analgesic, anti-inflammatory, antibacterial, and antifungal purposes. Here we look at its ability to be a producer of naturally occurring medicinal molecules, by conducting a systematic analysis of its biological activity. **Objective:** The present investigation was carried out to investigate the analgesic, antioxidant, antibacterial, thrombolytic, cytotoxic, and anti-inflammatory potentials of the methanolic extract of *Trichosanthes dioica* leaves and to evaluate its phytochemical composition. **Materials and Methods:** The methanolic extract of the *T. dioica* leaves was obtained *a*nd screened for various phytochemicals. The total phenolic, flavonoid, and tannin contents were measured by the colorimetric method. The antioxidant activity was evaluated by the DPPH free radical scavenging assay. The analgesic activities of these extracts were assessed in mice by acetic acid-induced writhing and tail-flick tests. The antibacterial activity was evaluated by disc diffusion against a panel of bacteria, and the results indicate that it, in fact has antisolvent activity. Thrombolytic potential was tested by the in vitro clot lysis method and cytotoxicity using the brine shrimp lethality bioassay. The anti-inflammatory activity was examined using Human Red Blood Cell (HRBC) membrane stabilization in hypotonic and heat-induced conditions. **Results:** Phytochemical screening of *Trichosanthes dioica* indicated that the extract had high concentrations of phenolics (181 ± 0.52 mg GAE/g), flavonoids (202 ± 0.77 mg QE/g), and tannins (187 ± 0.37 mg GAE/g). The extract demonstrated potent antioxidant activity with the IC_{50} value of 42.92±0.89µg/mL in the DPPH assay. Analgesic analysis revealed that the extract significantly and dose-dependently inhibited the peripheral (33.95%, 47.71% and 58.72% at doses of 200, 400, and 600 mg/kg) as well as central pain at 30,60, and 90 minutes, respectively. Moderate antibacterial activity was observed for the extract against *Staphylococcus aureus* and *Pseudomonas aeruginosa* with an inhibition zone of 10±0.09mm at 500µg/disc. Low thrombolytic (11.73±2.31%) activity compared to streptokinase (68.82±5.53%) and absence of cytotoxicity (LC_{50}=0µg/mL) were observed. A small to modest anti-inflammatory effect was detected by nearly all tests, inhibition of hypotonic and heat-induced hemolysis (6.48 and 15.47%) compared with the standard drug, acetylsalicylic acid, which yielded an inhibition of 87.53% and 12.35%, respectively. **Conclusion:** The methanolic extract of *T. dioica* leaves exhibited significant analgesic and antioxidant activity, a mild anti-inflammatory effect, and moderate antibacterial activity, with a low rate of thrombolysis. On the other hand, it is nontoxic at all when tested for cytotoxic activities. These results justify the known use of this plant in traditional medicine and indicate the need for further study to isolate and characterize active constituents.

Keywords: *T. dioica*, Folin-Ciocalteu, *P. aeroginosa*, Thrombolytic activity, Antinociceptive, Cytotoxic.

INTRODUCTION

Recently, the use of medicinal plants for mitigating disease is increasing day by day around the world [1], especially in developing countries where a significant number of people rely on traditional plants due to their primary healthcare needs [2]. About 87% of all types of human disorders, including Bacterial infection, cancer, immunological disease, etc., are treated by naturally derived products [3]. About 25% of prescription drugs come from natural sources, with many synthetic drugs based on natural precursors. Medicinal plants are crucial in healthcare, especially in Asia and Africa, where approximately 80% of the population relies on traditional medicine, mainly plant-based, for primary healthcare [4]. Herbal remedies are especially important in rural and underdeveloped areas with limited access to modern medicine[5]. The popularity of medicinal plants is rising due to their fewer side effects. Many existing

drugs are ineffective against diseases due to drug resistance, and new diseases are emerging. Therefore, identifying new compounds from natural plants is increasingly important [6]. Among the various medicinal plants used in traditional systems, *Trichosanthes dioica* Roxb., commonly known as pointed gourd or "patol," holds considerable ethnomedicinal importance in South Asian countries such as India, Bangladesh, and Nepal [7]. Belonging to the Cucurbitaceae family, *T. dioica* is a dioecious, herbaceous climbing plant that thrives in tropical and subtropical regions. It is predominantly cultivated for its edible fruits, which are widely used in Asian cuisines and traditional healing systems [8].

Tricosanthes, an herb in the Cucurbitaceae family, grows in Tropical Asia and Australia. *Tricosanthes dioica* is usually cultivated using vine cuttings or root suckers, as seed propagation is unreliable due to poor germination. T. dioica contains various phytochemicals, including Vitamin A, Vitamin C, tannins, saponins, alkaloids, and others. The fruits, leaf juice, and tender shoots of *T. dioica* have been used in traditional folk medicine for a long time. This plant has been employed to treat various conditions, including constipation, fever, skin disorders, and wounds, since ancient times. Additionally, it is known to boost appetite and aid digestion [8].

Plants, having numerous structural diversities and phytochemicals, are a great source of medicine for the pharmaceutical industry [11]. Phytochemicals, for example, polyphenols, flavonoids, isoflavonoids, phytoestrogens, terpenoids, carotenoids, etc., have great health benefits. Researchers suggest that flavonoids, terpenoids, essential oils, and alkaloids show antimicrobial activity [12]. Flavonoids, terpenoids, glycosides, saponins, and alkaloids exhibit antidiarrheal activity, while alkaloids, saponins, and steroids show anthelmintic properties. Lectins and polypeptides have antiviral activity, and saponins possess anticancer properties. Due to their efficacy against various diseases, researchers regularly conduct phytochemical screening and pharmacological investigations to discover new, effective, and safe therapeutic agents [13].

Accumulation of free radicals in the body can lead to various diseases. Natural antioxidants from plants can help minimize their impact by supplying electrons to damaged cells, protecting and stabilizing them [14]. Analgesics, or painkillers, are substances that alleviate pain without causing loss of consciousness. The term originates from the Greek words an- meaning "without" and algos meaning "pain." [15]. Researchers have been focused on discovering new analgesics due to the multi-billion-dollar pharmaceutical market and the side effects of current drugs. Historically, natural analgesics have been used with fewer severe side effects, suggesting that new and better compounds may be found from natural sources [9]. To investigate new leads of analgesic drugs from plant origin is a regular practice among researchers to find better painkillers with minimal side effects.

The discovery of penicillin by Alexander Fleming in 1928 marked a significant milestone in medical history. Although antibiotics initially promised effective treatment for bacterial infections, the development of antibiotic resistance has complicated these treatments. Therefore, researchers must continue to search for new antibiotics, including plant-derived options like caffeic acid, catechol, and eugenol, to combat bacterial infections and prevent resistance [16].

Thrombus formation in blood vessels can lead to diseases like heart attacks, strokes, and hypertension by blocking blood flow. Thrombolytic drugs are used to treat cerebral venous sinus disorders, but have limitations. Compounds from plant sources have shown thrombolytic activity [17].

Cancer is a widespread and serious disorder. While chemotherapy is commonly used to treat it, it often has unwanted side effects. Natural medicines derived from plants may have fewer adverse effects. Recently, some plant compounds have shown significant anti-cancer activity in vitro, indicating the potential for discovering new natural chemotherapeutic drugs [18]. Many medicinal plants are traditionally used in cancer treatment due to their phytochemicals, such as vitamins, flavonoids, and alkaloids, which have important antioxidant activity [19, 20].

The term "inflammation" describes the body's immune response a rapid, coordinated reaction to foreign stimuli, such as biological, chemical, and physical substances [21]. Inflammation helps remove harmful stimuli from the body and initiates healing. Anti-inflammatory drugs reduce this inflammation [22]. Developing a safe anti-inflammatory drug poses challenges for researchers. Synthetic drugs like NSAIDs can have severe side effects, including joint cartilage erosion and risks of kidney and liver disease with long-term use [23]. Herbal medicines from natural sources have minimal side effects, primarily due to phenolic compounds that possess anti-inflammatory properties. These compounds can help reduce inflammation in chronic and degenerative disorders [24]. So drugs from natural sources can play a significant role in the treatment of Inflammatory disease.

Tricosanthes, an herb from the Cucurbitaceae family, is found in Tropical Asia and Australia. *T. dioica* is known to contain various phytochemicals such as Vitamin A, Vitamin C, tannins, saponins, and alkaloids [7]. The fruits, leaf juice, and tender shoots of *T. dioica* have long been used in folklore medicine to treat various ailments, including constipation, fever, skin disorders, and wounds [8]. There is limited scientific information about the pharmacological activity of *T. dioica* leaves. Therefore, we conducted a study to identify the phytochemicals in *T. dioica* leaves and their pharmacological effects, and to explore the scientific basis for their use in traditional medicine.

MATERIAL AND METHODS

Study Site

The present research work was conducted from January to November 2023 in the Department of Pharmacy, Pabna University of Science and Technology, Rajapur, Pabna-6600, Bangladesh.

Chemicals and Reagents

All reagents used to perform the experiments were of analytical grade. The reagents such as methanol (MERCK Germany, 99.99%), ethanol (Sigma-Aldrich, 99.5%), Nutrient Agar (Sigma-Aldrich), NaCl (Loba, India), NaOH (Loba, India), Na_2CO_3 (Loba, India), and sodium hypochlorite (Loba, India), kanamycin 30 µg/disc (Oxoid, UK), nutrient Agar media (Oxoid, UK), Sodium Phosphate Buffer solution (Biosolution, Korea), Di-sodium EDTA (Chemx Chemicals, India), Streptokinase (STK, Incepta Pharma), Brine Shrimp eggs (Aquirium Co., USA), and also Morphine and formalin were obtained from Gonoshasthaya Pharmaceuticals Limited for central analgesic activity screening [25].

Experimental animals:

Swiss albino mice (4-5 weeks old, 25-30 grams) were sourced from icddr,b and housed in polypropylene cages with controlled temperature (24 ± 2ºC), humidity (60-70%), and a 12-hour light-dark cycle. They had access to icddr,b formulated rodent food and water ad libitum, and were acclimated for one week before testing [26]. Following FELASA (Federation of European Laboratory Animal Science Associations) guidelines, the mice were divided into five groups: positive control (STD), negative control (CTL), and three test groups receiving *T. dioica* leaf extract at doses of 200 mg/kg (ME 200), 400 mg/kg (ME 400) and 600 mg/kg (ME 400).

Collection of plant sample and preparation of extract

The leaves of *Trichosanthes dioica* were collected from Monohorpur, Chuadanga, in January 2022. After cleaning and air-drying for 15 days, the leaves were ground into powder and sieved. 800 g of this powder was soaked in 2 liters of methanol for 7 days with occasional

stirring. The mixture was then filtered, and the extract was concentrated using a water bath and hot air oven (below 45°C), followed by drying at room temperature. The final extract was weighed and stored for phytochemical and pharmacological analysis.

Determination of Total Phenolic Content (TPC)

A slightly modified Folin-Ciocalteu method [27, 28] was employed to determine the total phenolic content of *Trichosanthes dioica* methanolic leaf extract. In this method, 0.5 mL of the extract (1 mg/mL) was mixed with 5 mL of diluted Folin-Ciocalteu reagent and 4 mL of 7.5% sodium carbonate. After vortexing for 15 seconds, the mixture was incubated at 40°C for 30 minutes for color development. Absorbance was measured at 765 nm using a spectrophotometer. A standard curve was prepared using gallic acid solutions (0.1–0.5 mg/mL), and the total phenolic content was calculated using the calibration equation: y=1.578x+0.0487 (R^2 = 0.9857), illustrated in Figure 1. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry extract.

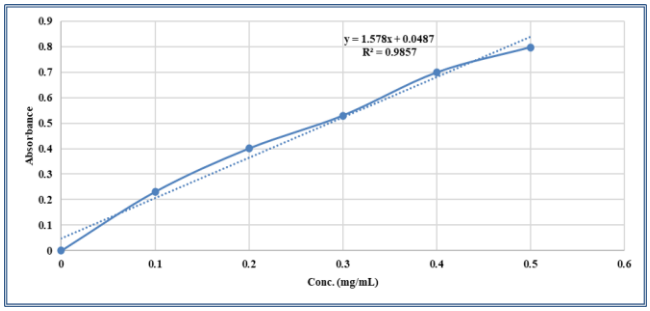


Figure 1: Total phenolic content determination from the gallic acid standard calibration curve

Determination of Total Flavonoid Content (TFC)

The total flavonoid content of *T. dioica* methanolic leaf extract was determined using the aluminum chloride ($AlCl_3$) colorimetric method [29, 30]. One milliliter of the extract (1 mg/mL) was mixed with 400 mg of sodium acetate and 2.5 mL of $AlCl_3$ reagent (prepared by dissolving 133 mg of $AlCl_3$ in 100 mL of methanol). The mixture was left at room temperature for 30 minutes. Absorbance was then measured at 510 nm using a spectrophotometer, with a blank prepared from methanol and $AlCl_3$ reagent. A standard calibration curve was generated using quercetin solutions (0.1–0.5 mg/mL), plotting absorbance versus quercetin concentration, as visualized in Figure 2. The total flavonoid content was calculated using the regression equation: y = 1.0734x + 0.0161 (R^2 = 0.9972). Results were expressed as milligrams of quercetin equivalents (QE) per gram of dry extract.

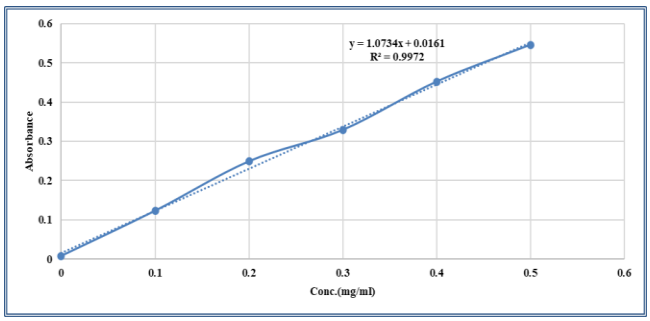


Figure 2: Total flavonoid content determination from the Quercetin standard calibration curve

Determination of Total Tannin Content (TTC)

The total tannin content in *Trichosanthes dioica* methanolic leaf extract was determined using a modified Folin-Ciocalteu method [31]. A mixture of 0.1 mL of extract, 7.5 mL of distilled water, 0.5 mL of Folin-Ciocalteu reagent, and 1 mL of 35% sodium carbonate was prepared. After adding 10 mL of distilled water for dilution, the mixture was shaken and left to stand for 30 minutes at room temperature. Absorbance was measured at 725 nm, with a blank prepared using water instead of the sample. Standard solutions of gallic acid (0.1–0.5 µg/mL) were used to create a calibration curve visualized in Figure 3. The total tannin content was calculated using the regression equation: y = 1.7797x + 0.0612 (R^2 = 0.9581). Results were expressed as milligrams of tannic acid equivalents per gram of dry extract.

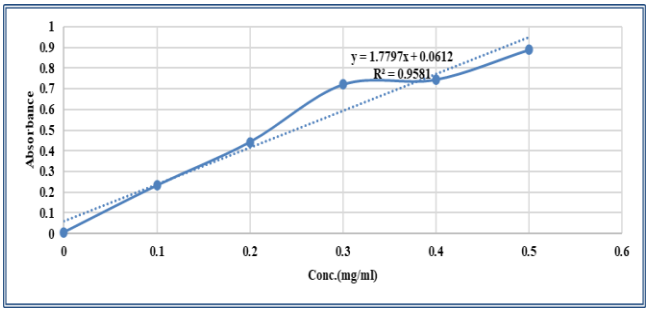


Figure 3: Total tannin content determination from the gallic acid standard calibration curve

DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical scavenging assay:

The quantitative antioxidant capacity was assessed using the DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical scavenging assay method with minor modifications [27, 32]. Different concentrations of sample extracts (100-1.5625 µg/mL) and ascorbic acid used as positive controls (100-1.5625 µg/mL) were prepared through serial dilution. For each concentration of the sample extract, 2 mL (2000 µL) was combined with 3 mL (3000 µL) of DPPH solution in methanol. This mixture was then incubated at room temperature (25°C) in the absence of light for 30 minutes. The absorbance of the reaction mixture was measured using a Thermo Scientific Multiskan Ex microplate photometer set at a wavelength of 517 nm, with measurements taken against a blank. A calibration curve plotting log concentration versus the percentage of scavenging activity was constructed from the absorbance readings, which is shown in Figure 4. The scavenging activity is denoted as SC_{50}, which refers to the concentration required to scavenge 50% of free radicals. The inhibition of DPPH radicals by the samples was calculated using the following equation:

$$\text{Percentage of (\%) Scavenging} = \left\{1 - \left(\frac{A_{Sample}}{A_{DPPH}}\right)\right\} \times 100$$

Here,
A_{Sample} = Absorbance of the Sample.
A_{DPPH} = Absorbance of the DPPH.

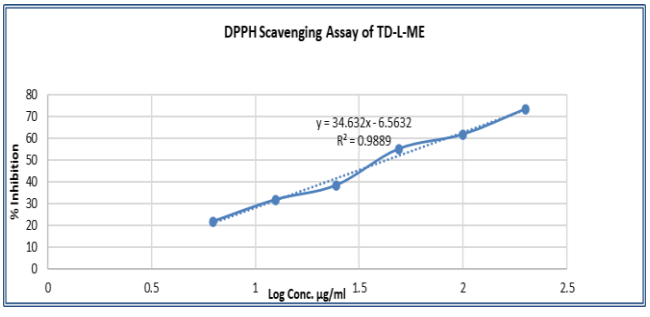


Figure 4: DPPH Scavenging Assay of TD-L-ME

Antibacterial assay by disc diffusion method

The modified agar disc diffusion method was used to conduct an in vitro antibacterial activity test [33, 34]. Firstly, the Agar plates were

injected with a standardized inoculum of the test microorganism throughout this method. Following that, 6 mm-diameter filter paper discs containing the test substance at concentrations of 250 and 500 µg /disc each were placed on the agar surface. Positive control, such as antibiotic discs containing 30 µg/disc of kanamycin, was used to confirm that the standard antibiotic exhibited its activity against the test organisms and to compare the response of the known antibacterial agent with that of the test samples. Filter paper discs made of sterile paper were used as samples, and to prepare a control disc, 10 µL of methanol was poured into the blank discs. In the proper environments, the petri plates were incubated, and two gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*), two gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*), and one fungus (*Candida albicans*) were assessed by measuring the zones of inhibition around each disc.

Evaluation of analgesic activity of methanolic extract of *T. dioica* leaves

Peripheral Analgesic Test

The acetic acid-induced writhing test was used to assess the antinociceptive property [35-37]. A total of 25 albino mice of both sexes were randomly divided into five groups (A–E), each containing five mice. To prepare test samples, 30 mg of the methanolic extract of *Trichosanthes dioica* leaves was dissolved in 1% Tween-80 to make 10 mL of solution. From this, doses of 200, 400, and 600 mg/kg were administered orally to Groups C, D, and E, respectively, via gastric gavage. Group A received 1% Tween-80 solution (negative control), and Group B was given 0.1 mL of a prepared sodium diclofenac solution (standard, positive control) via peritoneal injection. One hour after treatment, all mice received 0.7% glacial acetic acid intraperitoneally to induce pain. After 30 minutes, the number of abdominal writhes was counted for each mouse. The percentage inhibition of writhing was calculated using the formula:

$$\% \text{ Inhibition of writhing} = \left(\frac{Mean\ no.\ of\ writhing\ (Control)\ -Mean\ no.(test)}{Mean\ no.\ of\ writhing\ control}\right) \times 100\ \%$$

Central Analgesic Test

The central analgesic efficacy of the methanolic extract of *T. dioica* was assessed using the tail flick immersion method [25, 37]. The negative control group received a 1% Tween 80 in saline solution orally, while morphine was used as the reference standard. Heat stress was applied to the mice's tails as a pain stimulus, and licking times were recorded at 0, 30, 60, and 90 minutes, which is represented as early and late phases after administering the plant materials (ME 200, ME 400, and ME 600). The percent of pain inhibition was calculated using the following formula:

$$\text{Pain inhibition percentage (PIP)} = \left(\frac{Post-Drug\ Latency-Pre\ Drug\ latency}{Pre\ Drug\ latency}\right) \times 100$$

Evaluation of Cytotoxicity of the methanolic extract of *T. dioica* leaves

To assess the cytotoxicity of *T. dioica* methanolic leaf extract, a brine shrimp lethality assay was performed [38, 39]. A stock solution (800 µg/mL) was prepared by dissolving 16 mg of the extract in DMSO and seawater, and serial dilutions were made to obtain lower concentrations. Vincristine sulfate served as the positive control, and DMSO with seawater was used as the negative control. Brine shrimp (*Artemia salina*) eggs were hatched in artificial seawater under light and aeration, and 10 nauplii were added to each test tube. Each concentration of extract was tested by adding 2.5 mL to the tubes, and after 24 hours, the number of dead nauplii was recorded to calculate the percentage mortality. From this data, the percentage (average) of mortality of brine shrimp nauplii was calculated at each concentration of the sample by using the following formula, and the LC_{50} was determined:

$$\% \text{ Mortality} = \frac{\text{Number of dead nauplii}}{\text{Intial number of live nauplli}} \times 100$$

Evaluation of Thrombolytic activity of methanolic extract of *T. dioica* leaves by *in vitro* clot lysis model on human blood

The in vitro thrombolytic activity of methanolic extracts of *Trichosanthes dioica* leaves was assessed using a method reported by Prasad et al [40]. Blood from five healthy volunteers was collected and transferred into pre-weighed microcentrifuge tubes. After 45 minutes of incubation at 37 °C to allow clot formation, the serum was removed, and the tubes were reweighed to determine clot weight. Each clot-containing tube received 100 µL of either the plant extract (1 mg/mL), streptokinase (positive control), or sterile distilled water (negative control), and was incubated for another 90 minutes at 37 °C. Clot lysis was evaluated by measuring the decrease in clot weight, and the percentage of clot lysis was calculated to assess the thrombolytic potential expressed as a percentage of clot lysis, as shown below:

$$\% \text{ clot lysis} = \frac{\text{Weight of the lysis clot}}{\text{Weight of clot before lysis}} \times 100$$

Evaluation of membrane-stabilizing activity of the methanolic extract of *T. dioica* leaves.

The membrane-stabilizing (anti-inflammatory) activity of *Trichosanthes dioica* methanolic extract was assessed using human red blood cells through hypotonic and heat-induced hemolysis assays [41, 42]. Blood from a healthy volunteer was treated with EDTA, and RBCs were isolated, washed, and suspended in isotonic buffer. In the hypotonic assay, RBCs were exposed to a hypotonic solution with or without the plant extract or standard (salicylic acid), and hemolysis was measured via absorbance at 540 nm. In the heat-induced assay, RBCs with extract or standard were incubated at 54 °C or in an ice bath, and hemolysis was similarly measured. The extract showed potential membrane-stabilizing effects by reducing hemolysis, indicating anti-inflammatory properties.

% Inhibition of Hypotonic Solution-Induced Hemolysis

$$\% \text{ Inhibition} = \left(1 - \frac{\text{O.D.(Optical Density) of test sample in hypotonic solution}}{\text{O.D.(Optical Density) of hypotonic buffered saline solution alone}}\right) \times 100$$

% Inhibition of Heat-Induced Hemolysis

$$\% \text{ Inhibition} = \left(1 - \frac{\text{O.D. of heated test sample} - \text{O.D. of unheated test sample}}{\text{O.D. of heated control sample} - \text{O.D. of unheated test sample}}\right) \times 100$$

Statistical Analysis

The results were expressed as Mean ± Standard Error Mean (SEM) and p value. P value = *P<0.05, **P<0.01, ***p<0.001, ***P<0.0001 was taken into account as significant. Statistical data analyzed by SPSS 25.0.

RESULTS

Quantification and Determination of phytochemical content in methanolic extract of *T. dioica* leaves

The total phenolic content of the methanolic extract of *T. dioica* was determined to be 181±0.52 mg GAE per gram of dry extract. The total flavonoid content of the methanolic extract of *T. dioica was 202±0.77 mg QE pe*r gram of dry extract. The total tannin content of the methanolic extract of *T. dioica* was found to be *187±0.*36 mg GAE per gram of dry extract.

Quantitative analysis of antioxidant activity

The methanolic extract of *T. dioica* leaves showed concentration-dependent antioxidant activity with an IC_{50} value of 42.98±0.88

µg/mL against DPPH free radical, while the IC_{50} value of the standard natural antioxidant was 19.23 ± 0.95 µg/mL.

Evaluation of antibacterial activity of methanolic extract of *T. dioica* leaves

The antimicrobial activity of the methanolic extract of *Tricosanthes doica* was studied at different concentrations (250 and 500 µg/disc) against four pathogenic bacterial strains, two gram-positive (*Staphylococcus aureus, Bacillus subtilis*) and two gram-negative (*Escherichia coli, Pseudomonas aeruginosa*), as well as one fungal strain (*C. albicans*), using the disc diffusion method. The results showed that methanolic extracts of *Tricosanthes doica* leaves had less significant activity against *S. aureus* and *P. aeruginosa*, with zones of inhibition measuring 10±0.09 mm. and 08±0.05 mm respectively demonstrated in Table 1.

No activity was found against other bacteria and *C. albicans* fungus, and a comparative visualization among these bacterial strains is shown in Figure 5.

Evaluation of analgesic activity of the methanolic extract of *T. dioica* leaves

Peripheral Analgesic Activity

The peripheral analgesic activity of methanolic extracts of *T. dioica* leaves was evaluated using the acetic acid-induced writhing method. The results indicated that the methanolic extracts of *T. dioica* leaves, at doses of 200, 400, and 600 mg/kg, along with the standard Diclofenac sodium, significantly reduced abdominal writhing in mice compared to the 1% Tween-80 solution used as a negative control. The observed writhing frequency was 21.8±0.86 for the negative control, 3.8±0.37 for the standard with significance value (P<0.001), and 9±0.83 for the plant extract at the 600 mg/kg dose (P<0.001), as shown in Table 2. This study demonstrated a concentration-dependent increase in the analgesic activity of the methanolic extracts of *T. dioica* leaves, with the percentage inhibition of writhing rising from 33.95% at the 200 mg/kg dose to 58.72% at the 600 mg/kg dose, as depicted in Figure 6.

Central Analgesic Activity

The analgesic potential of *Trichosanthes dioica* (*T. dioica*) leaf extract was assessed using the tail flick test in mice, as presented in Table 3 and Figure 7. Reaction times were measured at 0, 30, 60, and 90 minutes after administering doses of 200, 400, and 600 mg/kg, along with positive and negative controls. A dose- and time-dependent increase in tail flick latency was observed. At the lowest dose (200 mg/kg), significant reaction time increases started at 30 minutes (3.41 ± 0.09 s, p<0.001) and reached 5.67 ± 0.16 s at 90 minutes. The 400 mg/kg dose resulted in a reaction time of 6.43 ± 0.17 s at 90 minutes (p<0.001), while the 600 mg/kg dose produced the highest increase to 8.50 ± 0.11 s (p<0.001). The positive control showed the largest increase (15.80 ± 0.52 s at 90 minutes), whereas the negative control showed no significant changes. These results indicate that *T. dioica* leaf extract has significant central analgesic activity, particularly at higher doses, supporting its ethnomedicinal use for pain relief and suggesting further research into its active components.

Evaluation of Cytotoxic activity of methanolic extract of *T. dioica* leaves

The cytotoxicity activity of the methanolic extract of *T. dioica* leaves was evaluated by Brine shrimp lethality bioassay, as presented in Table 4. The study showed that no Brine shrimp were killed at different concentrations of the methanolic extract of *T. dioica leaves.* So, no cytotoxicity activity of the methanolic extract of *T. dioica* leaves was found by this study.

Evaluation of Thrombolytic activity of methanolic extract of *Tricosanthes dioica* leaves by *in vitro* clot lysis model on human blood.

When 500 µL of distilled water was added to the control clot, little clot lysis with 12.25 ±2.07 % was observed. On the other hand, when streptokinase (standard) was added, extremely significant (P<0.001) clot lysis 68.82±5.53% was observed. But in case of methanolic extracts of *T. dioica* leaves, % of clot lysis was 11.73±2.31%, which was very insignificant compared to streptokinase, as demonstrated in Table 5 and elucidated in Figure 8.

Evaluation of membrane-stabilizing activity of the methanolic extract of *T. dioica* leaves

Hypotonicity-induced hemolysis test

To investigate the anti-inflammatory activity of the Methanolic extracts of *T. dioica leaves,* a hypotonicity-induced hemolysis test was done as presented in Table 6 and Figure 9. This study showed that Methanolic extracts of *T. dioica leaves had small anti*-inflammatory activity (6.48%) compared to acetyl salicylic acid (87.53%).

Heat-induced Hemolysis test

To investigate the anti-inflammatory activity of the methanolic extracts of *T. dioica* leaves, Heat induced hemolysis test was done. This study showed that Methanolic extracts of *T. dioica* leaves had promising anti-inflammatory activity (15.47%) compared to standard Acetyl Salicylic Acid (12.35%) as presented in Table 7 and Figure 10.

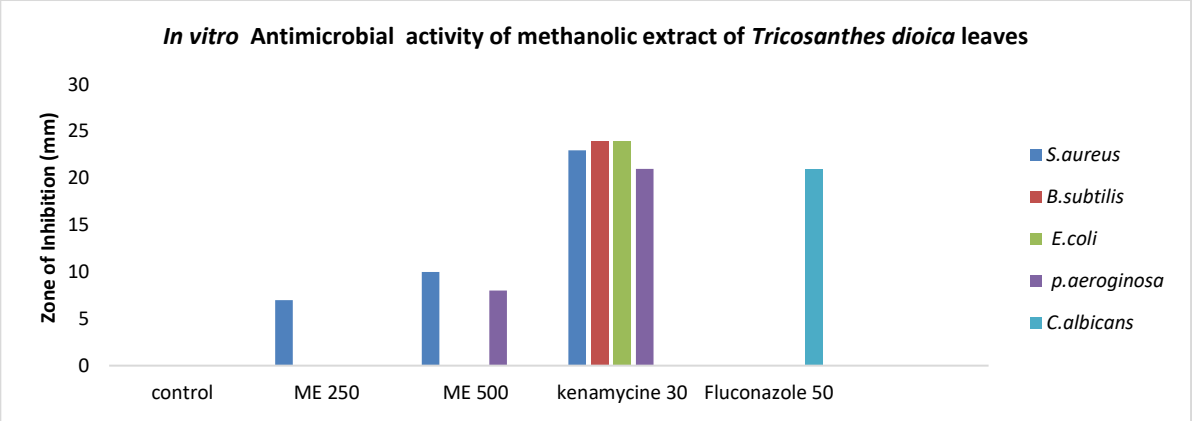


Figure 5: Graphical representation of antimicrobial activity of methanolic extract of *T. dioica* leaves by disc diffusion method

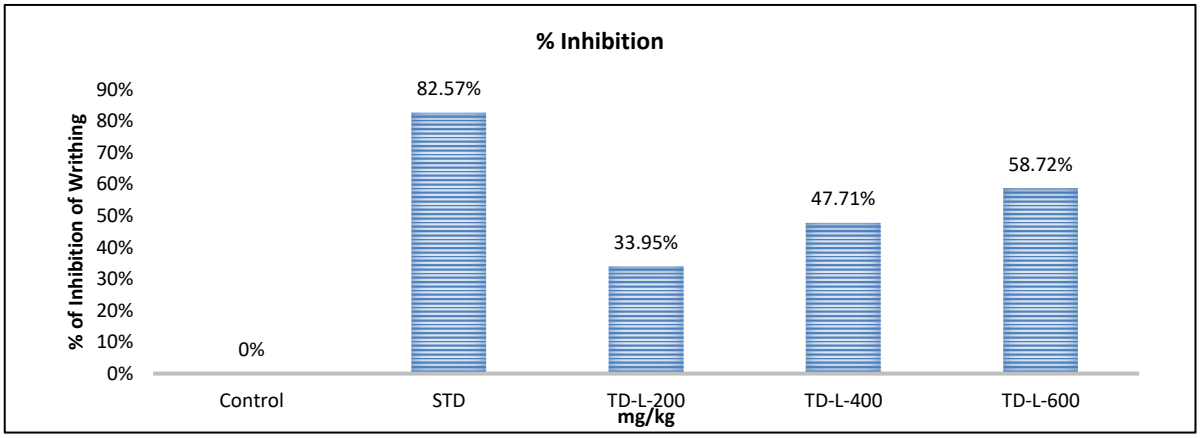


Figure 6: Percentage inhibition of writhing at various doses of methanolic leaves extract of *T. dioica* by the acetic acid induced writhing method

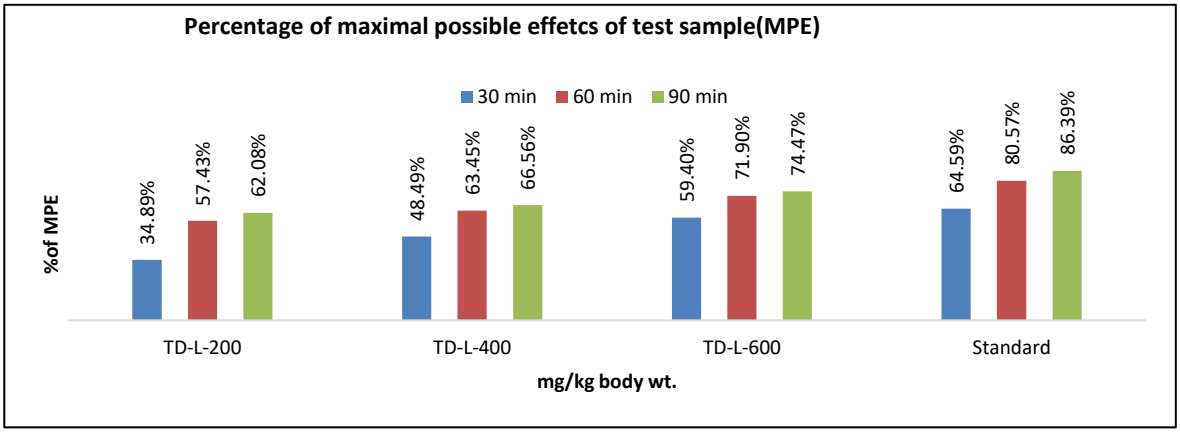


Figure 7: Percentage of maximal possible analgesic effects of the test sample and the standard at 0, 30, 60, and 90 minutes

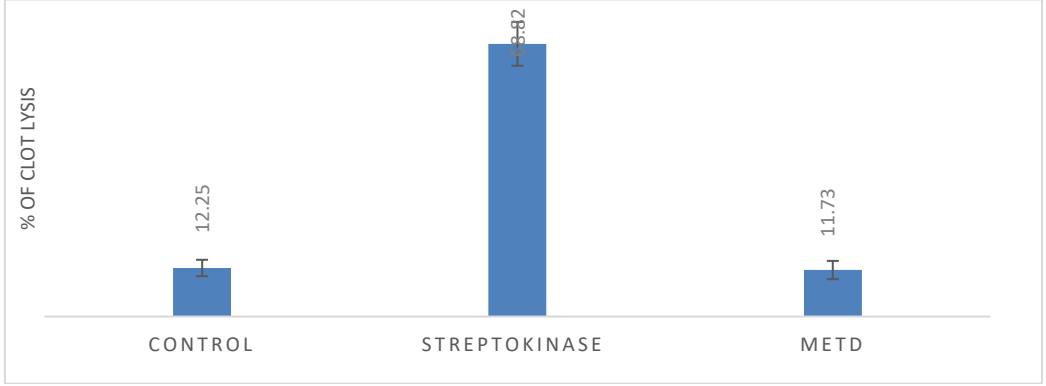
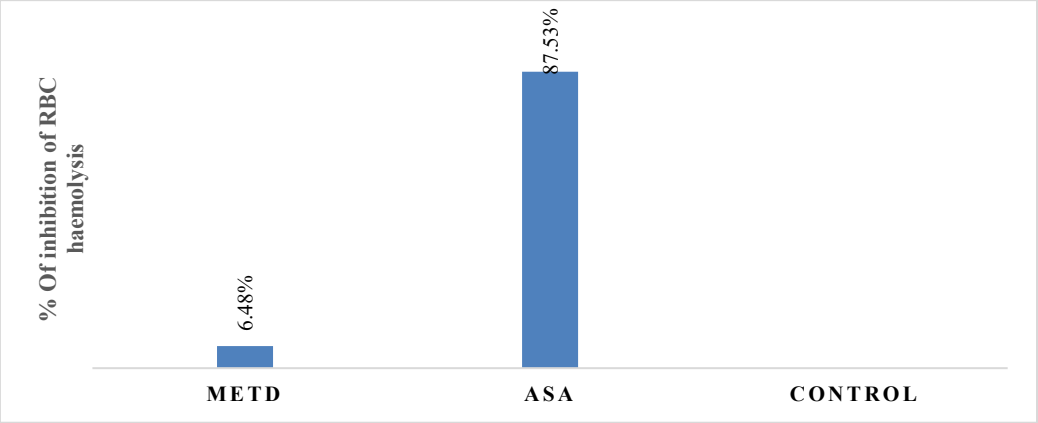


Figure 8: Thrombolytic effect of methanolic extracts of *T. dioica* leaves compared to Standard streptokinase



METD= Methanolic extracts of *T. Dioica.* and ASA= Acetyl salicylic Acid

Figure 9: % of Inhibition of RBC hemolysis of methanolic extract of *T. Dioica* and Acetyl salicylic Acid

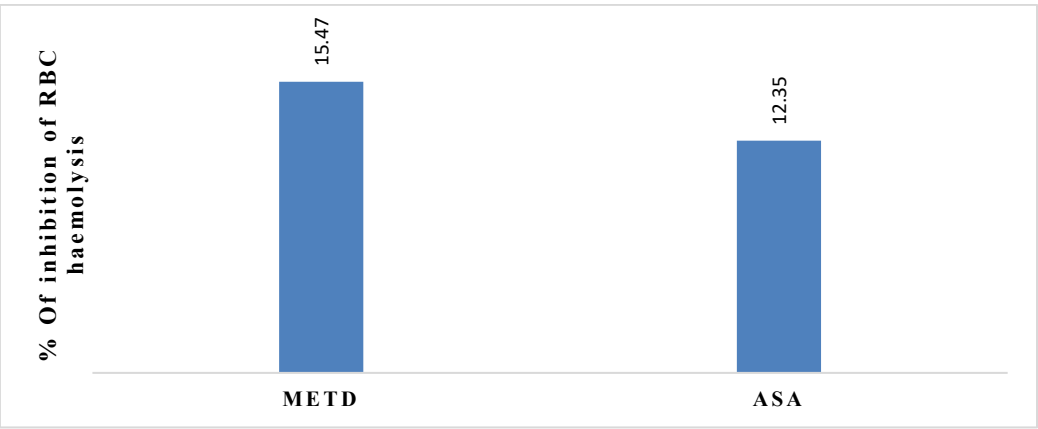


Figure 10: % of inhibition of RBC hemolysis of methanolic extracts of *T. dioica* and acetyl salicylic acid by heat induced hemolysis test

Table 1: Evaluation of antimicrobial activity of methanolic extract of *T. dioica* leaves by disc diffusion method

Methanolic extract of *T. dioica* leaves	**Bacterial Strains**				**Fungal strain**
	S. aureus	*B. subtilis*	*E. coli*	*P. aeroginosa*	*C. albicans*
Control	0 ±0.000	0±0.000	0±0.000	0±0.000	0±0.000
METD 250 µg/disc	7±0.0456	0±0.000	0±0.000	0±0.000	0±0.000
METD 500 µg/disc	10±0.0912	0±0.000	0±0.000	8±0.0567	0±0.000
Kanamycin (30 µg/disc)	23 ±0.0278	24±0.0465	24±0.0456	21 ±0.1233	-
Fluconazole (50 µg/disc)	-	-	-	-	21±0.123

Table 2: Percentage inhibition of writhing at various doses of methanolic extract of *T. dioica* leaves by the Acetic acid induced writhing method in mice

Dose	**Frequency of Writhing (Mean ± SEM)**	**% of Writhing**	**% Inhibition of writhing**
Control	21.8±0.86	100%	0
STD	3.8±0.37***	17.43%	82.57%
TD-L-200	14.4±1.02**	66.05%	33.95%
TD-L-400	11.4±.050***	52.29%	47.71%
TD-L-600	9±0.83***	41.28%	58.72%

Table 3: Analgesic effect of various concentrations of *T. dioica* leaves, negative control, and positive control on the elongation of the reaction time of the tail flick in mice

Dose	**0 minutes**	**30 minutes**	**60 min**	**90 min**
TD-L-200	2.02±0.15*	3.41 ±0.09***	4.37±0.12***	5.67±0.16***
TD-L-400	2.46±0.10	4.31±0.08***	5.09±0.10***	6.43±0.17***
TD-L-600	2.37±0.06*	5.51±0.14***	6.62±0.33***	8.50±0.11***

Positive Control	2.36 ±0.04**	6.27± 0.09**	9.56±0.13***	15.80±0.52***
Negative Control	1.92±0.04	2.22±0.073	1.86±0.07	2.15±0.09

Table 4: % of mortality of Brine shrimp nauplii at different concentrations of methanolic extract of *T. dioica leaves*

Concentration(µg/mL)	Log C	Number of nauplii	Alive	Dead	% Of mortality
400	2.602	10	10	00	0
200	2.301	10	10	00	0
100	2	10	10	00	0
50	1.699	10	10	00	0
25	1.398	10	10	00	0
12.5	1.097	10	10	00	0

Table 5: Effect of methanolic extracts of *T. dioica* leaves in clot lysis of human blood *in vitro*

Sample	% of clot lysis (Mean ± SEM)
Control	12.25 ±2.07
Streptokinase	68.82 ±5.53***
METD	11.73 ±2.31

Table 6: Effect of control and methanolic extracts of *T. dioica* as well as acetyl salicylic acid (standard) on Membrane stabilization of Human RBC.

Sample	Concentration	Absorbance	% of inhibition of RBC haemolysis
METD	2 mg/mL	1.688	6.48%
ASA	0.1 mg/mL	0.225	87.53%
CONTROL		1.805	

Table 7: Effect of control and methanolic extracts of *T. dioica* as well as acetyl salicylic acid (standard) on Membrane stabilization of Human RBC.

Sample	Concentration	Absorbance Cold	Absorbance Heat	% of inhibition of RBC hemolysis
METD	2 mg/mL	0.260	1.735	15.47
ASA	0.1 mg/mL	0.022	1.760	12.35
CONTROL			2.005	

METD= Methanolic extracts of *T. Dioica. And* ASA= Acetyl salicylic Acid

DISCUSSION

Plants provide diverse structural and phytochemical sources vital for pharmaceuticals. Notable phytochemicals like polyphenols, flavonoids, and terpenoids offer various health benefits. Recent studies indicate that the methanolic extract of *T. dioica* leaves contains high levels of phenolic compounds (181 ±0.05 mg GAE/g), flavonoids (202 ±0.77 mg QE/g), and tannins (187 ±0.37 mg GAE/g). These compounds exhibit antimicrobial, antidiarrheal, anthelmintic, antiviral, and anticancer properties, highlighting their potential therapeutic applications [13]. Researchers found that flavonoids and tannins have pharmacological activities, including antioxidative effects, free radical scavenging, and anticancer properties, along with anti-inflammatory benefits [43]. *T. dioica* leaves may help treat inflammatory diseases, cancer, and coronary heart disease, but further studies are required. Antioxidants from plants can minimize the impact of free radicals by stabilizing damaged cells [14].

The methanolic extract of *T. dioica* leaves exhibited concentration-dependent antioxidant activity, with an IC50 of 42.98±0.89 µg/mL against DPPH free radicals, compared to 19.23±0.95562 µg/mL for the standard natural antioxidant. Previous research indicates that phytochemicals like phenolics and flavonoids contribute to this antioxidant activity. Due to its significant antioxidant properties, *T. dioica* leaves may be beneficial in treating conditions like arthritis, cancer, diabetes, ischemia, and atherosclerosis. Further research is needed to identify the specific compounds responsible for this activity and their mechanisms [44, 45].

The antimicrobial activity of the methanolic extract of *T. dioica* leaves was evaluated by the Kirby–Bauer disc diffusion method. This study showed that the plant extract had narrow-spectrum antimicrobial activity. Plant extract showed only dose-dependent antibacterial activity against *S. aureus* and *P. Aeroginosa*. But antimicrobial activity was not significant against *S. aureus* and *P. aeroginosa* at high dose (500 µg/disc) compared to kanamycin 30 µg/disc. The major finding of this study was that the methanolic extract of *T. dioica leaves was not promis*ing for new broad-spectrum antimicrobial drugs [46]. Further study should be done by using other solvent extracts of *T. dioica* leaves to determine whether other solvent extracts of *T. dioica leaves have antimicro*bial activity or not.

Research suggests that various plants have analgesic activity, such as *Manikara zapota, Ficus racemosa, Bauhionia racemosa,* etc., which show analgesic activity [15]. The methanolic extract of *T. dioica* was evaluated for analgesic activity using the tail flick method. The results indicated significant dose-dependent analgesic effects ($p \leq .05$) compared to the standard diclofenac sodium. Phytochemical screening revealed the presence of flavonoids, tannins, and phenolic compounds, while alkaloids, terpenoids, and flavonoids are suggested to contribute to the analgesic effects [47, 48]. So, the analgesic activity of *T. dioica* leaves might be due to the presence of flavonoids. The

study suggests that *T. dioica* leaves can be used to relieve pain in injury, headache, arthritis, etc [49]. Further research is needed to identify the phytochemical components responsible for the analgesic activity of *T. dioica* leaf extract and to understand its mechanism of action.

This study evaluated the analgesic effects of methanolic extracts from *T. dioica leaves using the acet*ic acid-induced writhing method in mice. Acetic acid caused 100% abdominal writhing. Diclofenac sodium reduced the writhing rate by 17.43%, with an overall inhibition of 83.57%, which was statistically significant. In contrast, *T. dioica* extracts at doses of 200 mg/kg, 400 mg/kg, and 600 mg/kg resulted in writhing inhibition of 33.95%, 47.71%, and 58.72%, respectively, showing significant analgesic effects that were dose-dependent compared to diclofenac sodium. The 200 mg/kg dose was less effective [47, 49, 50].

A brine shrimp lethality bioassay showed no cytotoxic activity from methanol extracts of *T. dioica* leaves. However, Bhattacharya and Haldar reported moderate cytotoxic effects from ethanolic extracts, with an LC_{50} of 26.89 µg/mL compared to vincristine sulphate at 0.9 µg/mL. This discrepancy may arise from the different solvents used for extraction [51, 52]. Further studies are needed to clarify whether methanol extracts possess cytotoxic effects and the reasons for the differing results [8].

Current commercially available thrombolytic agents have numerous limitations, such as a large dose required for showing maximum effectiveness and short fibrin specificity, as well as a tendency to bleed [26]. Research on plant-based compounds is increasing due to their potential effectiveness against diseases. A study evaluated the thrombolytic activity of methanolic extracts of *T. dioica leaves.* Results showed that streptokinase (positive control) achieved 68.82±5.53% clot lysis, while normal saline (negative control) showed 12.25±2.07%. The *T. dioica extracts demonstrated* only 11.73±2.31% clot lysis, which was lower than the negative control. The difference between streptokinase and the negative control was statistically significant (p-value <0.001) [53]. This study suggested that methanolic extracts of *T. dioica* leaves had no thrombolytic activity.

Developing effective anti-inflammatory drugs poses challenges due to the severe side effects of synthetic options like NSAIDs, which can erode joint cartilage and harm the kidneys and liver with long-term use. In contrast, herbal medicines from natural sources often have minimal side effects, primarily due to their phenolic compounds that exhibit anti-inflammatory properties. Research shows that at a concentration of 2 mg/mL, methanolic extracts of *T. dioica* showed only slight *re*sistance to hypotonicity-induced lysis of human RBCs (6.18%), while acetylsalicylic acid at 0.1 mg/mL significantly resisted lysis (87.53%). Additionally, in the case of heat-induced lysis, the extracts displayed a notable effect (15.47%) compared to acetylsalicylic acid (12.35%) [8, 23, 42]. The plant extract's membrane-stabilizing effect varies with concentration, indicating the need for further studies on *T. dioica* leaf methanolic extract. The heat-induced hemolysis test suggests promising anti-inflammatory activity compared to acetylsalicylic acid, revealing significant opportunities for future research despite some procedural concerns.

Our findings indicate that the antioxidant activity of *T. dioica* leaves is linked *to i*ts phenolics, flavonoids, and tannins, suggesting potential therapeutic effects against oxidative stress-related diseases such as arthritis, cancer, diabetes, and atherosclerosis. The extract also showed significant analgesic activity, likely due to bioactive compounds like flavonoids and alkaloids, supporting its use in pain management. Antibacterial analysis revealed effectiveness against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, highlighting its potential for treating infections. However, minimal cytotoxic, thrombolytic, and anti-inflammatory activities were observed. Overall, *T. dioica* shows promise, but further research is needed to isolate specific phytochemicals and understand their mechanisms.

CONCLUSION

This study thoroughly evaluated the pharmacological properties of the methanolic extract from the leaves of *Trichosanthes* dioica, a plant traditionally used for medicinal purposes. The extract revealed a high content of phytochemicals, particularly phenolics (181±0.0.52 mg GAE/g), flavonoids (202±0.77 mg QE/g), and tannins (187±0.36 mg GAE/g), which supports its antioxidant and therapeutic potential. The antioxidant activity was significant, with a DPPH IC_{50} value of 42.98±0.88 µg/mL, indicating its potential utility in alleviating conditions related to oxidative stress, such as arthritis, cancer, and cardiovascular diseases. The extract also exhibited notable analgesic activity in both peripheral (acetic acid-induced writhing) and central (tail-flick test) models, demonstrating dose-dependent efficacy comparable to standard analgesic drugs. This finding supports the traditional use of *T. dioica for pain management.* However, the extract showed limited antibacterial efficacy, displaying modest activity only against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, without broad-spectrum activity. Cytotoxicity assays indicated no toxicity in brine shrimp lethality tests, suggesting the extract is safe for therapeutic use. The thrombolytic activity was minimal (11.73%), significantly lower than that of standard streptokinase (68.82±5.53%), indicating limited potential for treating thrombosis. In terms of anti-inflammatory effects, the extract displayed mild to moderate activity, performing better in heat-induced hemolysis (15.47%) than hypotonic-induced hemolysis (6.48%), though still less effective than standard acetylsalicylic acid. In conclusion, the methanolic extract of *T. dioica* leaves shows promising antioxidant and analgesic properties, with mild anti-inflammatory effects, limited antibacterial activity, and no observed cytotoxicity. These findings align with its traditional medicinal uses and suggest the potential for developing safer analgesics and antioxidant agents. However, its thrombolytic and antimicrobial effects remain limited. Further research is essential to isolate and characterize the bioactive compounds and validate these effects through advanced pharmacological models and clinical studies.

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Conflict of interest

The authors declared no conflict of interest.

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