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Comparative evaluation of anti-inflammatory activity and acute toxicity of *Alysicarpus vaginalis* (L.) DC. and *Desmodium gangeticum* (L.) DC. whole plants and their roots

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Author contributions

Rajapakshe T conducted the experiments, analyzed the data and wrote the manuscript. Jayasuriya B, Herath D, Karunarathna U and Dahanayake J provided technical and material support and were involved in conceptualization and designing the study, methodology interpretation, supervision, as well as editing the manuscript and approval of the final version.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

PBS, phosphate buffer saline solution; IC_{50}, 50% inhibition concentration; LC_{50}, 50% lethal concentration; GAE, gallic acid equivalents; QE, quercetin equivalents.

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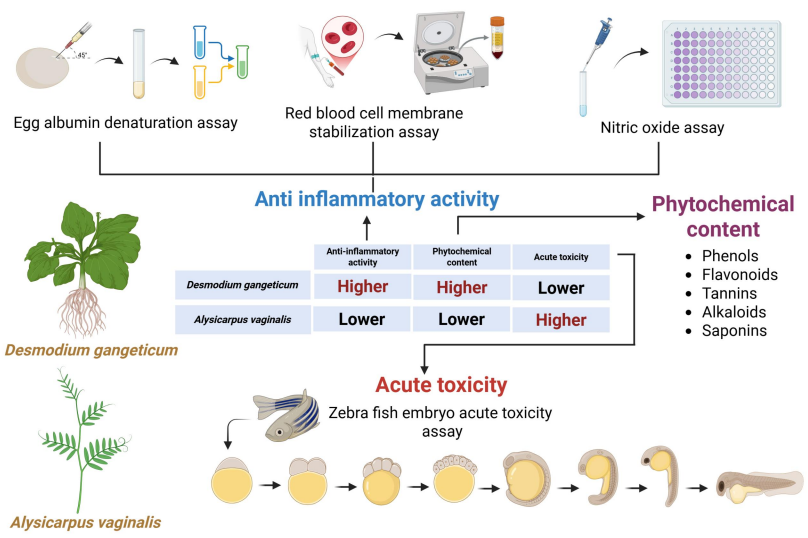
Abstract

Background: The root of *Desmodium gangeticum*, a key component of "Dashamoola," is a significant Ayurveda remedy for inflammatory conditions. Due to the less availability of *D. gangeticum* in Sri Lanka, indigenous physicians often substitute *Alysicarpus vaginalis* root without scientific validation. Further, no comparative study has been conducted on the whole plants of these species. This research is focused on comparing the aqueous extracts of roots and whole plants of *A. vaginalis* and *D. gangeticum* for anti-inflammatory activity, acute toxicity, and to quantify major phytochemicals. **Methods:** Freeze-dried aqueous extracts were prepared and assessed for anti-inflammatory potency using the egg albumin denaturation assay, heat-induced red blood cell membrane stabilization assay, and nitric oxide assay. Acute toxicity was evaluated using the zebrafish embryo assay, and major phytochemicals, were quantitatively screened. **Results:** In egg albumin denaturation assay, *D. gangeticum* whole plant (IC_{50} 107.89 ± 0.71 µg/mL) and root (IC_{50} 210.37 ± 0.39 µg/mL) exhibited superior anti-inflammatory potency compared to diclofenac sodium (IC_{50} 826.04 ± 0.27 µg/mL) and *A. vaginalis* (whole plant IC_{50} 1,336 µg/mL, root IC_{50} 3,162.28 µg/mL). In red blood cell membrane stabilization assay, *D. gangeticum* (whole plant IC_{50} 47.86 ± 0.52 µg/mL, root IC_{50} 331.13 ± 0.83 µg/mL) showed the highest activity. Quercetin (IC_{50} 285.01 µg/mL) was the most potent, with *D. gangeticum* (IC_{50} 2,080.03 µg/mL) and *A. vaginalis* (IC_{50} 7,183.87 µg/mL) showing weaker inhibition in the nitric oxide assay. Regarding toxicity, lower toxicity showed for *D. gangeticum* (LC_{50} 2,570.39 µg/mL) compared to *A. vaginalis* (LC_{50} 1,348.96 µg/mL). In phytochemical analysis, phenols, flavonoids, tannins, alkaloids and saponins were quantified. Statistical analysis revealed significant differences between effects of all extracts and reference drugs ($P < 0.05$). **Conclusion:** Comparative analysis revealed, *D. gangeticum* has higher anti-inflammatory activity and lower toxicity than *A. vaginalis*, suggesting its suitability over *A. vaginalis* in Sri Lankan practices.

Keywords: *Alysicarpus vaginalis*; *Desmodium gangeticum*; root; whole plant; anti-inflammatory activity; acute toxicity; phytochemicals



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Highlights

Desmodium gangeticum, a key component of "Dashamoola," is a significant Ayurvedic remedy for inflammatory conditions. In Sri Lanka, its less availability leads Indigenous physicians to substitute *A. vaginalis* without scientific validation. This research focused on the comparison of the anti-inflammatory activity, acute toxicity, as well as phytochemical content of aqueous extracts from both species. Findings indicated that *D. gangeticum* has superior anti-inflammatory activity and lower toxicity than *A. vaginalis*, highlighting its suitability for use in Sri Lankan practices.

Medical history of objective

Alysicarpus vaginalis and *D.gangeticum* originate from D.M.A Jayaweera's *Medicinal Plants (Indigenous and Exotic) used in Ceylon – Part III* (1981 C.E.). *A. vaginalis*, is valued in traditional medicine for treating various ailments, including kidney issues, respiratory problems, and skin conditions. Its roots and whole plant contain bioactive compounds with potential anti-inflammatory and diuretic properties. *D. gangeticum*, is a key component of "Dashamoola" in Ayurveda, used for urinary tract infections, arthritis, and enhancing immune function, and is noted for its antioxidant, antimicrobial, and analgesic properties.

Background

Alysicarpus vaginalis (L.) DC (Aswenna in Sinhala), commonly known as the Alyce clover, a member of Fabaceae family is well known in both traditional and contemporary medicinal uses. It is native to Asian regions and also found in certain parts of Africa and Oceania. Throughout history, various proportions of the plant have been involved in folk medicine in order to treat a range of ailments, including cytotoxicity, kidney stones, and infections like sepsis. The leaves of the plant have been used in remedies for eye conditions and earaches, while the roots are particularly valued for their diuretic properties and have been applied in the treatment of renal disorders, skin diseases, leprosy, and respiratory issues. Additionally, a decoction made from the plant's roots is commonly consumed to alleviate coughing, and manage urinary tract infections, leprosy, and lung-related conditions [1].

Desmodium gangeticum (L.) DC (Shalparni in Sinhala), on the other hand, is classified under Fabaceae family and naturally occurs in Indian subcontinent, commonly known as Sal-Leaved [2]. With a history deeply rooted in traditional medicine, *D. gangeticum* has found applications of its potential anti-inflammatory, anti-asthmatic, as well as hepatoprotective properties. Its root is one of the components of "Dashamoola," an important Ayurveda herbal remedy that is frequently prescribed for a variety of health conditions including urinary tract infections, gout, arthritis, fever, cough, bronchitis, general weakness and neuropathy [2].

Both of these plants are medicinal herbs with anti-inflammatory antibacterial, antifungal, and diuretic properties used in traditional medicine [1–3]. In Sri Lanka, *A. vaginalis* is primarily used for its lithotriptic properties, particularly in dissolving bladder stones. Furthermore, it has been used as an antiperiodic in chronic malarial fever and is recognized for its diuretic, diluent, and demulcent effects on the urinary tract. Traditional practitioners also employ it in the treatment of cardiac, renal, and hepatic dropsy, chronic pyelitis, cystitis, gonorrhea, and strangury [4]. Meanwhile, *D. gangeticum* holds significant value in Sri Lankan traditional medicine for treating chronic liver congestion, jaundice, gallbladder stones, laryngitis, bronchitis, pneumonia, typhoid, and other fevers [4].

Inflammation can be defined as a complicated biological mechanism triggered by various harmful stimuli and is typically characterized by pain, warmth, erythema, edema, as well as impaired capability at the inflamed area. Currently, diverse anti-inflammatory drugs are available to manage inflammatory conditions, but these treatments are associated with several limitations, including adverse effects. As a result, it's critical to identify as well as develop new anti-inflammatory agents to address these concerns effectively [5].

A research using the red blood cell membrane stabilization assay reported negligible anti-inflammatory activity for *D. gangeticum*, suggesting that its pharmacological effects may involve mechanisms beyond membrane stabilization [6]. Moreover, another species of *Desmodium* has demonstrated anti-inflammatory activity when evaluated using the egg albumin denaturation assay [7].

Phytochemical investigations have revealed that both plants contain bioactive compounds linked to anti-inflammatory activity. The root of *A. vaginalis* has been found to contain flavonoids, glycosides, alkaloids, steroids, terpenes, sugars, saponins and tannins [8]. Emphasizing the extract derived from the aerial parts of *A. vaginalis*, the study employed high-performance liquid chromatography (HPLC) techniques. Findings demonstrated the existence of steroidal compounds, including oleanolic acid, lupeol, as well as β-sitosterol in the chloroform fraction. Furthermore, catechin and gallic acid were detected in the n-butanol and acetone fractions, respectively [9].

Similarly, *D. gangeticum* is rich in alkaloids, flavonoids, carbohydrates, phenolics, saponins, and phytosterols [10]. Phytochemical screening studies have demonstrated that the *D. gangeticum* root and leaves contain a wide range of biologically active substances, such as, carbohydrates, phenols, flavonoids, alkaloids, tannins, terpenoids, steroids, phenylpropanoids, coumarins, pterocarpans and volatile oils. Between these, alkaloids, flavonoids, and pterocarpans are the primary bioactive constituents found throughout several components derived from the plant. Additional compounds including desmodin, hordenine, and gangetin contribute significantly to its extensive therapeutic potential. Desmodin, classified as a pterocarpan, has demonstrated antifungal and antibacterial properties, while gangetin, another pterocarpan, has been identified for its anti-inflammatory and analgesic activities. FTIR spectroscopy analysis has further revealed that *D. gangeticum* plant fractions are particularly abundant in phenol-derived substances [11]. Despite these findings, there is a lack of comparative studies evaluating the anti-inflammatory activity of these plants using standardized *in vitro* assays, such as the egg albumin denaturation assay, red blood cell membrane stabilization assay, and nitric oxide assay. Given the traditional medicinal value of both species and the variability in reported pharmacological effects, further research is needed to conduct to evaluate their exact anti-inflammatory mechanisms and therapeutic potential.

The distribution of *A. vaginalis* is prevalent in coastal regions in Sri Lanka, specially in dry regions and distribution of *D. gangeticum* is apparently very rare in Sri Lanka but can be found in Montane Zone. Due to that reason, in Sri Lanka, the roots of *A. vaginalis* are used mistakenly for the roots of *D. gangaticum* by wrong translation of the Sanskrit word "Sala-Parni" into Sinhalese [4].

There is no scientific validation to consider the roots of *A. vaginalis* can be used instead of *D. gangeticum*. Conversely, the complete botanical composition of *A. vaginalis* has been employed in traditional medicine as an anti-inflammatory remedy for stomachaches, in snakebite antidotes, and for the management of asthma and colds. However, no comparative studies have been carried out on the whole plants of these species.

Thus, the current research performed a comparison of the anti-inflammatory activity of *A. vaginalis* and *D. gangeticum* whole plants and their roots, using *in vitro* assays. The both plants acute toxicity was assessed with zebra fish embryo acute toxicity test.

Methods

Sourcing of plant materials

The entire plant of *A. vaginalis* was gathered from Wellawa, Kurunegala, Sri Lanka (North Western province 7.7833 °N, 80.3667 °E) and the whole plant of *D. gangeticum* was collected from the medicinal garden of the Faculty of Indigenous Medicine, University of Colombo, Colombo, Sri Lanka (Western province 6.9271 °N, 79.9478 °E) in

September, 2023. Authentication of the herbarium specimens of the whole plants was conducted at the Bandaranayaka Memorial Ayurveda Research Institute, Nawinna, Sri Lanka (Accession number 4149 and 4150 respectively).

Extraction of plant materials

The whole plants underwent washing followed by air drying for two weeks with occasional flipping. Dried plant materials were separated as roots and whole plants before they were sliced into small pieces. Then, they were ground to form coarse powder by using a mechanical grinder (Havells Supermix Mixer Grinder, Haridwar, India) and stored in glass bottles without exposing to direct sunlight and humid conditions. Four decoctions of whole plants and roots were prepared separately as per the Ayurveda pharmacopeia. In brief, 60 g of grinded plant materials were decocted with a measured volume of 1,920 mL of distilled water, until the volume is decreased to a final volume of 240 mL (1/8). The resulting solutions were filtered. The filtrates were concentrated using rotary evaporator and then freeze-dried to produce the resulting aqueous root and whole plants extracts of *A. vaginalis* and *D. gangeticum*.

Evaluation of anti-inflammatory activity of aqueous extracts of *Alysicarpus vaginalis* and *Desmodium gangeticum* whole plants and their roots

The anti-inflammatory activity of the whole plants and roots of *A. vaginalis* and *D. gangeticum* was assessed through three *in vitro* techniques: the egg albumin denaturation assay, the heat-induced red blood cell membrane stabilization assay and the nitric oxide assay.

Egg albumin denaturation assay

Inhibition of protein denaturation assay was performed following an earlier established procedure with slight adjustments [5].

Phosphate buffer saline solution at pH 6.4 was produced by dissolving 0.25 g of sodium monohydrogen phosphate (Na_2HPO_4), 0.25 g of sodium dihydrogen phosphate (NaH_2PO_4), and 0.82 g of sodium chloride (NaCl) in 100 mL of distilled water. The pH of the resulting solution was adjusted to 6.4 by using either 1 M hydrochloric acid (HCl) or 1 M sodium hydroxide (NaOH), as necessary. Diclofenac sodium was used as the positive control. All chemicals used for the preparation, including Na_2HPO_4, NaH_2PO_4, NaCl, HCl, NaOH, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diclofenac sodium was obtained from the State Pharmaceutical Manufacturing Corporation of Sri Lanka (Ratmalana, Sri Lanka) as a gift sample. Concentration series (39.06, 78.12, 156.25, 312.50, 625.00, 1,250.00, 2,500.00, 5,000.00 µg/mL) were prepared separately for each aqueous extract and for diclofenac sodium. PBS (phosphate buffer saline solution) was used as the solvent. The reaction mixtures for test samples were prepared with 0.2 mL volume of egg albumin, 2.8 mL volume of PBS and 2 mL volume of each concentration of plant extract. Instead of plant extract, 2 mL of diclofenac sodium or distilled water was introduced as the respective positive and negative controls. All the assay mixtures were exposed to a water bath temperature of 37 ˚C for a duration of 15 min. The reaction mixture temperature was increased gradually up to 70 ˚C with continuous stirring. Test tubes were kept at a temperature of 70 ˚C for a duration of 5 min and cooled until it reached room temperature. The absorbance of each mixture was checked at 660 nm with the aid of spectrophotometer (Multiskan Sky Spectrophotometer, Vantaa, Finland). Triplicate determinations were performed for each sample. Based on the results, the percentage inhibition was determined using the Equation (1):

Percentage of Inhibition = (Absorbance of Negative Control − Absorbance of Sample) / (Absorbance of Negative Control) × 100 (1)

Means of triplicates were used to plot graphs, log concentration versus percentage of inhibition and the concentration which gives 50% of inhibition (IC_{50}) was determined.

Heat-induced red blood cell membrane stabilization assay

Heat-induced stabilization of red blood cell membrane assay was performed following an earlier established procedure with slight adjustments [12, 13].

Ethical approval was granted by the Ethics Review Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka (ERC/B. Pharm 06). Human blood was obtained from five healthy Sri Lankan healthy male and female volunteers aged 20–50 years who abstained from taking non-steroidal anti-inflammatory drugs for at least two weeks before the experiment. Participants were provided with an information sheet in their preferred language, outlining the study's purpose, sample collection methods, and potential outcomes. The information was presented in an easily understandable format, and further clarification was offered as needed. Opportunities to ask questions were given at any point during the study. Participants were informed of their right to decline participation or withdraw at any time without penalty. Written informed consent was obtained voluntarily, with consent forms provided. The researcher's contact details were shared for any inquiries or complaints regarding the study. Ethical guidelines were strictly followed throughout the process. Subsequently, the blood samples were transferred into centrifuge tubes. These tubes were subjected to centrifugation at 3,000 revolutions per minute (rpm) for a duration of 10 min. Afterwards, all blood samples underwent three wash cycles, each utilizing an equivalent amount of normal saline. Then the blood volume was measured. Finally, it was reconstituted with normal saline to produce a suspension with a concentration of 10% (v/v). Phosphate buffer saline solution at pH 7.4 was produced by dissolving 0.26 g of NaH_2PO_4, 1.15 g of Na_2HPO_4 and 9 g of NaCl in 1,000 mL volume of distilled water. Then the pH of the resulting solution was adjusted to 7.4 using either 1 M NaOH or 1 M HCL, as necessary. Diclofenac sodium was used as the positive control. Concentration series 39.06, 78.12, 156.25, 312.50, 625.00, 1,250.00, 2,500.00, 5,000.00 µg/mL were prepared separately for each aqueous extract and for diclofenac sodium. PBS was used as the solvent. The reaction mixtures for test samples were prepared by mixing 3 mL of each concentration of plant extract with a volume of 300 µL of the prepared 10% red blood cell suspension. Instead of plant extract, 3 mL of diclofenac sodium or PBS was introduced as respective positive and negative controls. All the assay mixtures were exposed to a water bath temperature of 56 ˚C and incubated for a duration of 30 min. Then, all the tubes containing the reaction mixture were allowed to cool under tap water flow. Subsequently, the assay mixture underwent centrifugation at a speed of 2,500 rpm for a duration of 5 min to separate. In the final step, the absorbance of the final resulting solution was measured at 560 nm with the aid of spectrophotometer. Triplicate determinations were performed for each sample. Based on the results, the percentage inhibition was determined using the Equation (2):

Percentage of Inhibition = (Absorbance of Negative Control − Absorbance of Sample) / (Absorbance of Negative Control) × 100 (2)

Means of triplicates were used to plot graphs, log concentration versus percentage of inhibition and the concentration which gives IC_{50} was determined.

Nitric oxide assay

This assay was performed following an earlier established procedure with slight adjustments [14–16].

The phosphate buffer saline solution at pH 7.4 was produced by dissolving 1.3 g of NaH_2PO_4, 5.75 g of Na_2HPO_4 and 45 g of NaCl in 1,000 mL of distilled water. The pH of the resulting solution was adjusted to 7.4 using either 1 M NaOH or 1 M HCL as necessary. Sodium nitroprusside (10 mM) was produced by dissolving 0.76 g of sodium nitroprusside in 250 mL of PBS. In the production of the Griess reagent, 0.10 g of N-(1-naphthyl)-ethylene diaminedihydrochoride in 100 mL volume of distilled water and 1.01 g of sulphanilamide with 99% purity, 5.68 mL volume of phosphoric acid with 88% purity in distilled water were combined together (1:1 V/V) within 12 h prior to the analysis. Quercetin was used as a reference drug. A concentration

series (25–800 µg/mL) was prepared using quercetin. A concentration series was prepared for aqueous extracts of roots and whole plants of *A. vaginalis* and *D. gangeticum* which containing, 10,000, 9,000, 8,000, 7,000, 6,000, 5,000, 4,000, 3,000, 2,000, 1,000, 500, 250 µg/mL of four plant extracts. The reaction mixtures for each test sample were prepared with 2 mL of sodium nitroprusside (10 mM), 500 µL volume of 50 mM phosphate buffer saline solution at pH 7.4 and 500 µL of each test sample concentrations. For the blank of these test samples, 500 µL of each concentration of quercetin or plant extract was mixed with 250 µL of phosphate buffer saline. The reaction mixture for negative control was composed of 2 mL of sodium nitroprusside, 500 µL of 50 mM phosphate-buffered saline solution at pH 7.4, and volume of 500 µL of a solution consisting of isopropyl alcohol (IPA) and water in a ratio of 70:30. All the reaction mixtures were incubated exposing a temperature of 25 ˚C over a period of 150 min. Upon completion of the incubation period, 1 mL volume of the solution was extracted and combined with 1 mL volume of Griess reagent. Subsequently, the mixture underwent an additional incubation period of 30 min. Finally, the resulting solution absorbance was measured at 546 nm with the aid of a spectrophotometer. Triplicate determinations were performed for each sample. Based on the results, the percentage scavenging was determined using the Equation (3):

Percentage of Scavenging = (Absorbance of Negative Control − Absorbance of Sample) / (Absorbance of Negative Control) × 100 (3)

Means of triplicates were used to plot graphs, concentration versus percentage of scavenging and the concentration which gives IC_{50} was determined.

Evaluation of acute toxicity of aqueous extracts of *Alysicarpus vaginalis* and *Desmodium gangeticum* whole plants and their roots

Acute toxicity of the four plant extracts was assessed using zebra fish embryo acute toxicity test based on the Organization for Economic Cooperation and Development 236 guidelines with slight adjustments [17].

Zebrafish embryos for the study were procured from the Medical Research Institute, Colombo, Sri Lanka. Eggs were exposed to four concentration series (156.25, 312.50, 625.00, 1,250.00, 2,500.00, 5,000.00 µg/mL) of aqueous extracts of *A. vaginalis* and *D. gangeticum* roots and whole plants separately. As the negative control, distilled water was used and 50% ethanol solution was applied as the positive control [18]. During a period of 2 h of post-fertilization, individual embryos were placed in 20 wells in a 24-well plate, with each well with 2 mL volume of the designated drug solution, while the four wells which are remaining served as internal controls with only distilled water. For each concentration of the test compound, twenty embryos were subjected. Simultaneously, another group of twenty embryos was subjected to the positive control. The plates were kept at a temperature of 26 ˚C under a 14 h light and 10 h dark cycle for a duration of 96 h.

Apical observations (embryo coagulation, absence of heartbeat, non-detachment of the tail and lack of somite formation) were made on each tested embryo. The embryo of zebrafish is regarded as dead if any of these signs are observed. Morphological abnormalities, such as pericardial edema, spinal curvatures, yolk sac edema, and absence of eye buds, were also monitored throughout the test period. Observations were made and recorded once every 24 h until the test was completed. The results obtained after 96 h and based on the results, the percentage mortality was determined using the Equation (4):

$$\% \text{ of Mortality} = \frac{\text{Number of Dead Eggs}}{\text{Total Eggs}} \times 100 \quad (4)$$

Concentration versus % of mortality curves were plotted and the concentration which gives 50% of mortality (LC_{50}) was determined.

Quantitative determination of major phytochemicals of aqueous extracts of *Alysicarpus vaginalis* and *Desmodium gangeticum* whole plants and their roots

Phenolics, flavonoids, tannins, alkaloids and saponins were screened quantitatively using previously prescribed methods.

Total phenolic content. Total phenolic content in the aqueous extracts of *A. vaginalis* and *D. gangeticum* whole plants and their roots was assessed by Folin-Ciocalteu colorimetric procedure with slight adjustments [19].

Preparation of Calibration Curve and analysis of total phenolic content

A gallic acid stock solution (1 mg/mL) was produced by dissolving 50 mg of gallic acid in 50 mL volume of methanol. From this, varying concentrations (100–650 µg/mL) were obtained. Each concentration (1 mL) was combined with 5 mL volume of 10% Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) and 4 mL volume of 7% sodium carbonate. A blank reaction was prepared using methanol instead of gallic acid. The mixtures were shaken, incubated at 40 ˚C for a duration of 30 min, and finally absorbance was measured at 760 nm against the blank with the aid of spectrophotometer. Measurements were conducted in triplicate, and a calibration curve was graphed from the mean absorbance values.

For total phenolic content analysis, 1 mg of freeze-dried plant extracts were dissolved in a volume of 1 mL distilled water. Each extract (1 mg/mL) was combined with a 5 mL volume of 10% Folin-Ciocalteu reagent and 4 mL volume of 7% sodium carbonate. A blank was prepared using distilled water instead of the plant extract. All mixtures were shaken, incubated at a temperature of 40 ˚C for a duration of 30 min. Finally, the absorbance was measured at 760 nm against the blank with the aid of a spectrophotometer, with triplicate measurements. Total phenolic content was demonstrated as gallic acid equivalents (GAE) in mg/g of dry sample using the Equation (5):

$$C = \frac{c \times V}{m} \quad (5)$$

Where:

C = Total phenolic content (mg GAE/g)

c = Concentration of Gallic acid from the calibration curve

V = Extract volume (mL)

m = Extract mass (g)

Total flavonoid content. Total flavonoid content in the aqueous extracts of *A. vaginalis* and *D. gangeticum* whole plants and their roots was assessed by the aluminum colorimetric procedure with slight adjustments [20].

Preparation of Calibration Curve and analysis of total flavonoid content

A quercetin standard solution (1 mg/mL) was produced by dissolving 50 mg of quercetin in volume of 50 mL volume of methanol. From this, various concentrations (60–300 µg/mL) were produced. Every concentration (1 mL) was mixed with 3 mL of methanol and 0.2 mL of 10% aluminum chloride solution. After a duration of 5 min, 0.2 mL volume of 1 M sodium acetate was introduced, and the total volume of the resulting solution was adjusted to a volume of 10 mL with distilled water. A blank was produced with 0.2 mL of distilled water rather than aluminum chloride. The mixtures were thoroughly mixed, incubated at room temperature for a duration of 30 min. Absorbance was measured at 415 nm with the aid of a spectrophotometer. Measurements were done in triplicate, and a calibration curve was graphed using the mean absorbance values.

For total flavonoid content analysis, 1 mg of freeze-dried plant extracts were dissolved in a volume of 1 mL of distilled water. Each extract (1 mg/mL) was mixed with a volume of 3 mL of methanol and 0.2 mL of 10% aluminum chloride. After a duration of 5 min, 0.2 mL of 1 M sodium acetate was introduced, and the volume was adjusted to 10 mL with distilled water. A blank mixture was prepared using 0.2 mL of distilled water rather than aluminum chloride. All mixtures were incubated at room temperature for 30 min. Absorbance was measured at 415 nm with the aid of spectrophotometer, with triplicate measurements. The total flavonoid content of each plant extract was

determined using the calibration curve, demonstrated as quercetin equivalents (QE) in mg/g of dry sample using the Equation (6):

$$C = \frac{c \times V}{m} \quad (6)$$

Where:

C = Total flavonoid content (mg QE/g)
c = Concentration of quercetin from the calibration curve
V = Extract volume (mL)
m = Extract mass (g)

Total tannin content. Total tannin content in the aqueous extracts of *A. vaginalis* and *D. gangeticum* whole plants and their roots were determined by indigo carmine method [21].

An amount of 2 g from each plant extract were refluxed with 50 mL volume of petroleum ether for 30 min. After that period, petroleum ether was removed and the residue was boiled with 300 mL volume of distilled water for a period of 1 h. Afterwards prepared mixture was filtered and then filtrate was diluted up to 500 mL. From that solution, 25 mL volume was measured and 25 mL volume of indigo carmine solution and 750 mL volume of distilled water were introduced. The same reaction mixture with 25 mL of distilled water rather than the plant extract was utilized as the blank. Reaction mixture was titrated with prepared 0.1 M potassium permanganate solution until the test solution colour transfer from blue to green and eventually to golden yellow. The results were made in triplicate and finally the tannin content in the sample was calculated using the Equation (7):

$$\%\ \text{Tannin Content} = \frac{(V - V_0) \times 0.0004157 \times V_E \times 100}{g \times V_s} \quad (7)$$

Where:

V = 0.1 M $KMnO_4$ aqueous solution volume for the titration of the sample in mL, V_0 = 0.1 M $KMnO_4$ aqueous solution volume for the titration of the blank sample in mL, g = sample mass taken for the analysis in g, V_E = volume of the extraction solvent for the sample (500 mL), V_s = sample volume.

0.0004157 – tannins equivalent in 1 mL of 0.1 M $KMnO_4$ solution.

Total alkaloid content. Total alkaloid content in the aqueous extracts of *A. vaginalis* and *D. gangeticum* whole plants and their roots were determined by based on previously published procedure with slight adjustments [22].

Approximately 5 g of each sample and 200 mL of 20% acetic acid solution was introduced to a beaker. Afterwards, the sealed beaker was left to stand for 4 h. Following the incubation period, the mixtures were filtered, and the resulting extracts were concentrated to a quarter of their actual volume using a water bath. Throughout this process, ammonium hydroxide concentrated solution was carefully introduced drop by drop to each extract until completion. To facilitate the collection of precipitates, the whole solution was kept to settle before being filtered through filter paper of precise weight. Subsequently, the alkaloid present in the filtrate was adjusted and dried in an oven for duration of four hours. Total alkaloid amount was demonstrated as mg per g of air-dried material using equation given below.

$$\%\ \text{Alkaloid Content} = \frac{W_1 - W_2}{g} \times 100 \quad (8)$$

Where:

W_1 = precipitate weight with the filter paper
W_2 = the empty filter paper
g = the mass of the sample used for the analysis

Total saponin content. Total saponin content in the aqueous extracts of *A. vaginalis* and *D. gangeticum* whole plants and their roots were determined by based on previously published procedure with slight adjustments [23].

Amount of 5 g of each plant extract was precisely measured and added to a conical flask with a volume of 250 cm^3, containing exactly 100 cm^3 of 20% aqueous ethanol. The mixture was then heated up to 55 ˚C using a hot water bath and stirred continuously for 4 h. The mixture was then filtered, and the residue was subjected to re-extraction with another 100 cm^3 of 20% aqueous ethanol. This second extraction was also heated for 4 h at a constant temperature of 55 ˚C while being stirred continuously. Upon completion, the combined extracts were heated to 90 ˚C to evaporate the solvent, resulting in a final volume of 40 cm^3. Subsequently, 20 mL volume of diethyl ether was vigorously stirred into the concentrate using a 250 cm^3 separator funnel and thoroughly mixed. The ether layer was then discarded. Next, a volume of 60 cm^3 of n-butanol and 10 cm^3 of 5% sodium chloride solution were added to the remaining solution and mixed thoroughly. This step was conducted twice. Subsequently, the aqueous layer was removed. The residual solution was boiled in a water bath for 30 min and transferred to a pre-weighed crucible. The crucible was dried in an oven until it reach a consistent weight was achieved. The saponin content was determined as percentage using the Equation (9):

$$\%\ \text{Saponin} = \frac{\text{Weight of Saponin}}{\text{Weight of Sample}} \times 100 \quad (9)$$

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) and analyzed with the aid of GraphPad Prism version 8. Statistical comparisons were conducted using the independent t-test, among two groups, and differences among multiple groups were assessed with one-way analysis of variance. Pearson correlation coefficient was used to conduct the correlation analysis among multiple groups. P-value which is less than 0.05 was deemed statistically significant, and results were demonstrated with a 95% confidence interval for IC_{50} and LC_{50} values.

Results

Evaluation of anti-inflammatory activity of aqueous extracts of *Alysicarpus vaginalis* and *Desmodium gangeticum* whole plants and their roots

Egg albumin denaturation assay. Mean percentage of inhibition of egg albumin denaturation of aqueous extracts of *A. vaginalis* and *D. gangeticum* whole plants and their roots and diclofenac sodium is shown in Figure 1.

Values are demonstrated as mean ± SEM. With the increasing concentrations of each plant extract, the inhibition increased dose-dependently ($r = 0.84$, $P < 0.05$) (Figure 1) according to the Pearson correlation.

The highest percentage of inhibition was observed with the whole plant extract of *D. gangeticum* (IC_{50} 107.89 ± 0.71 µg/mL), followed by the root extract of *D. gangeticum* (IC_{50} 210.37 ± 0.39 µg/mL), *A. vaginalis* (IC_{50} 1,336.59 ± 0.14 µg/mL) whole plant extract and *A. vaginalis* root extract (IC_{50} 3,162.28 ± 0.63 µg/mL). The reference drug, diclofenac sodium, exhibited an IC_{50} value of 826.04 ± 0.27 µg/mL. Statistically significant differences ($P < 0.05$) were identified between the plant extracts and the reference drug.

Heat-induced red blood cell membrane stabilization assay. Mean percentage of inhibition of heat-induced hemolysis of erythrocytes by aqueous extracts of *A. vaginalis* and *D. gangeticum* whole plants and their roots at various concentrations is shown in Figure 2. *D. gangeticum* whole plant extract demonstrated the highest percentage of inhibition of heat-induced hemolysis of erythrocytes, which have IC_{50} value of 47.86 ± 0.52 µg/mL. The IC_{50} values of root extract of *D. gangeticum*, the whole plant extract of *A. vaginalis*, and the root extract of *A. vaginalis* were 331.13 ± 0.83 µg/mL, 912.01 ± 0.76 µg/mL and 1,380.38 ± 0.18 µg/mL respectively. The reference drug, diclofenac sodium, exhibited an IC_{50} of 741.31 ± 0.34 µg/mL. Significant differences ($P < 0.05$) were found within the plant extracts and the reference drug, as well as among the plant extracts themselves.

Values are demonstrated as mean ± SEM. With the increasing concentrations of each plant extract, the inhibition of hemolysis increased dose-dependently ($r = 0.87$, $P < 0.05$) (Figure 2) according to the Pearson correlation.

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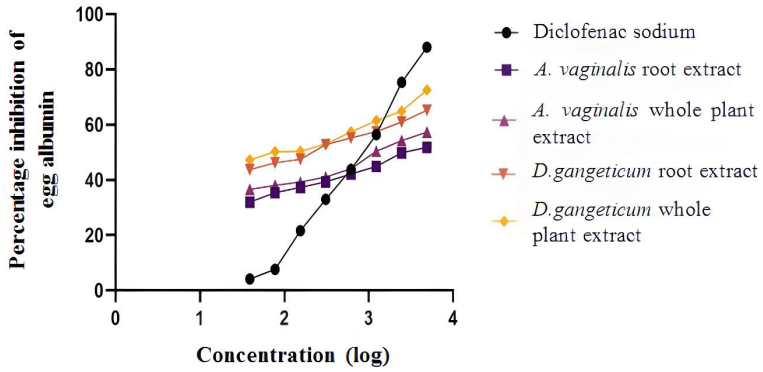

Figure 1 Mean percentage inhibition of egg albumin denaturation of aqueous extracts of *Alysicarpus vaginalis* and *Desmodium gangeticum* whole plants and their roots at different concentrations

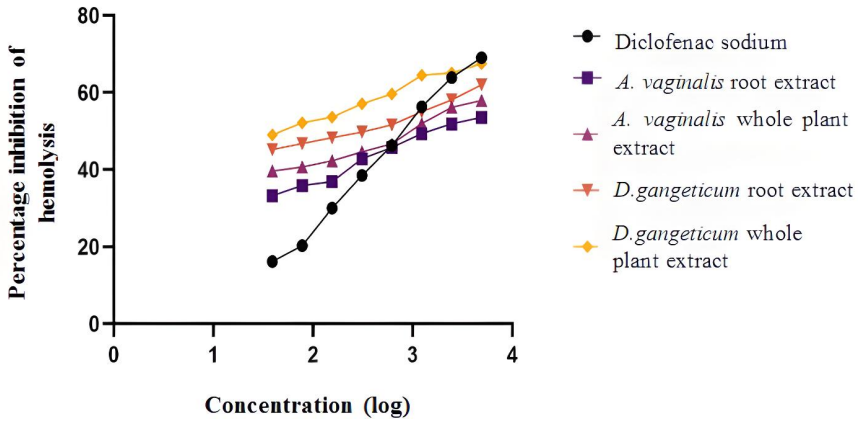

Figure 2 Percentage of inhibition of heat-induced hemolysis of erythrocytes by aqueous extracts of *Alysicarpus vaginalis* and *Desmodium gangeticum* whole plants and their roots at different concentrations

Nitric oxide assay. The IC_{50} values of quercetin and aqueous extracts of *A. vaginalis* and *D. gangeticum* whole plants and their roots at various concentrations are exhibited in Figure 3. The reference drug, quercetin, exhibited the highest activity of scavenging in the nitric oxide assay, with an IC_{50} value of 285.01 ± 0.96 µg/mL. The IC_{50} values of whole plant extract of *D. gangeticum*, the whole plant extract of *A. vaginalis*, the root extracts of *D. gangeticum*, and the root extracts of *A. vaginalis* were 2,080.03 ± 0.85 µg/mL, 7,183.87 ± 0.18 µg/mL, 7,551.24 ± 0.95 µg/mL and 8,150.09 ± 0.61 µg/mL. Significant differences ($P < 0.05$) were identified between the extracts and the reference drug, as well as among the plant extracts themselves.

Values are demonstrated as mean ± SEM. With the increasing concentrations of each plant extract, the inhibition of hemolysis increased dose-dependently ($r = 0.79$, $P < 0.05$) (Figure 3) according to the Pearson correlation.

Evaluation of acute toxicity of aqueous extracts of *Alysicarpus vaginalis* and *Desmodium gangeticum* whole plants and their roots

The results of the zebrafish embryo acute toxicity assay, expressed as the percentage of mortality is shown in Figure 4. The positive control, 50% ethanol caused 100% and negative control caused 0% of mortality. Among the four plant extracts tested, the root extract of *D. gangeticum* demonstrated the lowest acute toxicity with an LC_{50} value of 3,801.89 µg/mL, followed by the whole plant extract of *D. gangeticum* (LC_{50} 2,570.39 µg/mL), *A. vaginalis* root extract (LC_{50} 2,187.76 µg/mL), and *A. vaginalis* whole plant extract (LC_{50} 1,348.96 µg/mL). Statistically significant differences ($P < 0.05$) in toxicity were identified between the plant extracts.

Quantitative determination of major phytochemicals of aqueous extracts of *Alysicarpus vaginalis* and *Desmodium gangeticum* whole plants and their roots

Total phenolic, flavonoid, tannin, alkaloid and saponin contents of aqueous extracts of *A. vaginalis* and *D. gangeticum* whole plants and their roots are represented by Table 1.

Total phenolic content. Based on the results, the whole plant extract of *D. gangeticum* exhibited highest phenolic content, (0.587 ± 0.69 mg GAE/g) and the root extract of *A. vaginalis* exhibited lowest phenolic content (0.280 ± 0.39 mg GAE/g) when comparing with all four plant extracts. The root extract of *D. gangeticum* showed higher phenolic content (0.476 ± 0.58 mg GAE/g) than root extract of *A. vaginalis* and the whole plant extract of *A. vaginalis* (0.400 ± 0.55 mg GAE/g). The whole plant extract of *A. vaginalis* exhibited higher phenolic content than the root extract and lower phenolic content than the root and whole plant extracts of *D. gangeticum*.

Total flavonoid content. According to the results, the whole plant extract of *D. gangeticum* exhibited highest flavonoid content, (0.196 ± 0.41 mg QE/g) and the root extract of *A. vaginalis* exhibited lowest flavonoid content (0.102 ± 0.17 mg QE/g) when comparing with all four plant extracts. The root extract of *D. gangeticum* showed higher flavonoid content (0.168 ± 0.16 mg QE/g) than root extract of *A. vaginalis* and the whole plant extract of *A. vaginalis* (0.122 ± 0.34 mg QE/g). The whole plant extract of *A. vaginalis* exhibited higher flavonoid content than the root extract and lower flavonoid content than the root and whole plant extracts of *D. gangeticum*.

Total tannin content. Based on the results, the whole plant extract of *D. gangeticum* exhibited highest tannin content, (0.416 ± 0.61%) and the root extract of *A. vaginalis* exhibited lowest tannin content (0.083 ± 0.06%) when comparing with all four plant extracts. The root extract of *D. gangeticum* showed higher tannin content (0.166 ± 0.57%) than root extract of *A. vaginalis* and lower tannin content than whole plant extract of *A. vaginalis* (0.249 ± 0.93%). The whole plant extract of *A. vaginalis* exhibited higher tannin content than the root extract of *A. vaginalis*, root extract of *D. gangeticum* and lower tannin content than the whole plant extract of *D. gangeticum*.

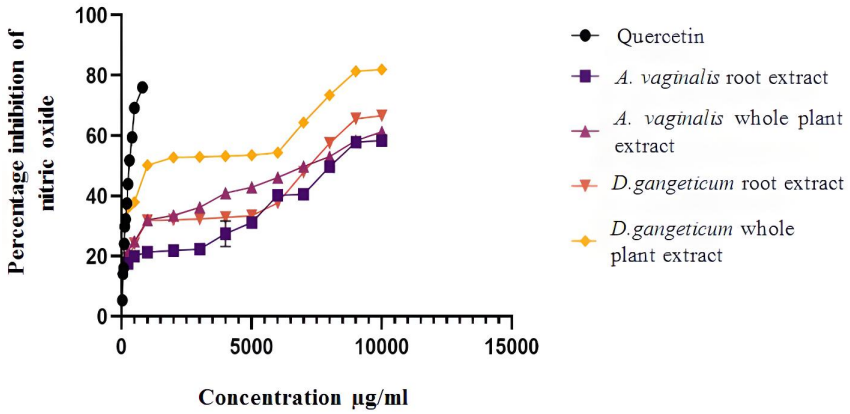

Figure 3 Percentage of inhibition of nitric oxide by aqueous extracts of *Alysicarpus vaginalis* and *Desmodium gangeticum* whole plants and their roots at different concentration

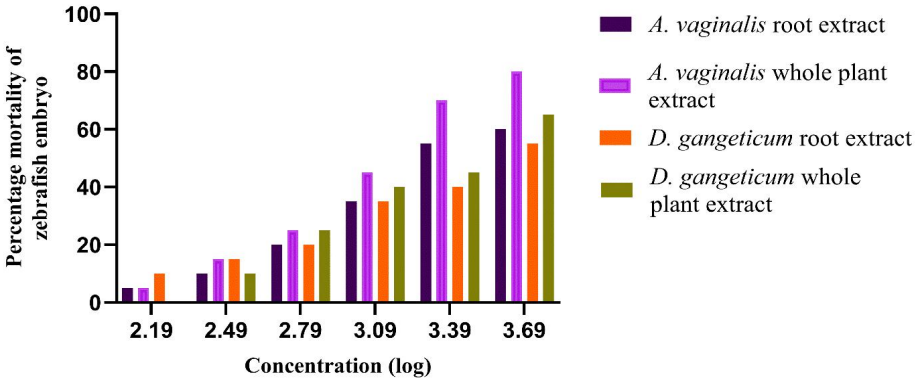

Figure 4 Percentage mortality of zebrafish embryo after the treatment with aqueous extracts of *Alysicarpus vaginalis* and *Desmodium gangeticum* whole plants and their roots

Table 1 Total phenolic, flavonoid, tannin, alkaloid and saponin contents of aqueous extracts of *Alysicarpus vaginalis* and *Desmodium gangeticum* whole plants and their roots

Phytochemical	Plant extract			
	***D.gangeticum* whole plant**	***D.gangeticum* root**	***A.vaginalis* whole plant**	***A.vaginalis* root**
Total phenolic content (mg GAE/g)	0.587 ± 0.007$^{\text{ǂǂ}}$	0.476 ± 0.005	0.400 ± 0.002$^{\text{ǂǂ}}$	0.280 ± 0.001$^{\text{ǂǂ}}$
Total flavonoid content (mg QE/g)	0.196 ± 0.001$^{\text{ǂǂ}}$	0.168 ± 0.011	0.122 ± 0.001$^{\text{ǂǂ}}$	0.102 ± 0.006$^{\text{ǂǂ}}$
Total tannin content (%)	0.416 ± 0.001$^{\text{ǂǂ}}$	0.166 ± 0.004	0.249 ± 0.005$^{\text{ǂǂ}}$	0.083 ± 0.015$^{\text{ǂǂ}}$
Total alkaloid content (%)	0.405 ± 0.004$^{\text{ǂǂ}}$	0.371 ± 0.004	0.193 ± 0.007$^{\text{ǂǂ}}$	0.149 ± 0.021$^{\text{ǂǂ}}$
Total saponin content (%)	0.241 ± 0.009$^{\text{ǂǂ}}$	0.308 ± 0.002	0.260 ± 0.007$^{\text{ǂǂ}}$	0.228 ± 0.001$^{\text{ǂǂ}}$

Values are demonstrated as mean ± SEM. Significantly different at $^{\text{ǂǂ}}P<0.05$ compared to *D.gangeticum* root. GAE, gallic acid equivalents; QE, quercetin equivalents.

Total alkaloid content. According to the results, *D. gangeticum* whole plant extract exhibited highest alkaloid content, (0.405 ± 0.91%) and the root extract of *A. vaginalis* exhibited lowest alkaloid content (0.149 ± 0.53%) when comparing with all four plant extracts. The root extract of *D. gangeticum* showed higher alkaloid content (0.371 ± 0.27%) than root extract of *A. vaginalis* and the whole plant extract of *A. vaginalis* (0.193 ± 0.02%). The whole plant extract of *A. vaginalis* exhibited higher alkaloid content than the root extract and lower alkaloid content than the root and whole plant extracts of *D. gangeticum*.

Total saponin content. Based on the results, *D. gangeticum* root extract exhibited highest saponin content, (0.308 ± 0.51%) and the root extract of *A. vaginalis* exhibited lowest saponin content (0.228 ± 0.38%) when comparing with all four plant extracts. The whole plant extract of *D. gangeticum* showed higher saponin content (0.241 ± 0.07%) than root extract of *A. vaginalis* and lower than the whole plant extract of *A. vaginalis* (0.260 ± 0.73%). The whole plant extract of *A. vaginalis* exhibited higher saponin content than the root extract of *A. vaginalis* and whole plant extracts of *D. gangeticum*.

Discussion

This research focused comparison on the anti-inflammatory activity and acute toxicity of aqueous extracts of *A. vaginalis* and *D. gangeticum* whole plants and their roots. The two plants historically used in traditional medicine. Through a series of *in vitro* assays, including the egg albumin denaturation assay, heat-induced membrane stabilization assay, and nitric oxide scavenging assay we found that *D. gangeticum* expressed significantly higher ($P < 0.05$) anti-inflammatory effects compared to *A. vaginalis*. Moreover, according to zebra fish embryo acute toxicity assay, *D. gangeticum* extracts demonstrated lower acute toxicity, which suggests that *D. gangeticum* may be a safer and more

effective option for anti-inflammatory treatments.

The anti-inflammatory activity of the plant extracts was assessed using three well-established *in vitro* procedures, which measure various aspects of the inflammatory response. In the egg albumin denaturation assay, *D. gangeticum* whole plant extract demonstrated the highest anti-inflammatory activity, significantly outperforming the other extracts, including the reference drug diclofenac sodium. However, findings from other studies demonstrated variations in the anti-inflammatory activity of *D. gangeticum*. One research that tested the egg albumin denaturation assay at a concentration range of 50–800 µg/mL found that *D. gangeticum* exhibited 23.32% to 78.82% inhibition, while diclofenac sodium showed a significantly higher inhibition ranging from 86.22% to 196.75% in the same concentration range [7].

Previous studies have shown the inhibition of protein denaturation plays an important role in anti-inflammatory mechanisms, with plant extracts consist in flavonoids and polyphenols exhibiting strong protein denaturation inhibition [24, 25]. This suggests that *D. gangeticum* contains potent bioactive compounds capable of inhibiting protein denaturation, a key event in inflammation. This result aligns with findings from other studies, where plants with high levels of flavonoids, alkaloids, and phenolics have shown strong protein-denaturation inhibition, contributing to their anti-inflammatory effects [2].

In the heat-induced red blood cell membrane stabilization assay, which reflects the stabilization of lysosomal membranes during inflammation, *D. gangeticum* again exhibited the highest potency. However, results from other studies indicate variations in its membrane stabilization activity. One study that tested the red blood cells membrane stabilization assay at a concentration range of 50–800 µg/mL found that *D. gangeticum* exhibited 6.2% to 38.2% inhibition, whereas diclofenac sodium demonstrated a significantly stronger inhibition of 47.8% to 78.9% in the same concentration range [7]. Additionally, another study showed 0% inhibition for *D. gangeticum*, suggesting minimal membrane stabilization activity [6].

The ability of *D. gangeticum* to stabilize cell membranes is indicative of its potential to prevent the release of harmful lysosomal enzymes during inflammatory processes, which could mitigate tissue damage. However, the lower inhibition values in heat induced red blood cells membrane stabilization compared to diclofenac sodium suggest that while *D. gangeticum* exhibits some degree of membrane stabilization, its potency in this mechanism is relatively weaker. This aligns with findings from studies on other medicinal plants such as *Centella asiatica*, which has been shown to exhibit membrane-stabilizing properties by inhibiting lysosomal enzyme release [26].

The nitric oxide scavenging assay revealed that *D. gangeticum* had the strongest ability to scavenge nitric oxide, a critical mediator in the inflammatory response. Elevated nitric oxide levels are commonly found in inflammatory conditions, and plants capable of inhibiting nitric oxide production may play a crucial role in managing inflammatory diseases [27]. These findings are consistent with research on *Moringa oleifera*, which is rich in phenolic compounds, have been reported to effectively scavenge nitric oxide and suppress inflammatory pathways [28]. The superior activity of *D. gangeticum* in this assay suggests that its active compounds may effectively modulate the nitric oxide pathway, similar to other plant extracts with known anti-inflammatory properties [29].

Acute toxicity was assessed using the zebra fish embryo model, which is an established method for evaluating the developmental toxicity of compounds due to the transparency of the embryos and their genetic similarity to humans. The final results exhibited that the *D. gangeticum* root extract exhibited the lowest toxicity, whereas *A. vaginalis* demonstrated higher toxicity, particularly in its whole plant extract. These findings are significant because they indicate that *D. gangeticum* may be a safer choice for therapeutic use, especially in applications requiring prolonged or higher doses.

The observed differences in toxicity between the extracts could be assigned to the varying concentrations of biologically active compounds in different plant parts. For instance, while the whole plant extract of *D. gangeticum* showed high anti-inflammatory activity, its toxicity profile was lower compared to *A. vaginalis*, suggesting that the bioactive compounds in *D. gangeticum* may be both effective and less harmful at therapeutic doses.

Several phytochemical studies have revealed that both *A. vaginalis* and *D. gangeticum* contain important biologically active compounds contributing to their pharmacological activities. *A. vaginalis* has been shown to contain flavonoids, tannins, saponins, alkaloids, and phenol derived compounds, which consist of antioxidant, anti-inflammatory, and hepatoprotective properties [1, 30]. *D. gangeticum*, on the other hand, has been extensively studied for its flavonoid and phenolic content, which play a role in its important anti-inflammatory and immunomodulatory effects [31, 32]. One study reported that FTIR analysis of *D. gangeticum* showed a broad band at approximately 3,311 cm^{-1} and 2,918 cm^{-1}, which corresponds to the existence of O-H active sites in phenolic compounds, suggesting that the plant extracts are predominantly rich in phenolic derivatives [33].

Recent pharmacological research has shown the flavonoids and polyphenols present in medicinal plants show their anti-inflammatory activities through suppression of nuclear factor kappa B (NF-κB) signaling and cyclooxygenase (COX) enzymes, thereby decreasing the generation of inflammation related compounds [34, 35]. Studies have suggested that the high phenolic content in *D. gangeticum* may contribute to its ability to modulate inflammatory responses by scavenging free radicals and regulating immune responses [31].

The screening of the phytochemicals of four plant extracts demonstrated that *D. gangeticum* contained the highest concentrations of total phenolics, flavonoids, tannins, alkaloids, and saponins, all of which are known for their higher anti-inflammatory properties that *A. vaginalis*. In comparison, *A. vaginalis* showed lower concentrations of these active compounds, particularly in the root extract. This likely explains the reduced anti-inflammatory efficacy of *A. vaginalis*, which may contain fewer active ingredients or have lower bioavailability. The lower phenolic and flavonoid content in *A. vaginalis* could account for its weaker anti-inflammatory activity, as these compounds are critical in modulating inflammatory processes.

This comparative analysis revealed that *D. gangeticum* exhibited the highest anti-inflammatory activity, while *A. vaginalis* showed the lowest. In terms of acute toxicity, *D. gangeticum* was least toxic, whereas *A. vaginalis* was most toxic. Overall, *D. gangeticum* demonstrated superior anti-inflammatory potency and lower toxicity than *A. vaginalis*.

Conclusion

In conclusion, this study provides valuable insight into the anti-inflammatory and acute toxicity effects of *A. vaginalis* and *D. gangeticum*. Upon comparison, it was observed that the *D. gangeticum* plant comprises with higher anti-inflammatory potency and lower acute toxicity activity compared with *A. vaginalis* plant. In conclusion, it can be affirmed that the use of *D. gangeticum* is more suitable than *A. vaginalis*. The Sri Lankan practice of employing *A. vaginalis* instead of *D. gangeticum* is deemed inappropriate based on these findings.

This research adds to the accumulating evidence that supports the therapeutic value of medicinal plants and highlights *D. gangeticum* as a promising candidate for further pharmacological development. However, additional studies are required to gain a comprehensive understanding of the pharmacological properties of both of these plants and for improving their use in clinical applications.

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