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RESEARCH ARTICLE

Phytochemical Analysis and *in vitro* Anti-inflammatory Activity of *Proboscidea louisianica* Leaf Extract Using HRBC Membrane Stabilization Assay

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Abstract: This research work involves investigation of the phytochemical composition and anti-inflammatory properties of *Proboscidea louisianica* leaf extract. Fresh leaves were collected, authenticated, shade-dried, and subjected to Soxhlet extraction using n-hexane as the solvent. Preliminary phytochemical screening revealed the presence of steroids, saponins, tannins, flavonoids, phenolic compounds, glycosides, proteins, and amino acids, while carbohydrates were absent. The anti-inflammatory activity was evaluated using the human red blood cell (HRBC) membrane stabilization method, with diclofenac sodium as the standard reference drug. The n-hexane extract demonstrated dose-dependent membrane stabilization effects at concentrations of 100, 200, 300, and 400 μg/mL, showing 67.85%, 76.71%, 83.28%, and 91.57% protection, respectively. These results were comparable to the standard drug diclofenac sodium, which exhibited 86.79%, 89.78%, 93.79%, and 96.76% protection at similar concentrations. The significant membrane stabilization effect of the extract suggests its potential as an anti-inflammatory agent, possibly due to the presence of beta-sitosterols and other bioactive compounds.

Keywords: *Proboscidea louisianica*; Anti-inflammatory activity; HRBC membrane stabilization; Phytochemical analysis; n-hexane extract.

1. Introduction

Plant-based medicines have served humanity since ancient civilizations, with their therapeutic applications documented across diverse cultures and geographical regions. Plants synthesize numerous chemical compounds for their metabolic functions, defense mechanisms, and protection against environmental threats [1]. The pharmaceutical significance of these compounds is highlighted by the fact that approximately 25% of modern drugs are derived directly or indirectly from plant sources [2]. The traditional medicinal systems worldwide have utilized various plant parts, including herbs and spices, for therapeutic purposes. These applications extend beyond mere medicinal use, as many plants were historically employed for food preservation, particularly in warmer climates where food spoilage posed significant challenges [3]. The documentation of plant-based medicines dates back to ancient civilizations, with the Egyptian Ebers papyrus listing over 800 plant medicines including aloe, cannabis, garlic, and juniper [4].

Figure 1. Leaves and flowers of *P. louisianica*

During the Early Middle Ages, European monasteries played a crucial role in preserving medicinal knowledge through the translation and documentation of classical texts while maintaining extensive herb gardens [5]. The Islamic world made significant contributions

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to herbal medicine, particularly in Baghdad and Al-Andalus, where scholars like Abulcasis and Ibn al-Baitar documented hundreds of medicinal herbs and their applications [6]. *Proboscidea louisianica*, commonly known as Devil's Claw or unicorn plant, belongs to the Martyniaceae family. Native to the Southwestern United States and Mexico, this annual herb can grow up to 900 cm in length with spreading stems [7]. The plant is characterized by opposite, ovate leaves reaching 30 cm in width, covered with glandular hairs containing essential oil droplets. The distinctive lavender-colored flowers and curved fruit capsules with horn-like projections are notable features of this species [8].

Inflammation represents a complex biological response to harmful stimuli, characterized by cardinal signs including redness, swelling, heat, pain, and loss of function [9]. The process involves intricate interactions between immune cells, blood vessels, and molecular mediators. At the cellular level, inflammation is mediated by various chemical compounds including prostaglandins, leukotrienes, histamine, bradykinin, and platelet-activating factor [10]. Despite the extensive traditional use of *P. louisianica*, systematic studies on its phytochemical composition and anti-inflammatory properties remain limited. Previous investigations have identified various bioactive compounds in the plant, including alkaloids, steroids, flavonoids, and phenolic compounds [11]. The present study aims to evaluate the phytochemical constituents of *P. louisianica* leaf extract and assess its anti-inflammatory potential using the HRBC membrane stabilization method, which serves as a reliable in vitro model for screening anti-inflammatory agents.

2. Materials and methods

2.1. Collection and Authentication of Plant Material

Fresh leaves of *Proboscidea louisianica* were collected from rural areas of Korangi, Andhra Pradesh, India. The plant material was authenticated by botanical experts, and voucher specimens were deposited in the herbarium. The leaves were thoroughly washed with distilled water to remove debris and soil particles, followed by shade drying at room temperature (25 ± 2°C) for 14 days. The dried leaves were pulverized using an electric grinder to obtain a fine powder and stored in airtight containers until further use [12].

2.2. Extract Preparation

The powdered leaf material (40 g) was subjected to Soxhlet extraction using n-hexane as the solvent. The extraction process was carried out for 48 hours at 68-70°C. The resulting extract was filtered through Whatman No. 1 filter paper and concentrated using a rotary evaporator under reduced pressure at 40°C. The concentrated extract was stored at 4°C in amber-colored bottles for subsequent analysis [13].

2.3. Phytochemical Screening

The n-hexane extract underwent comprehensive phytochemical screening following standard protocols to identify major bioactive constituents.

2.3.1. Test for Steroids

Liebermann-Burchard Test: Two milliliters of extract was mixed with 2 mL of acetic acid and concentrated sulfuric acid. The development of initial red coloration followed by blue and finally green color indicated steroid presence.

Salkowski Reaction: Two milliliters of extract was treated with chloroform and concentrated sulfuric acid. Red coloration in the chloroform layer and greenish-yellow fluorescence in the acid layer confirmed steroid presence [14].

2.3.2. Test for Tannins and Phenolic Compounds

Lead Acetate Test: Extract solution treated with lead acetate showed white precipitate formation, indicating tannins.

Ferric Chloride Test: Treatment with 5% ferric chloride solution resulting in bluish-black coloration confirmed phenolic compounds [15].

2.3.3. Test for Flavonoids

Alkaline Reagent Test: Extract treatment with sodium hydroxide solution produced intense yellow coloration that became colorless upon addition of dilute acid, confirming flavonoids.

2.3.4. Test for Saponins

Foam Test: Extract solution was vigorously shaken with water. Persistent foam formation for 10 minutes indicated saponins presence.

Froth Test: Extract diluted with distilled water was shaken in a graduated cylinder. Formation of 1 cm stable foam layer confirmed saponins [16].

2.4. Anti-inflammatory Activity using HRBC Membrane Stabilization Assay

2.4.1. Blood Collection and Preparation

Fresh human blood was collected from healthy volunteers under aseptic conditions. Blood samples were mixed with equal volumes of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% sodium chloride). The mixture was centrifuged at 3,000 rpm for 10 minutes, and packed cells were washed with isosaline solution. A 10% HRBC suspension was prepared using phosphate buffer saline (pH 7.4) [17].

2.4.2. Sample Preparation

Stock solutions of *P. louisianica* extract were prepared in concentrations of 100, 200, 300, and 400 μg/mL using acetone as solvent. Diclofenac sodium solutions of similar concentrations were prepared as standard reference.

2.4.3. Membrane Stabilization Assay

The reaction mixture contained: 1 mL phosphate buffer (0.15 M, pH 7.4), 2 mL hyposaline (0.36%), 0.5 mL HRBC suspension and Various concentrations of extract/standard drug [18]

Control samples were prepared similarly, excluding the extract. Samples were incubated at 37°C for 30 minutes and centrifuged at 3,000 rpm for 20 minutes. The supernatant absorbance was measured spectrophotometrically at 560 nm [18]. All experiments were performed in triplicate. [19] Results were expressed as mean ± standard deviation (SD). The percentage of membrane stabilization was calculated using the formula:

Percentage Stabilization = (1 - OD of test sample/OD of control) × 100

3. Results

3.1. Phytochemical Analysis

The preliminary phytochemical screening of n-hexane extract of *Proboscidea louisianica* leaves revealed the presence of several bioactive compounds. The results demonstrated positive tests for steroids (Liebermann-Burchard and Salkowski tests), tannins (Lead acetate test), flavonoids (Alkaline reagent and Shinoda tests), phenolic compounds (Ferric chloride test), saponins (Foam and Froth tests), glycosides (Borntrager's and Legal's tests), and proteins and amino acids (Million's and Xanthoproteic tests). Interestingly, the extract showed negative results for carbohydrates (Molisch test), indicating their absence. [20,21]

Table 1. Results of Phytochemical Screening of n-hexane extract of *Proboscidea louisianica* leaves

Test for	Name of the tests	Results
Steroids	Libermann-buchard test	+
	Salkowski reaction	+
Tannins	Lead acetate test	+
Flavonoids	Alkaline reagent test	+
	Shinoda test	+
Carbohydrates	Molisch test	-
Phenols	Ferric chloride test	+
Saponins	Foam test	+
	Froth test	+
Glycosides	Borntrager's test	+
	Legal's test	+
Proteins and amino acids	Million's	+
	Xanthoproteic test	+

3.2. Anti-inflammatory Activity

The anti-inflammatory activity of *P. louisianica* leaf extract was evaluated using the HRBC membrane stabilization method, with diclofenac sodium serving as the standard reference drug. The results demonstrated a concentration-dependent increase in membrane stabilization for both the test extract and the standard drug. [22]

At 100 µg/mL concentration, the extract showed 67.85% membrane stabilization, while diclofenac sodium exhibited 86.79% protection. The protective effect increased progressively with concentration.

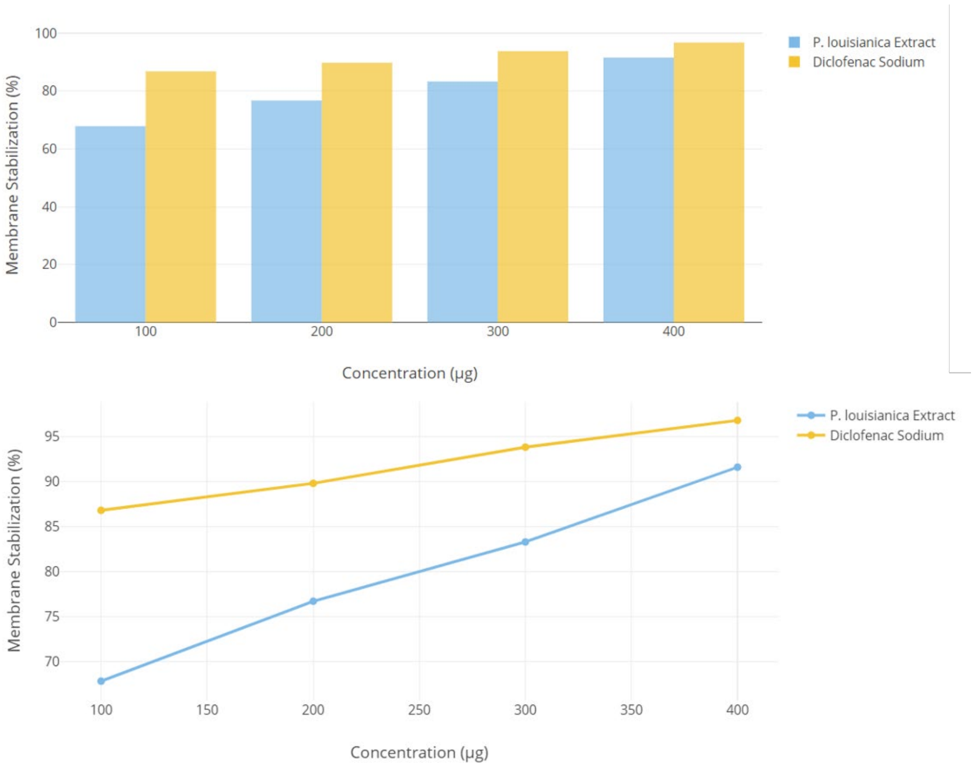

Figure 2. Comparative analysis of anti-inflammatory effect of *P. louisianica* extract vs standard diclofenace sodium

4. Discussion

The HRBC membrane stabilization method was selected due to the structural similarity between the HRBC membrane and lysosomal membrane. [23,24] The lysosomal membrane plays a crucial role in inflammation by releasing inflammatory mediators. When exposed to hypotonic solutions, excessive fluid accumulation leads to RBC membrane rupture and subsequent leakage of serum proteins and fluids into tissues. The ability of the extract to prevent this membrane lysis suggests its potential to inhibit the release of inflammatory mediators *in vivo*. While diclofenac sodium showed higher membrane stabilization percentages across all concentrations, the n-hexane extract demonstrated significant anti-inflammatory activity that increased proportionally with concentration. [25,26] The highest protection was observed at 400 µg/mL (91.57%), approaching the effectiveness of the standard drug (96.76%) at the same concentration.

The observed anti-inflammatory activity may be attributed to the presence of bioactive compounds, particularly beta-sitosterols, which were detected in the phytochemical screening. [27] These compounds, along with other identified phytoconstituents such as flavonoids and steroids, likely contribute to the membrane-stabilizing properties of the extract.

5. Conclusion

Plant-based medicines continue to offer therapeutic potential despite their generally milder effects compared to synthetic drugs. The n-hexane extract of *Proboscidea louisianica* leaves demonstrated significant phytochemical diversity, containing steroids, tannins, flavonoids, saponins, and glycosides, while showing the absence of carbohydrates. The HRBC membrane stabilization assay provided valuable insights into the extract's anti-inflammatory potential, showing dose-dependent protection ranging from 67.85% at 100 µg/mL to 91.57% at 400 µg/mL. The extract's effectiveness in preventing membrane lysis suggests its potential to inhibit inflammatory mediator release, possibly due to the presence of beta-sitosterols and other bioactive compounds.

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