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PHYTOCHEMICAL INVESTIGATION AND EVALUATION OF ANTI-INFLAMMATORY POTENTIAL OF *CENTROSEMA PUBESCENS* BENTH

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ABSTRACT

Objective: To evaluate the phytochemical screening and anti-inflammatory activity of ethanolic leaf extract of *Centrosema pubescens* Benth.

Methodology: The ethanolic leaf extract of *C.pubescens*, a member of the *Fabaceae* family, was extracted using ethanol**.** The extract was analysed for secondary metabolites through phytochemical screening. The anti-inflammatory activity of the extract was assessed using in-vitro assays, including inhibition of protein denaturation assay, heat induced haemolysis and hypotonic solution induced haemolysis.

Results: The results of the study revealed that *C. pubescens* is a source of various phytoconstituents such as alkaloids, glycosides, saponins, tannins, proteins and amino acids, phenolic compounds, carbohydrates, cardiac glycosides, flavonoids, phytosterols and terpenoids. The ethanolic leaf extract

of *C. pubescens* exhibited significant anti-inflammatory potential by inhibiting protein denaturation, inhibiting heat induced haemolysis and inhibiting hypotonic solution induced haemolysis.

Conclusion: These findings suggest that *C.pubescens* possesses promising anti-inflammatory activity, supporting its potential application in the development of herbal-based pharmaceuticals for inflammation management. Further research is required to elucidate its molecular mechanisms and active phytoconstituents responsible for its therapeutic effects.

Keywords: *C.pubescens*; Anti-inflammatory; Inhibition of protein denaturation assay; Heat induced haemolysis; Hypotonic solution induced haemolysis

1. INTRODUCTION:

Inflammation is defined as the local response of living mammalian tissues to injury caused due to any agent. It is a protective response by the body to variety of etiological agents may be Infectious or Non–Infectious **[1]**.

There are three basic stages of inflammation: Vasodilatation and increased permeability of blood vessels, Phagocyte migration, and tissue repair. Signs of inflammation: rubor(redness), tumor(swelling), calor (heat), dolor (pain), function laesa (loss of function) **[2]**.

The understanding of inflammation as a fundamental response to injury dates back thousands of years. Aulus Celsus (~25 BC–AD 38), in his work De Medicina, provided the earliest comprehensive description of its symptoms: rubor (redness), tumor (swelling), calor (heat), and dolor (pain). Galen of Pergamon added the fifth sign, functio laesa (impaired function), approximately a century later **[3]**. The primary triggers of inflammation often include mechanical stressors such as blunt trauma **[4]**, the presence of foreign bodies **[5, 6]**, vibrations **[7, 8]**, and chronic low-intensity pressure **[9]**.

Anti-inflammatory medications are broadly categorized into steroidal and non-steroidal anti-inflammatory drugs (NSAIDs). Some of these agents are no longer in use due to severe side effects **[10]**.

For millennia, traditional practices have incorporated potent anti-inflammatory plants into diets and medicinal remedies. Secondary compounds isolated from these plants represent a significant reservoir of potential anti-inflammatory agents. While the plant kingdom offers an abundance of secondary metabolites **[11]**, only a limited number have undergone screening for biological activity **[12]**. Consequently, numerous plants await investigation for their therapeutic potential. *C. pubescens*, a notable medicinal plant belonging to the Fabaceae family, is one such example. It is recognized for its traditional use in treating human skin infections **[13]**, promoting wound healing, serving as an anesthetic for scorpion and snake bites **[14]**, and assisting in post-delivery uterine cleansing **[15]**.

It is believed that current drugs available such as opoids and non –steroidal anti-inflammatory drugs (NSAIDS) are not useful in all cases of inflammatory disorders, because of their side effects and potency.1 The screening and development of drugs for their anti-inflammatory activity is still in progress and there is hope for finding anti-inflammatory drugs from indigenous medicinal plants **[16]**.

This study aims to assess the anti-inflammatory properties of *C. pubescens* extract through in-vitro assays, including the inhibition of protein denaturation assay, heat-induced haemolysis, and hypotonic solution-induced haemolysis. This research seeks to provide insights into its therapeutic potential and contribute to the growing body of knowledge on plant-derived alternatives to conventional anti-inflammatory drugs.

2. MATERIALS AND METHODS:

2.1. Collection of Plant Materials

Leaves of *C. pubescens* was gathered from the roadside of Priyo Path, Ahomgaon, Guwahati, Kamrup Metro District, Assam, India (PIN-781035), during November and December 2024. The plant's identity was confirmed by the Department of Botany, Gauhati University, Guwahati, as *Centrosema pubescens* Benth, a member of the *Fabaceae* family. Authentication was documented under Reference No. Herb/GUBH/2024/115, dated December 12, 2024.

2.2. Approval from the animal ethical committee:

Approval for conducting the tests was obtained from The Assam Royal Global University's Institutional Ethical Committee (IEC), under approval number RGU/IECHR/BPHARM/2025/01.

2.3. Preparation of alcoholic Extract of *C.pubescens*

The fresh *C. pubescens* leaves, once collected, underwent a thorough washing with water before being air-dried in the shade for approximately ten days at an ambient temperature of 25∘C. Following drying, the leaves were pulverized into a coarse powder using a blender. This powdered material was then subjected to cold maceration, employing 99.9% ethanol as the extraction solvent. The extraction ratio was set at 1:6, where 40 g of the *C. pubescens* powder was immersed in 240 mL of ethanol for a period of 72 hours to ensure comprehensive extraction of leaf constituents. Subsequently, the crude extract was initially filtered through a sieve cloth, followed by refinement using vacuum filtration. The obtained filtrate was then concentrated and brought to complete dryness utilizing a rotary evaporator, succeeded by a water bath. This final extract was prepared for subsequent analyses, focusing on its phytochemical composition and potential anti-inflammatory properties **[17]**.

2.3. Preparation of human erythrocytes

Blood was collected via venipuncture from healthy individuals using heparinized tubes. The collected blood was then centrifuged at 3000 rpm for 10 minutes to separate components. Following centrifugation, the plasma and buffy coat layers were carefully removed. The remaining red blood cells (RBCs) were subsequently washed twice with phosphate-buffered saline (PBS, pH 7.4), with each wash involving centrifugation at 3000 rpm for 5 minutes. After the final wash, the packed RBCs were resuspended in PBS to achieve a 10% v/v RBC suspension, which was then used for the assay **[18]**.

2.4. Phytochemical Screening

An extensive phytochemical analysis was carried out on the alcoholic extract derived from *C. pubescens*. This screening aimed to ascertain the presence of various bioactive constituents, specifically including alkaloids, glycosides, saponins, tannins, proteins and amino acids, phenolic compounds, carbohydrates, cardiac glycosides, flavonoids, phytosterols, and terpenoids **[19]**.

2.4.1. Determination of Total Phenolic Content and Total Flavonoid Content

Total Phenolic Content

The total phenolic content within the ethanolic extract of *C. pubescens* was quantified utilizing the Folin-Ciocalteu colorimetric method **[20]**. To perform the assay, different concentrations of the extract (100–1000 µg/mL) or a standard solution of gallic acid (10–100 µg/mL) were added into separate 25 mL volumetric flasks containing 9 mL of distilled water. A reagent blank was alongside prepared by replacing the sample with distilled water. After that, 1 mL of Folin-Ciocalteu phenol reagent was added to each mixture and thoroughly mixed. 10 mL of a 7% aqueous sodium carbonate solution was incorporated after 5 mins. The final volume was made up to 25 mL using double-distilled water. The resulting solutions were port to incubate at room temperature for 90 minutes. Absorbance readings were later taken at 760 nm, standardized against the reagent blank. The concentration of total phenolics was determined by referencing the gallic acid standard curve. Results are presented as gallic acid equivalents (GAE), expressed in milligrams per gram of the *C. pubescens* extract.

Total Flavonoid Content

The total flavonoid content in the ethanolic extract of *C. pubescens* was determined using the aluminum chloride colorimetric method[20]. To perform this assay, Different concentrations of extract (10–100 µg/mL) or a standard catechin solution (10–100 µg/mL) was added into separate 10 mL volumetric flasks, each containing 4 mL of double-distilled water. 0.3 mL of a 5% sodium nitrite solution was added to each volumetric flask. Then 0.3 mL of a 10%

aluminium chloride solution was introduced to each after 5 mins interval followed by addition of 2 mL of a 1M sodium hydroxide solution was added after 6 mins. The total volume was made up to 10 mL with double-distilled water, and the contents were systematically mixed. Then we have taken Absorbance at 510 nm against a reagent blank. The total flavonoid content was then calculated and presented as milligrams of catechin equivalents (mg CE) per gram of the extract.

2.5. Protein Denaturation Inhibition Assay

Bovine serum albumin (BSA) was utilized as the protein model in this assay, with denaturation initiated by maintaining the reaction mixture at 70◦C in a water bath for 10 minutes. Each reaction mixture comprised 1000 µL of various concentrations of the plant extract (100-500 µg/mL), 450 µL of a 5% w/v aqueous BSA solution, and 1400 µL of phosphate-buffered saline. A negative control was prepared by substituting the plant extract with distilled water in the same mixture. Subsequently, all mixtures were initially incubated at 37◦C for 15 minutes, then heated to 70◦C for an additional 5 minutes. After cooling under running tap water, their absorbances were recorded at 660 nm. Diclofenac sodium served as the positive control. The percentage inhibition of protein denaturation was calculated using the formula **[21]**.

% Inhibition of denaturation= (1−D/ C) ×100

Where D represents the absorbance of the test sample, and C denotes the absorbance of the negative control (lacking the test sample or reference drug).

2.6. Heat-Induced Haemolysis

This method involves inducing haemolysis through heat incubation. The 2 mL reaction mixture consisted of 1 mL of the test sample at various concentrations (100–600 µg/mL) or 5 mL of an isotonic buffer containing 2.0 mg/mL of different extractives, combined with either 1 mL or 30 µL of a 10% erythrocyte suspension. For the control tube, only the vehicle (solvent) was added instead of the test sample. Diclofenac sodium, at concentrations ranging from 50–400 µg/mL, was used as the standard drug. Each reaction mixture was gently inverted to mix.

All centrifuge tubes containing the reaction mixtures were then incubated in a water bath at 60◦C, 56◦C, or 54◦C for either 30 or 20 minutes. Some researchers also prepared duplicate sets, with one set maintained at 0−5◦C in an ice bath. Upon completion of the incubation period, the tubes were cooled under running tap water. The reaction mixtures were subsequently centrifuged at 3000 rpm for 5 minutes, 2500 rpm for 10 minutes, or 1300 rpm for 3 minutes, and the absorbance of the supernatants was measured at 560 nm. The experiment was conducted in triplicates for all tested samples. The percentage inhibition of

haemolysis was determined using the following formula **[22]**.

% Inhibition of haemolysis= (Absorbance control −Absorbance test) ×100/Absorbance control

2.7. Hypotonic Solution-Induced Hemolysis

This assay was performed using a hypotonic solution to induce haemolysis. Various agents can serve as hypotonic solutions, including hyposaline (50 mM NaCl in 10 mM sodium phosphate buffer saline, pH 7.4) and distilled water. Each reaction mixture contained erythrocyte suspension, the plant extract, and the selected hypotonic solution. A control was prepared by omitting the plant extract. Diclofenac sodium was employed as the reference standard drug.

The mixtures were incubated at 37∘C for 30 minutes and then centrifuged at 3000 rpm for 20 minutes. Finally, the haemoglobin content in the supernatant solution was quantified spectrophotometrically at 560 nm. The percentage of red blood cell membrane stabilization or protection was calculated using the equation (Sarveswaran *et al*., 2017) **[22]**:

% Protection=100−(Optical density of drug treated sample×100)/Optical density of con trol

3. RESULTS

Initial phytochemical analyses of the *C. pubescens* ethanolic extract identified a range of phytochemicals, which are presented in **Table 1**.

Preliminary phytochemical screening of the *C. pubescens* ethanolic leaf extract revealed various compounds. Alkaloids, carbohydrates, glycosides, proteins and amino acids, flavonoids, phenolic compounds, tannins, phytosterols, and triterpenoids were detected. Reducing sugars and anthraquinones were absent.

Table 1a: Results of preliminary phytochemical investigation

Class of chemical constituents	Ethanolic leaf extract of *C.pubescens*
1. Alkaloids:	
Dragendorff's test	+
Hager's test	+
Mayer's test	+
Wagner's test	+
Picric acid test	+
2. Carbohydrates:	
Barfoed's test	-
Molish's test	+
Seliwanoff's test	+
Resorcinol test	+
3. Reducing sugars:	
Benedict's test	-
Fehling's test	-

4. Glycosides:	
Legal's test	+
Aqueous NaOH test	-
Keller-Killiani test	+
Test for cardenolides	+
Bromine water test	+
5. Proteins and Amino acids:	
Millon's test	+
Ninhydrin test	-
Xanthoproteic test	+
6. Flavonoids:	
Alkaline reagent test	+
Conc. H2SO4	-
Lead acetate test	+
Aqueous NaOH test	+
7. Phenolic compounds:	
Ferric chloride test	+
Iodine test	+
Gelatin test	+
8. Tannins:	+
Gelatin test	+
10% NaOH test	+
Bromine water test	
9. Phytosterols:	
Sulphur test	+
Hesse's response	+
10. Triterpinoids:	
Hesse's response	+
11. Anthraquinones:	
Ammonium hydroxide test	-

+ : Present, - : Absent

Total Phenolic Content and Total Flavonoid Content:

The ethanolic leaf extract of *C. pubescens* exhibited a total phenolic content of 41.37±0.01 mg of Gallic Acid Equivalents per gram of extract (GAE/gm extract). The total flavonoid content was determined to be 7.812±0.372 mg of Catechin Equivalents per gram of extract (CE/gm extract) **(Table 1b)**.

Table 1b: Total Phenolic Content and Total Flavonoid Content

Ethanolic leaf extract of C.pubescens	Total phenolic content (mg of GAE/gm of extract)	Total flavonoid content (mg of CE/gm of extract)
	41.37 ± 0.01	7.812 ± 0.372

3.1. Inhibition of Protein Denaturation Assay:

As depicted in **Table 2**, the ethanolic extract of *C. pubescens* demonstrated a significant, dose-dependent inhibition of protein denaturation. At a concentration of 100 µg/mL, the extract showed 28.69% inhibition, which progressively increased to 94.48% at 500 µg/mL. Notably, at higher concentrations (400 µg/mL and 500 µg/mL), the extract's inhibitory effect (90.91% and 94.48% respectively) was comparable to, or even slightly surpassed, that of the standard drug, diclofenac sodium (88.45% and 93.98% respectively). Diclofenac sodium consistently exhibited strong inhibition across all tested concentrations, starting at 49.68% at 100 µg/mL.

Table 2: Percentage inhibition of protein denaturation by ethanolic extract of *C. pubescens* and Diclofenac sodium.

Concentration (µg/ml)	% inhibition (extract)	% inhibition (Diclofenac sodium)
100	28.69	49.68
200	54.78	61.92
300	65.51	74.12
400	90.91	88.45
500	94.48	93.98

3.2. Heat Induced Hemoysis:

As presented in **Table 3**, the *C. pubescens* extract demonstrated a dose-dependent inhibition of heat-induced hemolysis, with diclofenac sodium serving as the positive control. The extract's protective effect increased from 48.61% at 100 µg/mL to 58.29% at 500 µg/mL. Notably, the *C. pubescens* extract consistently exhibited higher inhibition percentages compared to diclofenac sodium across all tested concentrations. For example, at 500 µg/mL, the extract achieved 58.29% inhibition, whereas diclofenac sodium reached 47.24%.

Table 3: Percentage inhibition of heat induced haemolysis by ethanolic extract of *C. pubescens* and Diclofenac sodium

Concentration (µg/ml)	% inhibition of haemolysis (extract)	% inhibition of haemolysis (Diclofenac sodium)
100	48.61	24.03
200	48.74	32.98
300	56.13	36.61
400	56.23	46.84
500	58.29	47.24

3.3. Hypotonic Solution Induced Haemolysis:

As detailed in **Table 4**, the *C. pubescens* extract demonstrated a dose-dependent protective effect against hypotonic solution-induced hemolysis, with diclofenac sodium serving as the positive control. The extract's protection increased from 24.71% at 100 µg/mL to 28.13% at 500 µg/mL. While the extract showed consistent dose-dependency, its overall protective efficacy was considerably lower than that of diclofenac sodium. For instance, at 500 µg/mL, the extract provided 28.13% protection, whereas diclofenac sodium achieved a significantly higher 81.68% protection.

Table 4: Percentage protection of hypotonic solution induced haemolysis by ethanolic extract of *C. pubescens* and Diclofenac sodium

Concentration (µg/ml)	% protection (extract)	% protection (Diclofenac sodium)
100	24.71	46.65
200	27.35	51.68
300	27.59	63.02
400	27.97	69.48
500	28.13	81.68

4. DISCUSSION:

Preliminary phytochemical screening serves as a fundamental approach for identifying bioactive plant constituents and plays a significant role in the exploration and development of novel therapeutic agents. In addition, these screening methods support both the qualitative separation and quantitative estimation of pharmacologically important phytochemical compounds **[23]**. Earlier studies on *C. pubescens* have consistently documented the presence of diverse secondary metabolites, including tannins, flavonoids, saponins, terpenoids, steroids, cardiac glycosides, alkaloids, phenolic compounds, and **[24-29]**. These phytoconstituents are widely recognized for their broad spectrum of biological activities, such as antioxidant, antimicrobial, anti-inflammatory, cancer-preventive, antidiabetic, and antihypertensive properties **[30]**.

The current investigation offers insights into the anti-inflammatory potential of the ethanolic extract of *Centrosema pubescens* leaves, a property strongly supported by its rich phytochemical composition. Our preliminary phytochemical screening confirmed the presence of diverse bioactive compounds, including alkaloids, flavonoids, tannins, glycosides, cardiac glycosides, terpenoids, proteins and amino acids, phytosterols, carbohydrates, and phenolic compounds. These identified phytochemicals are widely recognized for their therapeutic roles, particularly their capacity to modulate inflammatory pathways **[31]**.

One of the documented causes of inflammatory disease is denaturation of tissue proteins. Agents that can prevent protein denaturation therefore, would be worthwhile for anti-inflammatory drug development **[32]**.

The anti-inflammatory efficacy was evaluated using three *in vitro* assays: inhibition of protein denaturation, heat-induced hemolysis, and hypotonic solution-induced hemolysis **[22]**. Each of these models simulates distinct facets of inflammatory processes, thereby providing a comprehensive analysis of the extract's anti-inflammatory capabilities.

Protein denaturation leads to the loss of a protein's biological properties and has been linked to the development of inflammatory conditions such as rheumatoid arthritis, diabetes, and cancer. Consequently, compounds that can inhibit protein

denaturation may contribute to the prevention of inflammatory disorders **[23]**. Indeed, protein denaturation is a well-established contributor to inflammation **[33]**, and substances capable of preventing this process are considered to possess anti-inflammatory attributes **[34]**. In the protein denaturation assay, our results indicated that the extract exhibited a significant, dose-dependent inhibition of protein denaturation, reaching 94.48% inhibition at 500 µg/mL. This effect was highly comparable to that of the standard drug, diclofenac sodium, which showed 93.98% inhibition. These findings suggest that the *C. pubescens* extract contains bioactive constituents that can stabilize proteins under stressful conditions, thereby mitigating their denaturation.

During inflammatory responses, the lysis of lysosomal membranes can occur, releasing enzymes that contribute to various pathological disorders. Similarly, injurious substances, such as hypotonic media, heat, methyl salicylate, and phenyl hydrazine, can cause the lysis of red blood cell membranes, leading to hemolysis and hemoglobin oxidation **[35, 36]**. Human red blood cell (HRBC) membranes are often used in anti-inflammatory studies due to their structural resemblance to lysosomal membranes **[37]**. The ability of a substance to prevent red blood cell lysis under hypotonic and heat-induced stress serves as an important indicator of its potential anti-inflammatory activity **[38]**.

In the heat-induced hemolysis assay conducted in this study, the ethanolic leaf extract of Centrosema pubescens demonstrated a notable protective effect on erythrocyte membranes. The membrane stabilization increased in a concentration-dependent manner, with inhibition percentages rising from 48.61% at 100 µg/mL to 58.29% at 500 µg/mL. Interestingly, the extract consistently outperformed diclofenac sodium, the standard reference drug, in this thermal stress model, indicating its strong ability to protect red blood cells from heat-induced damage.

The extract's effectiveness was also evaluated in a hypotonicity-induced hemolysis model. While the level of protection at 500 µg/mL was moderate (28.13%) compared to diclofenac sodium (81.68% at the same concentration), the extract still exhibited a dose-dependent protective effect. This suggests that although the membrane-stabilizing capacity of the extract under hypotonic conditions is less potent than that of the standard drug, it is nonetheless significant and may contribute to its overall anti-inflammatory profile.

The observed effects can likely be attributed to the presence of bioactive compounds such as flavonoids, tannins, and phenolic

compounds, which are known for their anti-inflammatory properties **[31]**. These findings are in line with previous research indicating the anti-inflammatory potential of plant-derived polyphenols in similar in vitro models **[22]**. Additionally, the traditional application of *C. pubescens* in treating skin inflammation and related conditions supports these laboratory findings **[13]**. However, while these in vitro results are promising, further validation through in vivo studies and mechanistic analyses is necessary to fully establish the therapeutic potential of the extract.

5. CONCLUSION:

This research confirms the anti-inflammatory capabilities of the *Centrosema pubescens* ethanolic leaf extract, underscoring its importance in traditional medicine. The extract effectively inhibited protein denaturation and stabilized cell membranes, showing efficacy comparable to conventional non-steroidal anti-inflammatory drugs (NSAIDs). These results establish a basis for further investigation, including the isolation of specific phytochemicals, *in vivo* studies, and the development of formulations for new, safe, plant-derived anti-inflammatory medications.

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