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Original Article

Unveiling the Anti-Inflammatory Potential of *Adhatoda vasica* (L) Root: An *in vitro, in vivo*, and *in silico* Correlation Study

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ABSTRACT

Objectives: The present study aimed to scientifically validate the traditional use of *Adhatoda vasica* root for inflammation management by evaluating the anti-inflammatory activity of its aqueous (AQERAV) and Ethanolic (EERAV) extracts through both *in vitro* and *in vivo* approaches**. Materials and Methods:** Root extracts were prepared using Soxhlet extraction with water and ethanol as solvents. The *in vitro* anti-inflammatory potential was assessed via the Bovine Serum Albumin (BSA) protein denaturation method. Acute toxicity studies were conducted in Swiss albino mice following OECD guidelines to determine safe dosage levels. *In vivo* anti-inflammatory activity was evaluated using the carrageenan-induced rat paw oedema model. Additionally, molecular docking studies were performed using PyRx with the AutoDock Vina algorithm to explore interactions of selected phytoconstituents with Cyclooxygenase Enzymes (COX-1 and COX-2). Toxicity predictions for ligands were conducted using the ProTox-III platform. **Results:** Toxicological assessments revealed that oral administration of both AQERAV and EERAV at 2000 mg/kg produced no mortality, confirming their safety. In the *in vitro* assay, AQERAV exhibited significant anti-inflammatory activity with an IC_{50} value of 28.82 µg/mL ($p<0.001$), comparable to the standard drug diclofenac. *In vivo* analysis showed a dose-dependent inhibition of paw oedema for both extracts, with AQERAV demonstrating statistically significant effects even at lower doses. EERAV at 100, 200, and 400 mg/kg resulted in oedema volumes of 0.435±0.039, 0.339±0.03, and 0.226±0.047 mL, respectively, at 1 hr post-administration. Docking studies revealed strong binding affinities of key phytoconstituents to COX-1, with scores ranging from -8.8 to -5.1 kcal/mol. **Conclusion:** The study provides strong pharmacological evidence for the anti-inflammatory potential of *Adhatoda vasica* root extracts. The combination of *in vitro, in vivo*, and *in silico* analyses suggests that especially the aqueous extract contains bioactive molecules capable of modulating inflammatory pathways, thereby reinforcing the plant's traditional use and highlighting its potential as a safer, natural alternative to synthetic anti-inflammatory agents.

Keywords: *Adhatoda vasica*, Root Extract, Docking, Anti-Inflammatory Activity, Acute Toxicity.

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INTRODUCTION

Inflammation is defined as the complex biological response when the body's immune system is exposed to harmful stimuli such as pathogens, damaged cells, toxic compounds or irradiation. It is an initiator of healing as it serves as a protective mechanism by removing the injurious stimuli. Activation of different immune cells, pro-inflammatory cytokines like IL-1β, TNF-α, release of reactive oxygen species, prostaglandins by Cyclooxygenase (COX) pathways are a result of well-coordinated cascade of molecular and cellular events (Medzhitov, 2008). However, diseases like arthritis, atherosclerosis, diabetes, cancer and various neurodegenerative disorders are contributed by chronic and uncontrolled inflammation (Chen *et al.*, 2018). The ongoing pharmacological management of inflammation generally includes Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) and corticosteroids. Despite their efficacy, these synthetic drugs often produce adverse effects like gastrointestinal irritation, ulceration, hepatotoxicity, renal dysfunction and cardiovascular risks (Vane and Botting, 1998). NSAIDs works by inhibiting COX-1 and COX-2 enzymes, which are responsible for prostaglandin synthesis. Although the inhibition of COX-1 enzyme alters the gastric mucosal protection,

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which requires the co-administration of antacids or proton pump inhibitors to decrease the gastrointestinal side effects (Laine, 2001). This safety concern gives rise to the exploration of safer alternatives of medicines from natural sources. Historically, it was proven that medicinal plants served as rich sources of bioactive compounds possessing anti-inflammatory properties and are known to have fewer side effects than the modern system of medicine. Phytocommicals like flavonoids, alkaloids, saponins and tannins exhibit their efficacy through various mechanistic pathways, likely by inhibiting COX and LOX (lipoxygenase), suppression of cytokine release and by their antioxidant properties (Calixto *et al*., 2004). In the present study, *Adhatoda vasica* Nees (commonly known as Vasaka or Malabar Nut), belonging to the family Acanthaceae, has drawn significant attention because of its diverse usage. In Ayurvedic and Unani medicine, this plant has been used widely for respiratory ailments. This plant is rich in alkaloids like vasicine and vasicinone, potential constituents that have bronchodilatory and anti-inflammatory effects (Dhuley, 1999). *Adhatoda vasica* Nees (syn. *Justicia adhatoda*), commonly known as Vasaka or Malabar nut, is a well-known medicinal plant extensively used in the Indian traditional system of medicine, particularly in Ayurveda, Siddha, and Unani. This evergreen shrub, belonging to the Acanthaceae family, is widely distributed across Southeast Asia and has been traditionally used to treat respiratory ailments such as asthma, bronchitis, and cough due to its bronchodilator and expectorant properties (Chakraborty *et al*., 2010). While the leaves of *A. vasica* have been predominantly studied for their pharmacological activities, including antitussive, anti-asthmatic, anti-bacterial, and anti-oxidant properties (Burman and Nayak, 2025; Ignacimuthu and Shanmugam, 2010), the therapeutic potential of its roots remain comparatively underexplored. Previous studies on different parts of the plant have demonstrated a broad spectrum of pharmacological activities. For instance, the leaf extract of A. vasica has shown significant anti-tubercular (Saeed *et al*., 2007), anti-ulcer (Gupta and Sharma, 2011) and hepatoprotective activities (Nadkarni, 2002). The flowers and bark have also been reported to possess antimicrobial and antioxidant effects (Venu *et al*., 2018). Despite this wide range of documented biological activities, very limited studies have focused specifically on the root, particularly in the context of inflammation. Therefore, this study bridges a crucial gap in existing literature and supports the traditional use of *A. vasica* in inflammatory conditions, opening new avenues for its therapeutic applications.

Therefore, in continuation with our previous work (Mondal *et al*., 2018) about the Nootropic activity of Ethanolic and Aqueous Root Extracts of *A. vasica*, the present study investigates the anti-inflammatory activity of aqueous and ethanolic extracts of *A. vasica* root using both *in vitro* and *in vivo* models. The rationale is to validate the traditional claims and scientifically evaluate the potential of *A. vasica* root as a safer, natural alternative for inflammation management. In the present study, both *in vitro* and *in vivo* investigations were undertaken to evaluate the anti-inflammatory activity of aqueous and ethanolic root extracts of *A. vasica*. The rationale behind focusing on the root extract stems from preliminary phytochemical evidence suggesting the presence of bioactive alkaloids, flavonoids, and polyphenols that could mediate anti-inflammatory effects. The *in vitro* assay was carried out using protein denaturation inhibition and membrane stabilization methods, while the *in vivo* evaluation involved carrageenan-induced paw edema in Wistar rats. Results from both studies indicated significant anti-inflammatory activity, suggesting the root extracts-especially the ethanolic one-possess potent bioactive constituents capable of modulating inflammatory pathways.

MATERIALS AND METHODS

Plant Material

A. vasica needs was purchased in the month of June from the Alva Pharmacy, Mangalore, and was dried in the shade at room temperature, then subjected to size reduction to a fine powder with the help of a mixer grinder. The figure of the plant and root of *A. vasica is* shown in Figure 1.

Preparation of ethanolic extract

The root powder (750 g) was packed in a Soxhlet apparatus and extracted (Mondal *et al*., 2018) with 1 L of ethanol (95%) for 18 hr at>78°C. Appearance of colourless solvent in the siphon tube was taken as the termination of extraction. The extract was then transferred into a previously weighed empty beaker and evaporated to a thick paste on the water bath, maintained at <50°C. The ethanolic extract of the root of *A. vasica* (EERAV) appeared dark brown and amorphous in nature with a percentage yield of 1%.

Preparation of aqueous extract

About 100 g of root powder was taken in a round-bottom flask (2000 mL) and macerated with 500 mL of distilled water for 24 hr with occasional shaking in a closed vessel. 10 mL of chloroform was added as a preservative. Then the marc was removed by filtering the extract and then concentrated in a water bath maintained at 50°C. The extract was finally dried thoroughly to remove all traces of the solvent. The aqueous extract of the root of *A. vasica* (AQERAV) appeared dark brown, sticky in nature, with a percentage yield of 1%. The two extracts were examined for their color and consistency. Their percentage yield was calculated with reference to air, air-dried sample used for extraction (Mondal *et al*., 2025), then stored in an air-tight container in a refrigerator below -4°C.

In vitro anti-inflammatory study

Serial dilution from 1000 µg/mL to 10 µg/mL was performed for both AQERAV and EERAV and for reference drug. All samples

contained 5.0 mL of total volume. Reaction mixtures were prepared using 1.2 mL of phosphate-buffered saline (pH 6.4) and 0.8 mL of Bovine Serum Albumin (BSA). Then 2 mL from each different concentration AQERAV and EERAV solution were mixed gently with reaction mixtures. A similar procedure was used for reference drugs, and they were used as positive controls for this study. Each concentration was evaluated in UV at 660 nm.

Each reaction mixture was incubated (Kola *et al.*, 2019) in a water bath at 37°C±2°C for 20 min, and later, it was heated at 60°C at which the reaction mixture was maintained for 15 min. Then, the reaction mixture was allowed to cool down at room temperature for 15 min. Absorbance of the reaction mixture before and after denaturation was measured for each concentration at 660 nm using a colorimeter. Each test was repeated thrice, and the mean absorbance was recorded. The percentage of inhibition of protein was determined on a percentage basis with respect to the control using the following formula:

$$\%\ Inhibition = \left(\frac{Absorbance of Control - Absorbance of Test}{Absorbance of Control}\right) \times 100$$

In silico studies

Ligands preparation and optimization

Fourteen phytoconstituents previously identified in the root of *A. vasica* were selected based on a comprehensive review of the literature. Their two-dimensional chemical structures were constructed using ChemDraw Professional 8.0. Subsequently, the three-dimensional conformations were generated and energy-minimized using Open Babel, and the resulting files were saved in Structure Data File (SDF) format for downstream ligand preparation and molecular docking studies (Nonglang *et al.*, 2024).

Drug-like properties of the ligands

The pharmacokinetic and physicochemical suitability of all ligand candidates was assessed using parameters such as aqueous solubility (LogS), lipophilicity (LogP), Lipinski's rule of five, and predicted oral bioavailability scores. Drug-likeness was further evaluated by analyzing key molecular descriptors, including Molecular Weight (MW), Hydrogen Bond Donors (HBD), Hydrogen Bond Acceptors (HBA), LogP, and LogS. These descriptors were computed using the SWISSADME platform (http://www.swissadme.ch/). For molecular docking studies targeting inflammation, the three-dimensional crystal structures of Cyclooxygenase-1 (COX-1; PDB ID: 6Y3C) and Cyclooxygenase-2 (COX-2; PDB ID: 5KIR) were retrieved from the Protein Data Bank. Prior to docking, water molecules were removed from the protein structures, and polar hydrogens were added to optimize ionization states of amino acid residues using BIOVIA Discovery Studio 2022 Client software (Palei *et al.*, 2025).

Molecular docking analyses and visualization

The protein structures, obtained in PDB format, were imported into the PyRx virtual screening tool, where docking simulations were conducted using the AutoDock Vina algorithm. A grid box with dimensions of 54.68 Å × 65.23 Å × 56.91 Å was defined to encompass the active sites. To identify the most reliable binding conformations, energy minimization and scoring were performed within PyRx. Subsequently, molecular interactions such as hydrogen bonds, hydrophobic contacts, and π-π stacking were analyzed and visualized using Discovery Studio 2021 Client, providing insight into the binding affinities and interaction profiles of the ligand-protein complexes (Samajdar and Kumar, 2023).

Toxicity prediction

The potential toxicity of all the ligands in human systems was evaluated using the ProTox-III online platform (https://tox-new.charite.de/protox_III/). This computational tool predicts toxicological endpoints across 14 distinct models, utilizing the compound's two-dimensional chemical structure as input. The platform generates toxicity profiles with associated confidence scores, enabling early-stage assessment of safety parameters during drug discovery (Samajdar and Mondal, 2023).

In vivo studies

Acute toxicity study

The acute toxicity experiments were performed on Swiss albino mice weighing 20-25 g, for the acute toxicity examination as per the guidelines of the Organization for Economic Cooperation and Development (OECD) (Mondal *et al.*, 2010). The selected male and female mice were then assigned to standard control and remedy groups (5/sex/ group). The research group rats obtained the suspension of AQERAV and EERAV using 0.5% CMC, once orally as a test sample at doses of 1000, 1500, and 2000 mg/kg body weight, which was prepared by suspending the compounds in 0.5% CMC solutions and blended thoroughly. CMC solution (0.5% v/v) was obtained as a vehicle for the control group animals. All the animals were weighed before the experiment started, marked for identification, and fasted overnight, but were given free access to water. After dosing, the animals fasted further for 4 hr, and for any mortality and irregular changes, observations were reported continuously for each individual mouse in their respective groups during the first 4 hr, and then, they were kept under observation up to 14 days.

In vivo Anti-inflammatory activity

Wistar rats weighing about 120-150 g were used for the anti-inflammatory study (Awady *et al.*, 2024; Khandelwal *et al.*, 2024). The animals were housed in a colony cage that had a 12-hr light and dark cycle, a temperature of 25±2°C, a relative humidity of 45 to 55%, and unrestricted access to water and standard

animal feed. The animal house where the animals were housed under normal circumstances was approved by the Committee for the Control and Supervision of Animal Experiments (CCSEA).

The Institutional Animal Ethics Committee of the School of Pharmacy, GITAM University, approved the experimental protocol (Approval number IAEC/GU-1287/SM-F/2/August 2024). A week was spent acclimating each animal before use.

Both AQERAV and EERAV were tested for their anti-inflammatory qualities using rat paw oedema. The first step of inflammation is oedema, and carrageenin-induced paw oedema is the quickest and most often used method for testing the anti-inflammatory property. This method is based on the plethysmography evaluation of acute carrageenan-induced rat paw oedema. In this study, ten groups of five Wister rats each, weighing between 110 and 150 g and of either sex, were employed. Group 1 received an oral dose of 0.5% carboxymethyl cellulose in ordinary saline as a solvent control. Group 2 was provided with diclofenac (10 mg/kg) in solvent. Both extracts were administered orally to groups 3-10 at a dose of 100 mg/kg. 1 hr before the carrageenan injection, these extracts were given.

After an hour, 0.1 mL of a carrageenan suspension in CMC solution was subcutaneously injected into the subplantar region of each animal's left hind paw. The volume of the paw was measured right away. Paw volumes were recorded for a maximum of 3 hr in the control, standard, and test groups. The percentage of paw volume inhibition was calculated. Using the formula:

% inhibition=100 x (1-(Mean paw volume of drug-treated group - Initial mean paw volume)/(Mean paw volume of control group -initial mean paw volume).

RESULTS

Extraction and Phytochemical Study

The aqueous (AQERAV) and ethanolic (EERAV) root extracts of *A. vasica* were obtained using the Soxhlet extraction method. Both extracts appeared dark brown and were amorphous in nature. The extracts were properly stored in airtight containers after concentration. The percentage yield of the ethanolic extract (EERAV) was calculated to be 1%. Yield of the aqueous extract was noted separately for comparison in subsequent analysis. Further studies were performed using both the aqueous (AQERAV) and ethanolic extract (EERAV) of *A. vasica* roots.

After a preliminary phytochemical screening (Table 1). Both extracts had high concentrations of alkaloids and flavonoids, which suggests that they are abundant and soluble in polar and semi-polar solvents. The ethanolic extract included more tannins and phenols than the aqueous one. While only weakly found in the ethanolic extract, saponins were abundant in the aqueous extract. Moderate levels of proteins, carbohydrates, and glycosides were detected in both extracts. Notably, terpenoids and steroids were absent in the aqueous extract but present in the ethanolic one, suggesting the role of ethanol in extracting less polar constituents. The variation in phytochemical presence is attributed to the polarity of the solvents used. Overall, the ethanolic extract exhibited a broader spectrum of phytoconstituents. This study supports the medicinal potential of both extracts, especially the ethanolic one, for further pharmacological evaluation (Gulfraz *et al.*, 2011).

Table 1: Preliminary Phytochemical Screening of Aqueous and Ethanolic Root Extracts of *A. vasica*.

Phytoconstituents	AQERAV	EERAV
Alkaloids	++ (Present)	++ (Present)
Flavonoids	++ (Present)	++ (Present)
Tannins	+ (Present)	++ (Present)
Glycosides	+ (Present)	+ (Present)
Saponins	++ (Present)	+ (Present)
Phenols	+ (Present)	++ (Present)
Carbohydrates	+ (Present)	+ (Present)
Proteins	+ (Present)	+ (Present)
Terpenoids	- (Absent)	+ (Present)
Steroids	- (Absent)	+ (Present)

Table 2: *In Vitro* anti-inflammatory activities of aqueous (AQERAV) and ethanolic extract (EERAV) of *A. vasica* roots.

Treatment	Concentration (mg/mL)	% Inhibition	IC_{50} value±SEM
Diclofenac	100	122.25	26.39±1.33
	200	138.87	
	400	228.53	
EERAV	100	62.35	29.54±1.02**
	200	131.57	
	400	210.44	
AQERAV	100	109.39	28.82±1.72***
	200	157.33	
	400	239.50	

Values are expressed as Mean±SE (n=6). *p<0.05, **p<0.01, and ***p<0.001 compared with vehicle control (ANOVA followed by Dunnett's t test).

In vitro anti-inflammatory activity

In vitro anti-inflammatory study of the aqueous and alcoholic extracts was carried out using the Bovine Serum Albumin (BSA) protein denaturation method. Inhibiting protein denaturation is a potential way to assess anti-inflammatory effectiveness (Vogl *et al.*, 2013). Almost all the synthesized compounds showed anti-inflammatory activity. When compared with the standard diclofenac, the extract AQERAV, $p<0.001$, with the IC_{50} values of 28.82, which is notably strong when compared with the standard value of 26.39 (IC_{50}), but not exceeding the standard value. The extract EERAV also showed good anti-inflammatory activity. Both compounds were found to be statistically significant. The anti-inflammatory efficacy of both extracts was comparable to that of the standard drug diclofenac (100 mg/kg). EERAV demonstrated significant inhibition primarily at higher doses, suggesting a concentration-dependent effect. Overall, AQERAV displayed consistent and significant anti-inflammatory activity across all dose levels. These findings support the therapeutic potential of *A. vasica* root extracts in managing acute inflammation. The results are displayed in Table 2 and Figure 2.

Figure 1: Roots and Plant of *A. Vasica.*

Molecular docking studies

The molecular docking study was conducted to evaluate the binding affinities and interaction profiles of phytoconstituents derived from *A. vasica* against Cyclooxygenase-1 (COX-1; PDB ID: 6Y3C) and Cyclooxygenase-2 (COX-2; PDB ID: 5KIR) receptor proteins using the PyRx virtual screening

Table 3: Docking score of Vasaka ligands.

Ligands	Binding Affinity (cΔG in kcal/mol)	
	6Y3C	5KIR
Peganine	-7.7	-7.7
Vasicinone	-7.5	-7.3
Anisotine	-7.6	-6.9
9-acetamido-3,4-dihydropyrido-(3,4-b)-indole	-7.7	-8.1
Deoxyvasicinone	-7.0	-7.1
Vasicinolone	-8.0	-7.9
b- Sitosterol	-6.5	-7.4
4-Hydroxychalcone	-8.0	-7.7
Ethyl a-D-Glucoside	-8.0	-7.1
beta-Sitosterol-beta-D-glucoside	-8.2	-7.4
D-glucoside	-8.8	-8.6
Triacontane	-6.5	-6.1
N-Benzylacetamide	-5.1	-5.4
Diclofenac	-7.3	-7.7

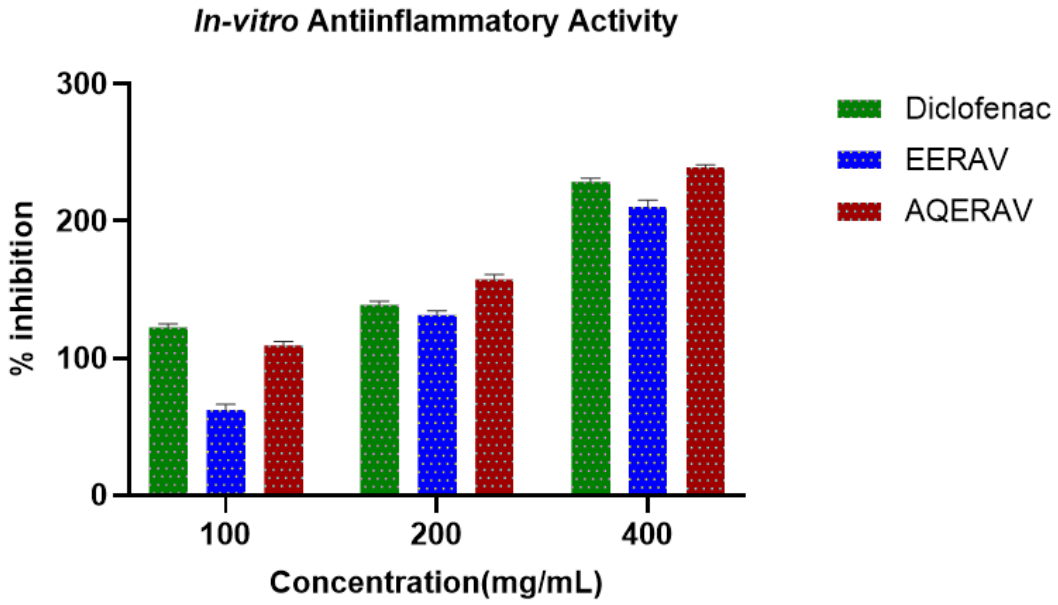

Figure 2: *In vitro* anti-inflammatory activity of AQERAV and EERAV.

tool. The binding energies of these phytochemicals were compared to those of Diclofenac, a widely used Non-Steroidal Anti-Inflammatory Drug (NSAID), to assess their potential as alternative anti-inflammatory agents. As presented in Table 1, the docking results demonstrated that *A. vasica* compounds exhibited significant binding affinities within the range of -8.8 to -5.1 kcal/mol for COX-1, with D-Glucoside showing the highest affinity (-8.8 kcal/mol), followed by β-sitosterol-β-D-glucoside. In the case of COX-2 (5KIR), the strongest interaction was observed for D-Glucoside (-8.6 kcal/mol), followed by 9-acetamido-3,4-dihydropyrido[3,4-b] indole (-8.1 kcal/mol). These values were notably more favorable than those of Diclofenac, which showed binding affinities of -7.3 kcal/mol and -7.7 kcal/mol for COX-1 and COX-2, respectively. Molecular interaction analysis, visualized using Discovery Studio 2021 Client, revealed stable and specific interactions between the top ligands and key amino acid residues within the active sites of both enzymes (Figures 3 and 4). These findings suggest that selected phytochemicals from *A. vasica* possess promising inhibitory potential against COX enzymes, supporting their role as natural anti-inflammatory agents. The results are shown in Table 3. Furthermore, *in silico* ADME and toxicity assessments were performed to evaluate the pharmacokinetic and safety profiles of these compounds (Gulfraz *et al.*, 2011).

ADMET studies

The pharmacokinetic and physicochemical properties of the selected ligands were assessed using Swiss ADME (Table 4A). The compounds exhibited a broad lipophilicity spectrum, with molecular weights ranging from 149.19 to 705.92 g/mol and Log P values between 1.3 and 8.69, indicating favorable membrane permeability and solubility in nonpolar environments such as lipids and oils with high drug likeness for all compounds except Triaconitane. Notably, almost all ligands adhered to Lipinski's Rule of Five, with no more than a single violation, and conformed to Ghosh's criteria for drug-likeness, underscoring their potential as orally bioavailable therapeutic agents. Toxicological profiling (Table 4B) via ProTox-III revealed that all tested compounds, including the lead molecule D-Glucoside, fell within toxicity classes 3 to 5, with predicted LD_{50} values spanning from 290 to 23,000 mg/kg. These findings suggest a favorable safety margin, supporting the suitability of these ligands for further preclinical development (Vogl *et al.*, 2013).

Acute toxicity study

In an acute toxicity study, oral administrations of the AQERAV and EERAV at 2000 mg/kg, p.o., did not produce any deaths and clinical signs of toxicity in mice. The dose induced sedation and mild diuresis with purgation at all tested dose levels was observed. There was no significant difference in body weights between the control and treatment groups. Food and water intake showed daily fluctuations within the range of control animals, which indicates that the dose was safe to a single dose of 2000 mg/kg body weight, and it indicates that the median lethal dose is higher than the tested dose level. The experimental dose was selected between the minimum effective dose and maximal non-lethal dose, i.e, 400 mg/kg (one-fifth), 200 (one 10^{th}), and 100 mg/kg (one 20^{th}) of the preceding dose p.o. The acute toxicity study revealed that oral administration of AQERAV and EERAV at 2000 mg/kg did not induce mortality or significant clinical toxicity in mice. Mild

Table 4A: ADME parameters of each ligand in Swiss ADME.

Ligands	Mol Wt. (g)	Log P	HBD	HBA	Violation	BB barrier Yes/No	GI Absorption	Log S
Peganine	188.23	1.64	1	2	0	No	High	-0.76
Vasicinone	349.43	3.37	0	3	0	Yes	High	-3.27
Anisotine	349.38	3.21	1	4	0	Yes	High	-4.22
9-acetamido-3,4-dihydropyrido-(3,4-b)-indole	227.26	1.74	1	2	0	Yes	High	-1.93
Deoxyvasicinone	186.21	2.09	0	2	0	Yes	High	-1.39
Vasicinolone	218.21	1.53	2	4	0	No	High	-1.16
b- Sitosterol	414.71	5.05	1	1	1	No	Low	-9.67
4-Hydroxychalcone	224.25	2.24	1	2	0	Yes	High	-3.16
Ethyl a-D-Glucoside	208.21	1.3	4	6	0	No	High	0.73
beta-Sitosterol-beta-D-glucoside	576.85	5.22	4	6	1	No	Low	-9.67
D-glucoside	705.92	4.39	5	11	1	No	Low	-7.69
Triacontane	464.89	8.69	0	0	1	No	Low	-17.7
N-Benzylacetamide	149.19	1.74	1	1	0	Yes	High	-1.15

sedation, diuresis, and purgation were observed but without adverse effects on body weight or feeding behavior. These findings suggest that both extracts are safe at the tested dose, with the median lethal dose (LD_{50}) exceeding 2000 mg/kg. The absence of severe toxicity supports the use of 100, 200, and 400 mg/kg as experimental doses. This selection aligns with standard safety margins in preclinical evaluations.

In vivo anti-inflammatory activity

The *In vivo* carragennen-induced rat paw edema model, which was carried out for both AQERAV and EERAV, was found effective. The percentage inhibition, in case of EERAV at the doses of 100, 200 and 400mg/kg was found to be 63.54, 69.11 and 79.68 at 1 hr, and at 3 hr, the percentage inhibition was 73.79, 72.58, and 85.81. Whereas the percentage inhibition, in case of AQERAV at the doses of 100, 200, and 400 mg/kg was found 82.53, 86.86, and 88.06 for 1 hr, and for 3 hr, the percentage inhibition was 86.06, 88.37, and 91.61, as shown in Table 5, and he percentage inhibition at the lower doses is found to be less and is not found to be significant in the case of EERAV, whereas at higher doses, it showed better anti-inflammatory potential. The anti-inflammatory potential of AQERAV for all types of dosages was found significant, when compared with the standard diclofenac (% Inhibition 0.215 mL±0.043) at 100mg/kg, at 1 hr, and % inhibition 0.234 mL±0.035mg/kg at 3 hr, shown in Figure 5. The results suggest that both extracts possess bioactive constituents capable of mitigating protein denaturation, a marker of inflammation. Statistical analysis confirmed the significance of both extracts' effects ($p<0.05$). These findings support the traditional use of the plant in inflammatory conditions and warrant further mechanistic investigations (Basit *et al.*, 2022).

Taken together, the correlation between the *in vitro* and *in vivo* models reinforces the potential of AQERAV as a more efficacious anti-inflammatory agent than EERAV. These findings support the pharmacological relevance of the extract's phytoconstituents in modulating key inflammatory pathways and warrant further phytochemical characterization and mechanistic exploration.

Table 4B: Toxicity prediction of ligands.

Ligands	Level of Toxicity (1=highly toxic; 6=safe)	Predicted LD_{50} (µg/mL)
Peganine	3	290
Vasicinone	4	1100
Anisotine	4	1100
9-acetamido-3,4-dihydropyrido-(3,4-b)-indole	4	445
Deoxyvasicinone	4	1100
Vasicinolone	4	1250
b- Sitosterol	4	890
4-Hydroxychalcone	4	1048
Ethyl a-D-Glucoside	6	23000
beta-Sitosterol-beta-D-glucoside	6	8000
D-glucoside	4	1230
Triacontane	3	750
N-Benzylacetamide	4	900

Table 5: *In vivo* anti-inflammatory activities of aqueous (AQERAV) and ethanolic extract (EERAV) of *A. vasica* roots.

Treatment	Dose/kg mg/kg	Group	Volume of Mercury Displaced (mL) (% Inhibition)		
			1 hr	2 hr	3 hr
Control (0.5% CMC)	-	1	0.320 mL±0.025	0.347 mL±0.021	0.303 mL±0.015
Standard	100	2	0.215 mL±0.043***(89.53)	0.209 mL±0.082***(91.54)	0.224 mL±0.035***(89.96)
	200	3	0.183 mL±0.021***(92.51)	0.202 mL±0.033***(91.87)	0.210 mL±0.055***(89.79)
	400	4	0.112 mL±0.016***(94.35)	0.183 mL±0.039***(93.65)	0.112 mL±0.042***(95.38)
EERAV	100	5	0.435 mL±0.039ns(63.54)	0.352 mL±0.025**(70.39)	0.351 mL±0.049**(73.79)
	200	6	0.399 mL±0.036ns(69.11)	0.385 mL±0.073**(71.37)	0.313 mL ±0.063**(72.58)
	400	7	0.296 mL±0.047***(79.68)	0.288 mL ±0.091***(84.69)	0.265 mL ±0.053***(85.81)
AQERAV	100	8	0.269 mL±0.029***(82.53)	0.289 mL±0.053*(83.95)	0.233 mL±0.044***(86.06)
	200	9	0.222 mL±0.051**(86.86)	0.203 mL±0.033**(87.49)	0.211 mL±0.039**(88.37)
	400	10	0.215 mL±0.036***(88.06)	0.201 mL±0.053***(90.49)	0.200 mL±0.041***(91.61)

In silico, in vitro, and *in vivo* correlation study

The correlation between *in silico, in vitro*, and *in vivo* studies is of paramount importance in various scientific fields, particularly in drug discovery and development, toxicology, and biomedical research (Ganesan *et al*., 2024). These three approaches offer complementary insights, and their integration significantly enhances the efficiency, accuracy, and ethical considerations of research. The molecular docking studies revealed that the bioactive constituents present in AQERAV exhibited promising binding affinities toward key inflammatory enzymes, COX-1 and COX-2 (PDB ID: 5KIR). Among the identified phytoconstituents, D-Glucoside demonstrated the highest binding affinity towards both COX-1 and COX-2, indicating a strong potential to inhibit these pro-inflammatory targets. β-Sitosterol-β-D-glucoside showed the second-highest affinity for COX-1, while 9-acetamido-3,4-dihydropyrido[3,4-b]indole ranked just below D-Glucoside in its affinity for COX-2. These results suggest that the aqueous extract of *A. vasica* contains potent COX inhibitors, supporting its role in modulating inflammatory processes. The *in vitro* anti-inflammatory activity of AQERAV was assessed using standard enzyme inhibition assays, where it demonstrated a significantly lower IC_{50} value in comparison to the standard drug (diclofenac), highlighting its superior inhibitory potential. The strong *in vitro* efficacy aligns with the docking results, especially the high binding scores of D-Glucoside and β-sitosterol derivatives, suggesting that these compounds may directly contribute to the suppression of inflammatory mediators by inhibiting COX enzymes.

In the *in vivo* anti-inflammatory model, AQERAV produced statistically significant inhibition of inflammation, even at lower doses, compared to both the ethanolic extract and control. The extract's percentage inhibition was comparable to the standard drug, confirming its efficacy. Notably, the activity was more pronounced with AQERAV than with the ethanolic extract, which suggests higher bioavailability or a better concentration of polar bioactive compounds in the aqueous medium.

A strong correlation was established across all three experimental approaches. The compounds showing the highest binding affinities in the docking studies (notably D-Glucoside) were consistent with the most effective *in vitro* and *in vivo* anti-inflammatory outcomes. This multi-tiered consistency confirms that the active phytochemicals in AQERAV not only interact strongly with inflammatory targets but also translate this interaction into functional biological effects, both *in vitro* and *in vivo*. The findings validate AQERAV as a potent, multi-targeted anti-inflammatory agent, supporting its traditional use and suggesting its potential for further development.

DISCUSSION

The current investigation offers compelling pharmacological evidence substantiating the traditional use of *A. vasica* root in inflammation management. A multi-tiered experimental approach encompassing *in vitro, in vivo*, and *in silico* models revealed that both aqueous (AQERAV) and ethanolic (EERAV) extracts exhibit substantial anti-inflammatory activity, with AQERAV emerging as more potent and consistent across assays.

In the BSA protein denaturation assay, AQERAV demonstrated an IC_{50} value (28.82 µg/mL) closely matching that of the standard diclofenac (26.39 µg/mL), indicating effective suppression of heat-induced denaturation of proteins, a hallmark of inflammation. This aligns with prior studies that have established the relevance of protein stabilization in anti-inflammatory screening (Calixto *et al*., 2004). The ethanolic extract, although active, exhibited weaker inhibition at lower concentrations,

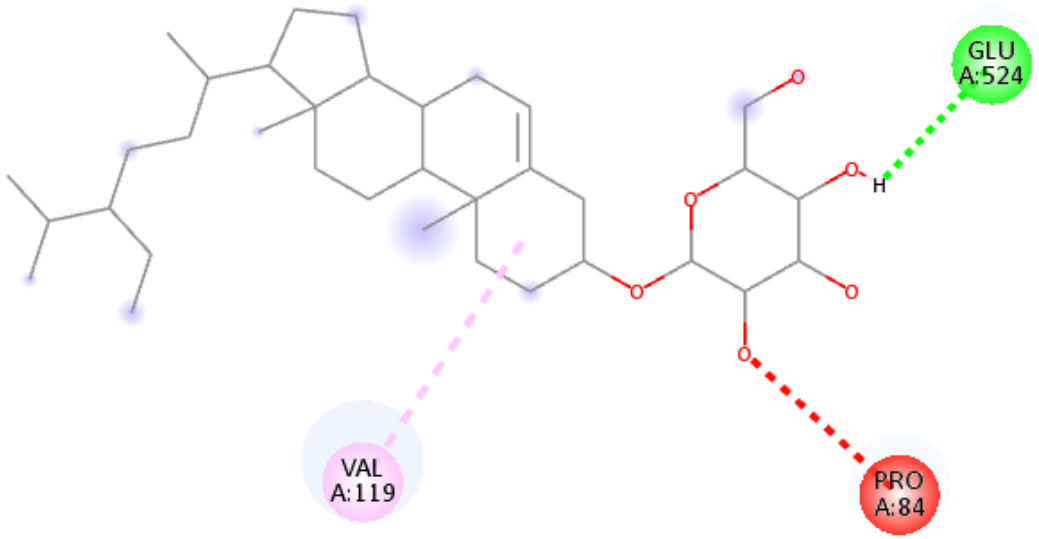

Figure 3: Interaction diagram of COX 1 protein with D-glucoside.

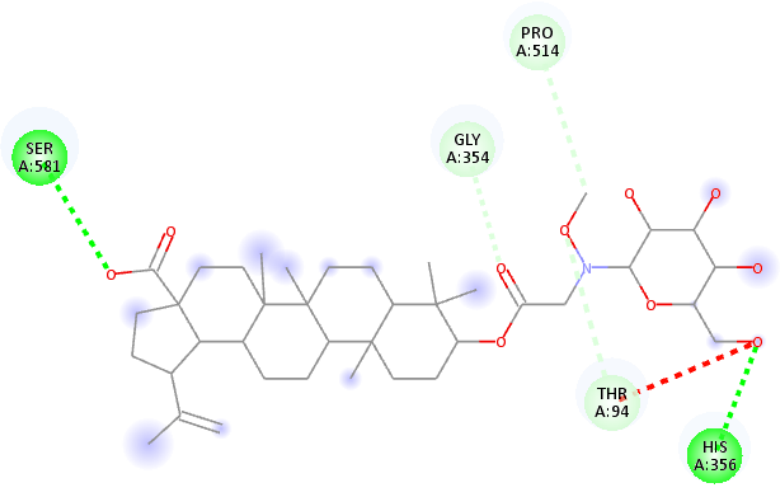

Figure 4: Interaction diagram of COX 2 protein with D- glucoside.

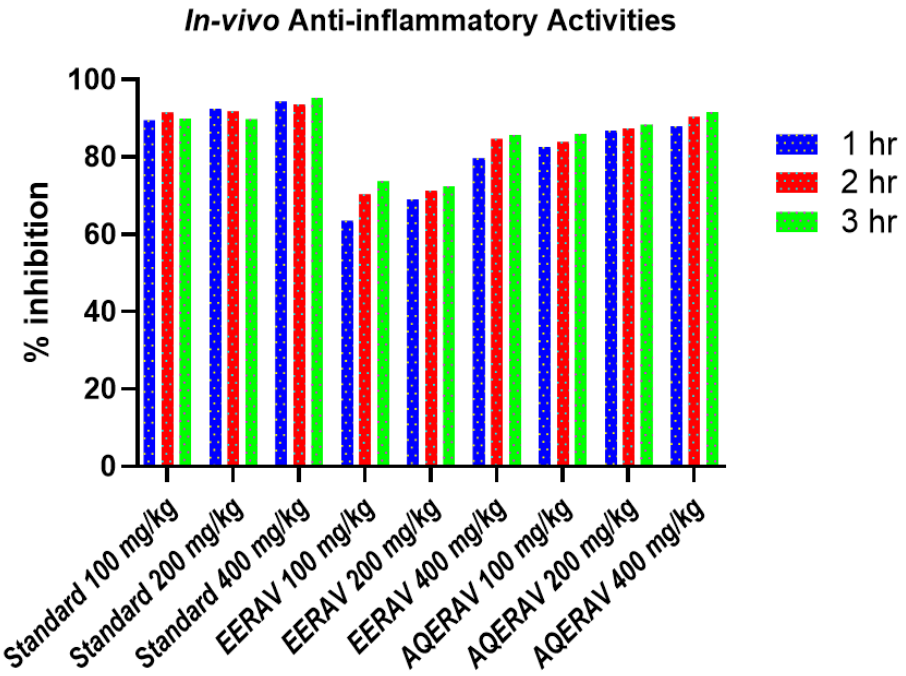

Figure 5: *In vivo* anti-inflammatory activity of AQERAV and EERAV.

suggesting that the polar phytoconstituents in AQERAV play a more central role in mediating the observed effects.

In vivo, AQERAV significantly attenuated carrageenan-induced paw oedema in a dose-dependent manner, achieving over 90% inhibition at 400 mg/kg, which is comparable to diclofenac (94.35% inhibition). Notably, this effect was sustained over 3 hr, indicating the presence of compounds with rapid onset and prolonged action. These results corroborate previous findings that support *A. vasica*'s efficacy in inflammatory disorders and expand the evidence to its root part, which has been underexplored relative to its leaves (Dhuley, 1999).

The phytochemical analysis revealed that AQERAV contains abundant alkaloids, flavonoids, saponins, and phenolics-classes of compounds previously shown to modulate key inflammatory mediators such as cyclooxygenases, cytokines, and reactive oxygen species (Calixto *et al.*, 2004). Notably, the presence of saponins and flavonoids, both known to exhibit membrane stabilizing and COX-inhibitory actions, may explain the high efficacy of the aqueous extract.

Supporting the biological results, molecular docking studies provided mechanistic insights. D-Glucoside, one of the prominent constituents of AQERAV, displayed the strongest binding affinity toward COX-1 (-8.8 kcal/mol) and COX-2 (-8.6 kcal/mol), surpassing diclofenac in docking scores. β-Sitosterol-β-D-glucoside and 9-acetamido-3,4-dihydropyrido[3,4-b]indole also exhibited notable interactions. These findings are consistent with other *in silico* investigations highlighting the COX-inhibitory

potential of plant-derived glycosides and alkaloids (Ganesan *et al*., 2024). Furthermore, ADMET predictions revealed favorable gastrointestinal absorption and low toxicity profiles for these compounds, reinforcing their suitability for oral administration.

A noteworthy aspect of this study is the congruence between the *in vitro, in vivo*, and *in silico* findings. The top docking ligands from AQERAV not only demonstrated strong interactions with COX isoforms but also corresponded to significant pharmacological effects *in vivo*. This translational correlation emphasizes the utility of *in silico* tools in predicting bioactivity and guiding phytochemical prioritization in natural product research (Palei *et al*., 2025).

Importantly, the acute toxicity study confirmed the safety of both extracts up to 2000 mg/kg, with no observed lethality or behavioral anomalies, supporting their therapeutic applicability. This is particularly relevant considering the adverse effects associated with chronic NSAID use, such as gastrointestinal and cardiovascular risks (Vane and Botting *et al*., 1998). Thus, *A. vasica* root extracts offer a safer, plant-based alternative for inflammation management.

In summary, this study bridges a crucial gap in existing ethnopharmacological literature by highlighting the potent anti-inflammatory activity of *A. vasica* root-traditionally recognized yet scientifically under-validated. It lays a solid foundation for future efforts in isolation of bioactive principles, chronic toxicity assessments, and eventual clinical translation.

CONCLUSION

The findings of the present study scientifically validate the traditional claims regarding the anti-inflammatory potential of *A. vasica* root. Both aqueous (AQERAV) and ethanolic (EERAV) extracts demonstrated significant anti-inflammatory activity in *in vitro* (BSA protein denaturation assay) and *in vivo* (rat paw oedema) models, with AQERAV showing efficacy even at lower doses. The extracts were found to be safe at an oral dose of 2000 mg/kg in acute toxicity studies. Furthermore, molecular docking analysis confirmed strong binding affinities of selected phytoconstituents toward the COX-1 enzyme, supporting their role in modulating inflammatory pathways. A strong correlation among *in vitro, in vivo*, and *in silico* results further substantiates the therapeutic relevance of these root extracts.

These results not only confirm the ethnomedicinal use of *A. vasica* but also highlight its potential as a safer, plant-based alternative to conventional anti-inflammatory drugs. Future studies may explore the mechanistic pathways involved, evaluate chronic toxicity and pharmacokinetic profiles, and investigate the synergistic effects of individual phytoconstituents. This study lays a robust foundation for advanced pharmacological research, formulation development, and potential clinical applications of *A. vasica* in the management of inflammatory disorders.

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ABBREVIATIONS

IL-1β: Interleukin-1 beta; **TNF-α:** Tumor Necrosis Factor alpha; **COX**:Cyclooxygenase; **NSAIDs:** Non-Steroidal Anti-Inflammatory Drugs; **LOX:** Lipoxygenase; **EERAV:** Ethanolic Extract of the Root of *Adhatoda vasica;* **AQERAV:** Aqueous Extract of the Root of *Adhatoda vasica;* **BSA:** Bovine Serum Albumin; **UV:** Ultraviolet; **SDF:**Structure Data File**;** **MW:** Molecular Weight; **HBD:** Hydrogen Bond Donors; **HBA:** Hydrogen Bond Acceptors; **SWISSADME:** Swiss Absorption, Distribution, Metabolism, and Excretion predictor; **PDB:** Protein Data Bank; **ADME:** Absorption, Distribution, Metabolism, and Excretion; **LD_{50}:** Median Lethal Dose (Lethal Dose for 50% of Test Subjects); **CMC:** Carboxymethyl Cellulose; **OECD:** Organization for Economic Cooperation and Development; **CCSEA:** Committee for the Control and Supervision of Animal Experiments; **IAEC:** Institutional Animal Ethics Committee; **ANOVA:**Analysis of Variance; **SEM:** Standard Error of Mean; **ADMET:** Absorption, Distribution, Metabolism, Excretion, and Toxicity; **IC_{50}:** Inhibitory Concentration 50 (Concentration Required to Inhibit 50% of target activity).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION

Teasha Chakraborty: Conceptualization, Data Curation; Prasenjit Mondal: Writing draft, Validation; Suman Acharyya: Resource, Validation; Saptarshi Samajdar: Software, Methodology; Mitali Sahoo: Review, Editing; Soubhanik Ghosh: Review, Editing; Sumanta Mondal: Resource, Methodology

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The study protocol was approved by Institutional Animal Ethics Committee (IAEC), GITAM (Deemed to be University).

SUMMARY

The present study aimed to scientifically validate the traditional use of *Adhatoda vasica* root for inflammation management. Aqueous (aqerav) and ethanolic (eerav) extracts were prepared using soxhlet extraction and evaluated for anti-inflammatory activity through *in vitro* bsa protein denaturation, *in vivo*

carrageenan-induced rat paw oedema, and molecular docking studies. Aqerav showed potent *in vitro* activity (IC_{50}: 28.82 µg/mL), comparable to diclofenac, and significant dose-dependent *in vivo* effects. Both extracts were safe up to 2000 mg/kg. Docking revealed strong binding to cox-1. The findings support the traditional use of *a. Vasica* and highlight its potential as a safe, plant-based anti-inflammatory agent.

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