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Evaluation of the Antioxidant and Anti-Inflammatory Potential of Hydroalcoholic Leaf Extract of *Leucaena Leucocephala* (Lam.) De Wit Using Complementary Experimental and Computational Approaches

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ABSTRACT

Inflammation is a common clinical and socioeconomic problem. Prolonged use of synthetic drugs may cause adverse effects, creating interest in safer plant-based alternatives. *Leucaena leucocephala* (Lam.) de Wit. is a member of the Fabaceae (pea) family. In folk medicine, the leaves of this plant were used to treat fevers, stomach disorders, and wounds. In this study, the anti-inflammatory activity of a hydroalcoholic (70:30 ethanol, water) leaf extract is evaluated. Extraction was done by the maceration process, yielding 9%. Preliminary phytochemical analysis showed the presence of Phenols, Flavonoids, alkaloids, and other phytoconstituents in the extract. Antioxidant activity was evaluated using the DPPH assay $IC_{50\ value}$ was 146.431 µg/mL. The extract showed concentration-dependent inhibition of BSA denaturation, giving % denaturation inhibition of 60.8% at 500 µg/mL, indicating *in vitro* anti-inflammatory potential. The extract exhibited dose-dependent anti-

inflammatory activity in a Carrageenan-induced inflammation, with reported inhibition of paw edema is 52.50% for 200 mg/kg and 58.14% for 400 mg/kg. In an *in silico* approach, network pharmacology and molecular docking supported interactions of phytoconstituents with selected inflammation-related target proteins.

Keywords: Inflammation; *Leucaena leucocephala* (Lam.) de Wit; Carrageenan-induced inflammation; network pharmacology; molecular docking.

ABREVATION

BSA	Bovine Serum Albumin
CCSEA	Committee for the Control and Supervision of Experiments on Animals
DPPH	2,2-Diphenyl-1-picrylhydrazyl
HE-LL	Hydroalcoholic Extract of *Leucaena leucocephala* leaves extract
NSAID	Non-Steroidal Anti-Inflammatory Drugs
TFC	Total Flavonoid Content
TPC	Total Phenolic Content

INTRODUCTION

Inflammation is a common clinical problem that affects quality of life and is considered a social and economic burden. Inflammation is a protective biological response of the body to harmful stimuli such as tissue injury, infection and foreign substances. Uncontrolled inflammation may contribute to the development and progression of several chronic disorders, including diabetes, atherosclerosis, obesity and cancer, which are the world's leading causes of mortality. It is believed that current drugs such as corticosteroids, NSAIDs and opioids' prolonged use may have harmful side effects. These limitations have encouraged the search for safer and effective therapeutic agents from natural sources (Afsar et al., 2015).

Leucaena leucocephala (Lam.) de Wit (family Fabaceae) grows naturally in most tropical areas of the world. Traditionally, the whole plant is used as fodder and green manure; decoctions of plant material are used to treat Fevers and stomach disorders, and leaves are used to reduce swellings in inflammation. Its leaves contain flavonoids and polyphenolic compounds that may contribute to its biological activities (Zayed et al. 2019). Based on its phytochemical similarity, folk medicine, ethnopharmacological evidence and traditional use for inflammation, *Leucaena leucocephala* (Lam.) de Wit leaves were selected to evaluate their anti-inflammatory activity.

MATERIALS AND METHODS

Collection of plant material

Leaves of *Leucaena leucocephala* (Lam.) de Wit were collected from Bapuji Pharmacy College, Davanagere, and authenticated by the Department of Studies in Botany, Davanagere University, Karnataka, and deposited in its herbarium under voucher no. HDUD193.

Preparation of plant extract:

The collected leaves were shade-dried, then coarsely powdered. The powdered leaves (800 g) were extracted by maceration using a hydroalcoholic solvent (ethanol, water 70:30 v/v) for 4 days with intermittent stirring followed by re-maceration. The combined extract was concentrated to obtain a semi-solid hydroalcoholic extract, then preserved in a desiccator.

Chemicals and drugs

DPPH, Carrageenan, quercetin, and Gallic acid were procured from S D Fine-Chem Limited, Mumbai. Indomethacin from S.M. Pharma and Chemicals, Mumbai. Analytical-grade solvents and chemicals were used throughout the study.

Qualitative and quantitative phytochemical investigation

The HE-LL was qualitatively screened for flavonoids, alkaloids, glycosides, phenols, steroids, tannins, and triterpenoids using standard methods. The total phenolic and flavonoid contents were quantitatively estimated using the Folin–Ciocalteu (Singleton VL et al. 1999) and aluminium chloride colourimetric methods (Zhishen J et al. 1999), using the formula TPC = $\frac{CV}{M}$ and TFC = $\frac{CV}{M}$ respectively, where C = Concentration (mg/mL), V = Volume of extract used (mL), M = Mass of extract used (g)

LC-MS Analysis

The extract was analysed by LC–MS at Chromatogen Analytical Solutions using a Waters UPLC system equipped with an ACQUITY BEH column (50 × 2.1 mm, 1.7 µm). The mobile phase comprised 0.1% formic acid in water (A) and acetonitrile (B), with a flow rate of 0.4 mL/min, an injection volume of 3 µL, and a 6-min gradient run. Detection was performed using a Waters SQ mass spectrometer in ESI mode over an *m/z* range of 50–2000. Data were acquired and processed using MassLynx software (Smyth WF et al. 2006).

Network Pharmacology

Phytochemicals identified from the extract were screened using IMPPAT and Dr. Duke's databases (https://cb.imsc.res.in/imppat/;https://phytochem.nal.usda.gov/), and their canonical SMILES were obtained from PubChem. Compound-associated targets were predicted using Molsoft and SuperPred, while inflammation-related targets were retrieved from GeneCards (https://pubchem.ncbi.nlm.nih.gov/;https://www.genecards.org/). Common targets were identified using Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/index.html), followed by protein–protein interaction analysis using STRING ((https://string-db.org/) and visualisation in Cytoscape. Hub targets were identified using CytoHubba, and pathway enrichment was performed using KEGG (Szklarczyk D et al. 2023; Shannon P et al. 2003)

Molecular Docking

Molecular docking was performed using AutoDock 4.2.6. The three-dimensional structures of the target proteins were retrieved from the RCSB Protein Data Bank (Burley SK et al.), while the structures of the selected ligands were obtained from ChemSpider. Protein preparation was carried out using AutoDock Tools, followed by grid-box definition and generation of grid maps using AutoGrid (Morris GM et al. 2009). Docking interactions were visualised using Discovery Studio Visualizer (Dassault Systèmes BIOVIA).

***In vitro* DPPH antioxidant assay.**

The in vitro antioxidant activity of HE-LL was evaluated using the DPPH radical scavenging assay. DPPH (0.1 mM) was prepared in methanol, and 1000 µg/mL stock solutions of HE-LL and ascorbic acid were prepared in methanol. Working concentrations (50–300 µg/mL) were prepared by dilution. Each concentration (1 mL) was mixed with 3 mL DPPH solution and incubated in the dark at room temperature for 30 min. Absorbance was measured at 517 nm using a UV–Visible spectrophotometer. DPPH–methanol served as the control, methanol as the blank, and ascorbic acid as the reference standard (John SN et al. 216). Radical-scavenging activity was calculated using the standard formula: % Scavenging activity $= \frac{A2-A1}{A2} \times 100$

Where A_1 = absorbance of control, A_2 = absorbance of standard/Test

***In vitro* protein denaturation assay**

A 1% BSA solution was prepared by dissolving 0.5 g BSA in 50 mL PBS (pH 6.4). Diclofenac sodium was used as the standard and prepared as a 1000 µg/mL stock solution, followed by serial dilution to 100–500 µg/mL. HE-LL was similarly diluted. The assay mixture contained 2.8 mL PBS, 2 mL test or standard solution, and 0.2 mL BSA. A control contained PBS. Mixtures were incubated at 37°C for 15 min, followed by heating at 70°C for 5 min to induce protein denaturation (Kumar et al. 2025). The percentage inhibition of BSA denaturation was calculated using the standard formula: % Inhibition of denaturation $= \frac{1-A2}{A1} \times 100$. Where A_1 = absorption of the control sample, A_2 = absorption of the test sample.

Experimental Animals

Healthy adult Wistar albino rats of either sex (180–250 g) were procured from Sri Sai Biosciences, Bengaluru, and housed under controlled conditions (22 ± 3°C; 30–70% relative humidity) with free access to standard pellet diet and water. The study was approved by the Institutional Animal Ethics Committee (IAEC), Bapuji Pharmacy College (Ref. No. BEA.B.Ph./2025/004), and conducted in accordance with CCSEA guidelines (Reg. No. 105/PO/Re/S/99/CCSEA).

Acute oral toxicity studies

Mathew et al. (2017) reported no toxicity or mortality following oral administration of ethanolic *Leucaena leucocephala* (Lam.) de Wit. leaf extract at 2000 mg/kg in albino mice, as per OECD Guideline 425. Based on

this reported safety, one-tenth and one-fifth of the tested dose were selected, and 200 and 400 mg/kg body weight were used as the lower and higher doses, respectively, in the present study.

Experimental design for anti-inflammatory Activity

Wistar albino rats (n=6/group) were divided into five groups: normal, disease control, standard, and HE-LL at 200 and 400 mg/kg. Carrageenan (1% w/v) was injected into the subplantar region, except in the normal group. Indomethacin (10 mg/kg) was administered to the standard group, and HE-LL were administered to the low-dose and high-dose groups orally after 30 min of induction. Paw volume was measured using a plethysmometer at 0–300 min and analysed by one-way ANOVA followed by Tukey's multiple comparison test (Ramakrishna G et al.)

The percentage inhibition of oedema was determined using a formula.

$$\% \text{ Inhibition} = \frac{\text{Mean}_{\text{Disease Control}} - \text{Mean}_{\text{Treatment}}}{\text{Mean}_{\text{Disease Control}} - \text{Mean}_{\text{Normal}}} \times 100$$

RESULTS

Leaf extract was subjected to a maceration extraction process, yielding 72 g of dark green, semi-solid extract, corresponding to a 9% yield.

Qualitative and Quantitative Phytochemical Analysis

Preliminary phytochemical screening of HE-LL revealed saponins, steroids, alkaloids, flavonoids, tannins, glycosides, cardiac glycosides, phenols, and triterpenes. The total phenolic and flavonoid contents were 13.1 mg GAE/g and 20.96 mg QE/g extract, respectively.

LC-MS Characterisation

LC–MS analysis of HE-LL revealed four major peaks, tentatively identified by retention times, observed m/z values in ESI-positive mode, theoretical masses, literature, and phytochemical databases. Definitive identification requires high-resolution MS/MS fragmentation and authentic reference standards.

Table 01: Tentative identification of compounds in HE-LL fraction by LC-MS.

Peak No.	RT (min)	Observed m/z	Theoretical m/z	Putative compound
P1	1.84	475.32	475.087	Chicoric acid
P2	1.94	487.26	487.085	Quercetin-O-hexoside
P3	2.48	304.25	304.058	Taxifolin
P4	1.95	340.3	339.30	Apigenin

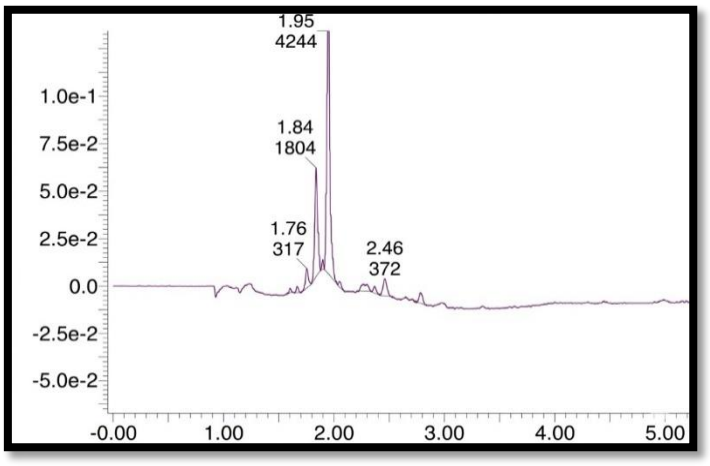


Fig. 01: **LC-MS Characterisation of HE-LL and its peak with retention time.**

Network Pharmacology

Thirty-three phytocompounds reported from *Leucaena leucocephala* (Lam.) de Wit were screened for drug-likeness using MolSoft, yielding 14 shortlisted compounds. Target prediction identified 361 compound-associated targets, while 299 inflammation-related targets were retrieved from GeneCards. Venny analysis identified 22 common targets, followed by STRING-based PPI and KEGG enrichment, which revealed 43 target–pathway associations across 25 pathways related to cytokine production, oxidative stress, inflammatory signalling, and immune-cell activation responses. Based on network connectivity and target relevance, Quercetin, Luteolin, and Apigenin were selected for molecular docking against NFKB-1, ALOX12, and STAT3.

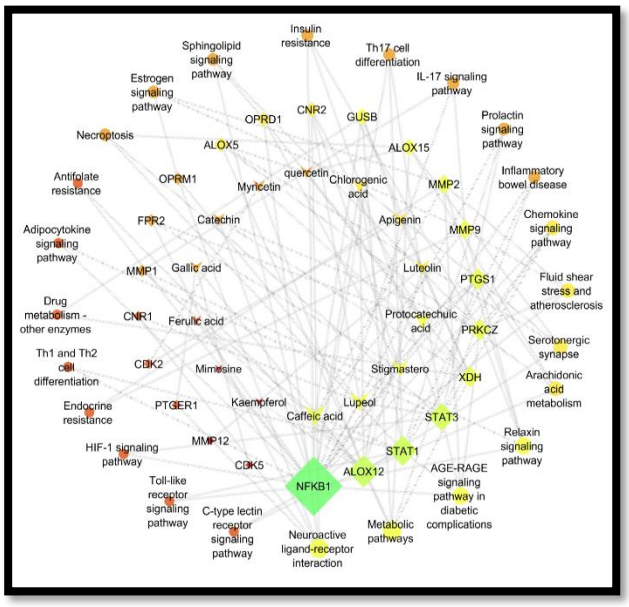


Fig. 2: Network construction of the phytocompounds with their respective targets and modulated pathways. Diamonds represent targets, circles represent pathways, and arrows represent compounds.

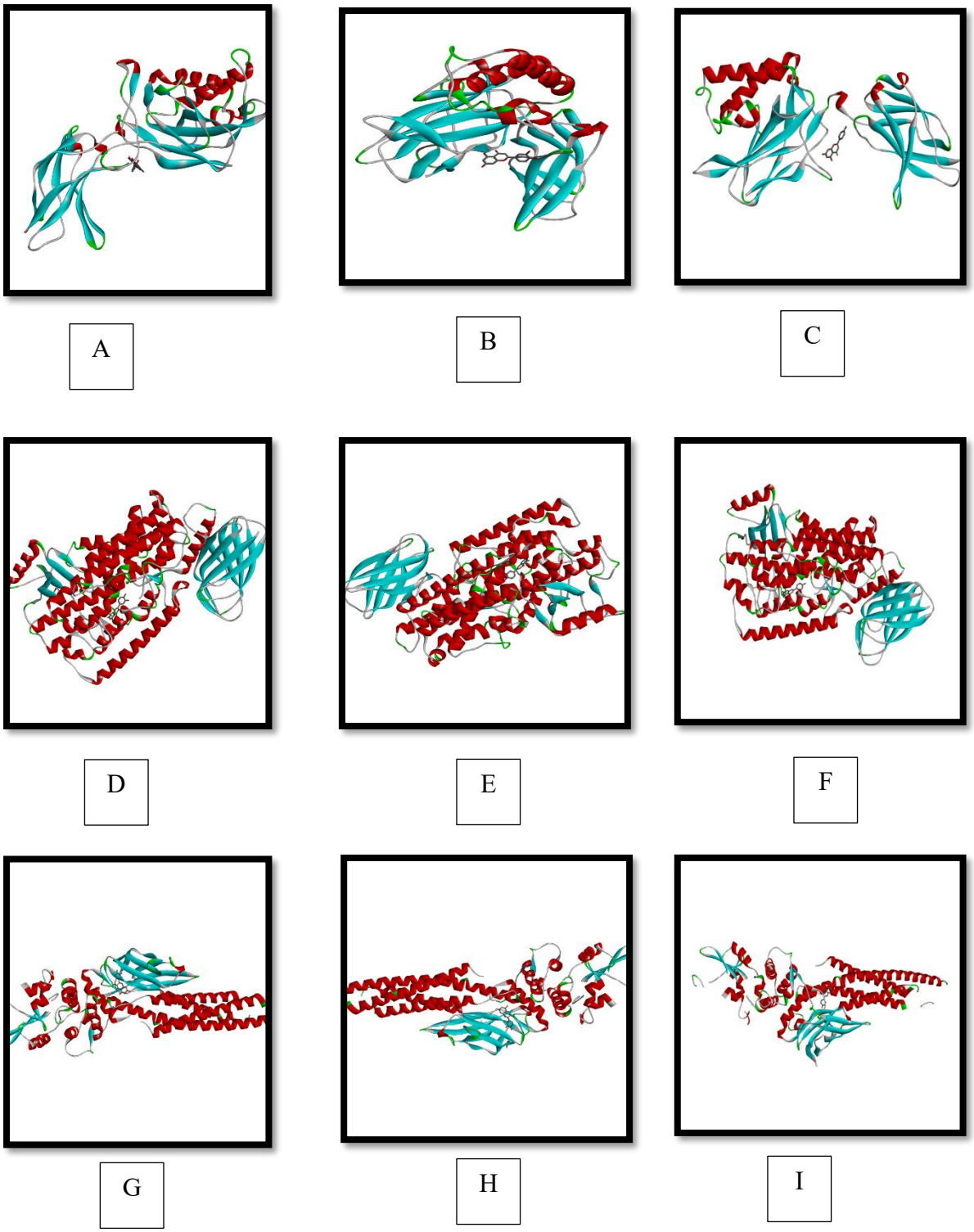
Molecular Docking.

Molecular docking evaluated interactions of LC–MS-identified HE-LL phytoconstituents Quercetin, Luteolin, and Apigenin with NFKB1, ALOX12, and STAT3. These targets were selected based on compound–target network relevance and their roles in inflammatory signalling and arachidonic acid metabolism. The crystal structures of NFKB1, ALOX12 and STAT3 were retrieved from the Protein Data Bank using PDB IDs 1SVC, 8GHD and 6NUQ, respectively.

For NFKB1 (PDB ID: 1SVC), luteolin exhibited the lowest binding energy (−5.69 kcal/mol). For ALOX12 (PDB ID: 8GHD), quercetin exhibited the lowest binding energy of −9.40 kcal/mol. For STAT3 (PDB ID: 6NUQ), quercetin exhibited the lowest binding energy of −7.80 kcal/mol. Overall, the docking findings demonstrated favourable predicted interactions of Quercetin, Luteolin and Apigenin with the selected inflammatory protein targets, supporting their further investigation in relation to the anti-inflammatory potential of HE-LL.

Table 2. Binding energy and molecular interactions of compounds with targets.

Gene	Phytochemicals	BE (kcal/mol)	Active Torsions	Hydrogen bond interactions	Non-Hydrogen bond interaction
NFKB-1	Quercetin	-5.37	1	ARG (54) GLN (53) GLU (341) ASP (339)	ALA (73) GLU (341)
	Luteolin	-5.69	1	ARG (54) GLN (53) ASP (339)	ALA (73) GLU (341)
	Apigenin	-5.57	1	ALA (73) ARG (54)	ALA (73) ALA (248)
ALOX12	Quercetin	-9.40	1	SER (594) GLN (590) HIS (365)	ILE (593) PHE (352) LEU (597)
	Luteolin	-9.29	1	SER (594) GLN (590) HIS (365)	ILE (593) PHE (352)
	Apigenin	-9.04	1	SER (594) GLN (590) HIS (360)	HIS (360) ILE (593) PHE (352)
STAT 3	Quercetin	-7.80	1	PRO (333) GLN (247) GLU (324) ARG (325) THR (346)	CYS (328) CYS (251)
	Luteolin	-7.45	1	GLU (324) GLN (247) THR (346)	CYS (328) CYS (251) ARG (325) ALA (250)
	Apigenin	-7.11	1	ASN (485) GLN (326)	LEU (459) LYS (244) LYS (244)



A- NFKB-1 with Quercetin, B- NFKB-1 with Luteolin, C- NFKB-1 with Apigenin, D- ALOX12 with Quercetin, E- ALOX12 with Luteolin, F- ALOX12 with Apigenin, G- STAT 3 with Quercetin, H- STAT 3 with Luteolin, I- STAT 3 with Apigenin

Fig 3. Molecular docking analysis of key targets with active compounds

DPPH Radical-Scavenging Activity

HE-LL showed concentration-dependent DPPH radical-scavenging activity, with inhibition increasing from 30% at 50 µg/mL to 76.63% at 300 µg/mL, compared with 52.7% to 72.12% for ascorbic acid. The IC_{50} values were 146.431 µg/ml for HE-LL and 69.989 µg/mL for ascorbic acid, with R^2 values of 0.9779 and 0.9991, respectively, confirming a concentration-dependent response.

Table 3. In vitro DPPH Antioxidant activity results.

Sl. NO	Concentration (µg/ ml)	Absorbance at 517 nm	% DPPH radical scavenging
1	Control	0.703	0.000
Standard (Ascorbic acid)			
1	50	0.37	47.937
2	100	0.33	52.77
3	150	0.29	58.04
4	200	0.26	62.87
5	250	0.22	67.99
6	300	0.19	72.12
Test (HE-LL)			
1	50	0.492	30.01
2	100	0.424	39.69
3	150	0.313	55.48
4	200	0.275	60.85
5	250	0.209	70.24
6	300	0.164	76.63

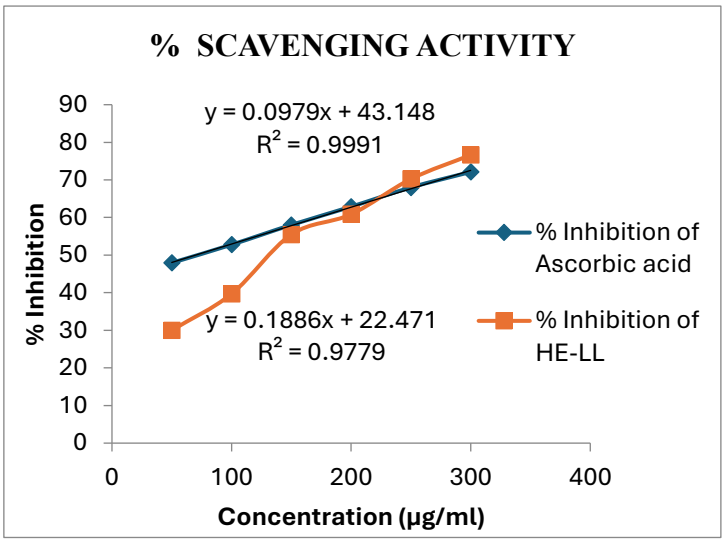


Fig. 4: Percentage scavenging of Standard and test compound linear graph

***In vitro* protein denaturation assay**

HE-LL showed concentration-dependent inhibition of BSA denaturation, increasing from 31.25% at 100 µg/mL to 60.8% at 500 µg/mL, compared with 36.36–79.54% for diclofenac sodium. HE-LL showed moderate anti-inflammatory activity, with an IC_{50} of 363.93 µg/mL versus 243.46 µg/mL for diclofenac sodium. Regression analysis showed strong linearity, with R^2 values of 0.9913 and 0.9903, respectively.

Table 4: *In vitro* anti-inflammatory activity by bovine serum albumin denaturation assay

Sl. NO	Concentration (µg/ ml)	Absorbance at 660 nm	% inhibition of denaturation
1	Control	0.176	0.000
Standard (diclofenac sodium)			
1	100	0.112	36.36
2	200	0.098	43.94
3	300	0.08	54.55
4	400	0.059	66.47
5	500	0.036	79.54
Test (HE-LL)			
1	100	0.121	31.25

2	200	0.112	36.36
3	300	0.099	43.75
4	400	0.082	53.41
5	500	0.069	60.8

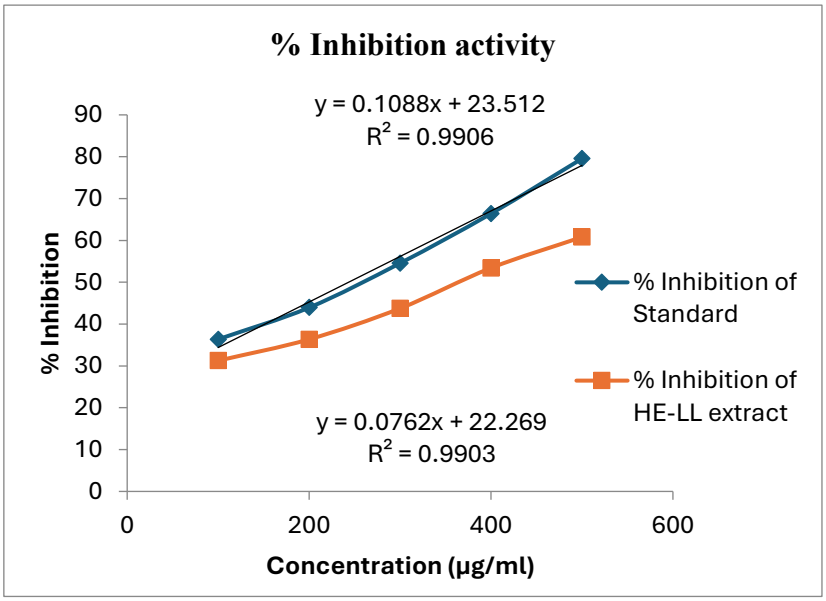


Fig. 5: Percentage inhibition of Standard and test compound (linear graph)

***In vivo* anti-inflammatory activity by the Carrageenan-induced paw edema model**

HE-LL demonstrated appreciable anti-inflammatory activity in carrageenan-induced paw edema. Indomethacin showed maximum inhibition of 69.77% at 180 min. HE-LL at 200 and 400 mg/kg achieved maximum inhibition of 52.50% at 240 min and 58.14% at 180 min, respectively. The higher dose showed greater inhibition than the lower dose, indicating a dose-dependent response, although inferior to indomethacin.

Table 5: Mean paw edema in different treatment groups using the carrageenan-induced inflammation method

Groups	0 min	30 min	60 min	120 min	180 min	240 min	300 min
Normal	0.300 ± 0.000	0.300 ± 0.000	0.300 ± 0.000	0.300 ± 0.000	0.300 ± 0.000	0.300 ± 0.000	0.300 ± 0.000
Disease Control	0.300 ± 0.000	0.400 ± 0.010[a]	0.480 ± 0.010[a]	0.680 ± 0.019[a]	0.730 ± 0.010[a]	0.700 ± 0.010[a]	0.640 ± 0.019[a]

Standard	0.300 ± 0.000	0.370 ± 0.016	0.410 ± 0.016**	0.450 ± 0.013****	0.430 ± 0.017****	0.440 ± 0.006****	0.420 ± 0.004***
HE-LL Lower dose (200 mg/kg)	0.300 ± 0.000	0.390 ± 0.029	0.460 ± 0.019	0.550 ± 0.019****	0.520 ± 0.010****	0.490 ± 0.010***	0.470 ± 0.019**
HE-LL Higher dose (400 mg/kg)	0.300 ± 0.000	0.380 ± 0.026	0.430 ± 0.019*	0.480 ± 0.016****	0.480 ± 0.008****	0.470 ± 0.010****	0.460 ± 0.0169***

All values were presented as a Mean ± SEM, One-way analysis of variance (ANOVA)followed by Multiple comparison of Tukey's test. [a]p<0.0001, as compared with the Normal group and **** p<0.0001, *** p<0.001, ** p<0.01, and *$p<0.05$ as compared with disease control group.

Table 6: %inhibition of anti-inflammatory activity

Group	0 min	30 min	60 min	120 min	180 min	240 min	300 min
Standard	—	30.00%	38.89%	60.53%	69.77**%**	65**%**	64.70%
HE-LL-Lower dose	—	10.00%	11.11%	34.21%	48.84%	52.5%	50%
HE-LL-Higher dose	—	20.00%	27.78%	52.63%	58.14%	57.5%	52.94%

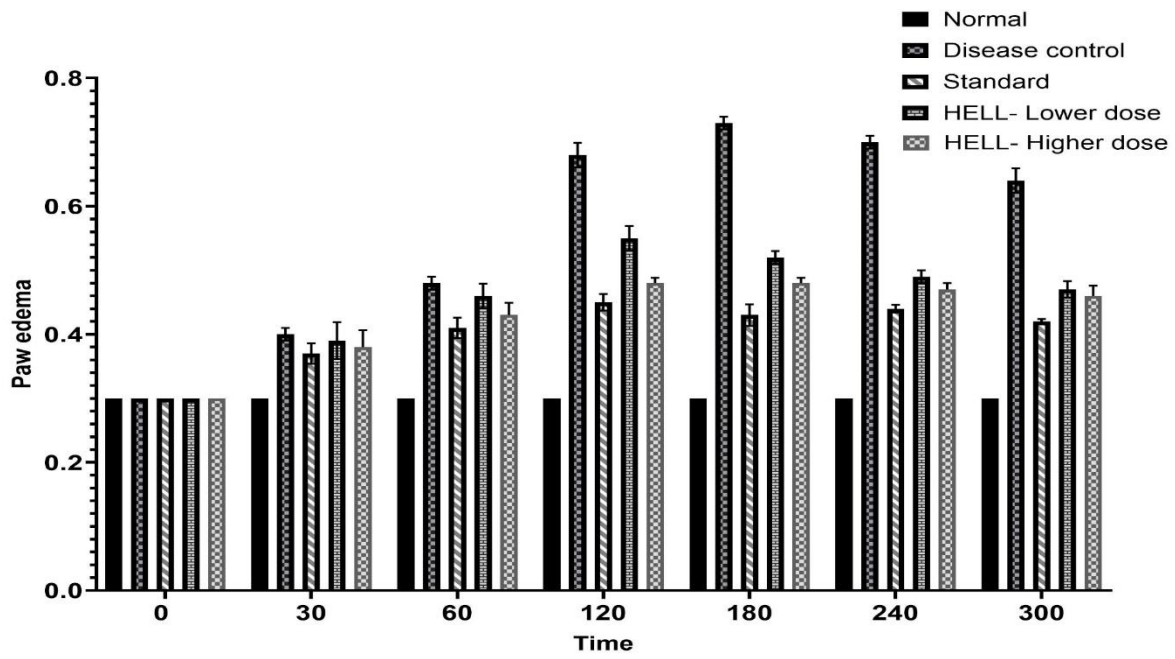


Fig. 6: Bar graph showing paw edema of each group at each time interval

DISCUSSION

The present study evaluated the antioxidant and anti-inflammatory potential of the hydroalcoholic extract of *Leucaena leucocephala* (Lam.) de Wit. leaves using pharmacological and computational approaches. The leaves were extracted by maceration using ethanol: water (70:30), yielding 72 g of semi-solid extract. Phytochemical screening revealed flavonoids, tannins, phenolics, saponins, glycosides, steroids, alkaloids and triterpenes. LC-MS tentatively identified Chicoric acid, quercetin-O-hexoside, taxifolin and an apigenin derivative. The extract exhibited concentration-dependent DPPH radical-scavenging activity, with an IC_{50} of 146.431 µg/mL compared with 69.989 µg/mL for ascorbic acid. This dose-dependent radical scavenging matches the study in which Leucaena flavonoid glycosides were isolated and attributed their chain-breaking capacity to available phenolic hydroxyl groups that donate hydrogen atoms to neutralise DPPH complexes (Mohammed et al. 2015). In the BSA denaturation assay, the extract showed 60.8% inhibition at 500 µg/mL, with an IC_{50} of 363.93 µg/mL versus 243.46 µg/mL for the standard; this was comparable to the 49–73% inhibition reported in a study done for several plant extracts (Shaw Y et al. 2017). In the carrageenan-induced paw edema model, the extract at 200 and 400 mg/kg reduced edema, with the 400 mg/kg dose showing maximum inhibition of 58.14% at 180 min compared with 69.77% for indomethacin. Similar time-dependent activity has been reported for *Cinnamomum longepaniculatum* leaf essential oil in the carrageenan-induced paw edema model (Guay J et al. 2004). Network analysis identified NFKB1, ALOX12 and STAT3 as important targets. Molecular docking showed the strongest predicted binding of quercetin to ALOX12 (−9.40 kcal/mol). These findings support the antioxidant and anti-inflammatory potential of *Leucaena leucocephala* leaves and suggest possible molecular mechanisms underlying these effects.

CONCLUSION

The present study demonstrated the potential anti-inflammatory activities of the hydroalcoholic extract of *Leucaena leucocephala* (Lam.) de Wit leaves. Preliminary phytochemical screening revealed flavonoids, tannins, phenolics, saponin glycosides, steroids, alkaloids and triterpenoids, while LC-MS analysis identified several bioactive constituents, including Chicoric acid, quercetin-O-hexoside, taxifolin and apigenin derivative. The HE-LL showed appreciable dose-dependent antioxidant activity in the DPPH assay, indicating its free radical scavenging potential and concentration-dependent inhibition of protein denaturation in the BSA assay, indicating its potential *in vitro* anti-inflammatory activity. *In vivo* studies demonstrated anti-inflammatory activity, with the 400 mg/kg dose producing greater effects than the 200 mg/kg dose. Network pharmacology identified multiple inflammation-related targets and pathways, while molecular docking suggested favourable interactions of quercetin, luteolin and apigenin with the selected targets. Overall, the findings support the antioxidant and anti-inflammatory potential of *Leucaena leucocephala* leaves and provide a basis for further investigation of their active constituents and mechanisms.

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Competing Interests

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Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Thanmayi M O, Dr Prakash Dabadi, Dr A.M. Krupanidhi, and Lakshmi K.C. The first draft of the manuscript was written by Thanmayi M O, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated and/or analysed during the current study, including the animal experiment data, in-vitro assay results, phytochemical analysis data, LC–MS records retained by the authors, and molecular docking outputs, are available from the corresponding author on reasonable request.

Ethics approval

The animal experimental procedures were reviewed and approved by the Institutional Animal Ethics Committee of Bapuji Pharmacy College, Davanagere (Approval No. BEA.B.Ph./2025/004). All animal experiments were conducted in accordance with the guidelines of the CCSEA and the applicable institutional requirements.