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Original Article

Investigation and Prediction of *Cassia auriculata* Synergistic Anti-inflammatory and Analgesic Activity against COX-1 (6y3c-COX1) and TNF (1rj8-TNF) Proteins

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ABSTRACT

Background: *Cassia auriculata* orthodoxly used as an herbal remedy for alleviating joint and muscle pain, and other circumstances. In contrast, the prescribed anti-inflammatory and analgesic medications are associated with a range of adverse effects, including cardiovascular issues. **Objectives:** To predict anti-inflammatory and analgesic effects of *C. auriculata* leaves and flower methanolic extract (CALME; CAFME). Further for a healthier understanding of binding communications of bioactive constituents, molecular docking were assessed through probable interactions between proteins, COX-1(6y3c-COX1), TNF (1rj8-TNF), and ligand quercetin ($C_{15}H_{10}O_7$), diclofenac and analgin. **Materials and Methods:** Phytoconstituents were assessed qualitatively, while quantitative analysis was conducted using a HPTLC method. CALME and CAFME have been studied for their anti-inflammatory and analgesic properties at doses of 200 mg/kg using mercury displacement and tail flick methods. **Results:** HPTLC analysis identified quercetin as the main polyhydroxy component, constituting 2.08% w/w. Both CALME and CAFME demonstrated a notable decrease in paw volume when compared to the control groups ($p < 0.05$). *In silico* investigation highlighted the possible inhibitory effect of quercetin on 6y3c- COX1 and 1rj8-TNF. Quercetin had the lowest negative value (-8.29 Kcal/mol), which was closest to that of diclofenac (-8.27 Kcal/mol), indicating that it was more powerful and had more active binding pockets. **Conclusion:** This research identified quercetin as the most promising target for COX-1 and TNF, leading us to confirm that quercetin significantly surpassed both diclofenac and analgin in terms of inflammation and various disorders. Consequently, this study highlights CALME's anti-inflammatory and analgesic properties as a viable alternative to current treatment methods.

Keywords: Analgesic, Anti-inflammatory, *Cassia auriculata*, Chromatography, Diclofenac, Docking.

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INTRODUCTION

The inflammation phenomenon is a biological reaction against aggressive agents namely hazardous chemical, tissue injury or uncontrolled cellular growth causing secretion of cellular fluid which lead inflammation in body tissues (Megha *et al.,* 2021). Though a bio-defensive mechanism, endogenous inflammatory mediators responsible to trigger inflammation can produce and aggravate many health disorders (Feldmann & Maini, 2010). The use of Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) in the treatment of diseases associated with inflammatory reactions have adverse effects which pose a major problem in their clinical use (Lin *et al.,* 2023). Hence, new anti-inflammatory and analgesic drugs lacking such effects are being searched as alternatives to NSAIDs. The available modern medicines are generally producing unpleasant health effect because they are chemically synthesizing and are fast reactive in human body (Nunes *et al.,* 2020). Anti-inflammatory and analgesic modern drugs like diclofenac and analgin is globally eye witnessing drug in the pharmaceutical markets. Although analgin (metamizole or dipyrone) have analgesic, antipyretic, and spasmolytic action, still it has limited clinical use due to agranulocytosis a distinguished side effect of analgin and led to its extraction from the bazaar in a number of countries (Miljkovic *et al.,* 2018). Similarly, diclofenac characterized by producing GIT discomfort, hepatotoxic, and anal bleeding severely limiting their solicitation (Altman *et al.,* 2015). Contrary to modern drugs, the consumption of herbs as medicine

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is age long and surprisingly the clinical acceptance of herbs by the earlier population lack scientific understandings (Alotiby & Al-Harbi, 2021), therefor presently a global phenomenal needs of natural floral medicines as an alternative source.

Cassia auriculata (Caisalpiniaceae), a medicinal herb generally discussed in traditional scriptures as biennial fauna and grow all over India in open plantations (Rahman *et al.,* 2024). This solitary natural herb that is commonly prescribing by traditional healers against inflammation and pain. The leaves are widely used as stomachic, gestroprotective, blood purifier, hepatoprotective and to treat the individual met with constipation (Khurm *et al.,* 2021); while the flower are medicinally used against diabetes, reduce high cholesterol level, and also as a beautifying agent in females (Rao *et al.,* 2000).

Over the past decade, numerous studies have concentrated on discovering natural bioactive substances with anti-inflammatory and analgesic properties, specifically phytoconstituents such as polypenolics, glycosides, and betasitosterol, which have been reported in different extracts (leaves and flowers) of *C. auriculata* (Salma *et al.,* 2020). Polyhenolic derivatives such as gallic acid, reutin, quercetin and proanthocyanins are the bioactive secondary phytometabolites that require to scavenge the Reactive Oxygen Species (ROS) and attending as a plant antioxidants (Stagos, 2019; Fasano *et al.,* 2016; Tournadre, 2014). It is conceivable that excessive exposure to pathogenic microorganism, stress, fracture, wrong exercises or yoga, trauma and sport injuries, can target inflammatory and pain mediators where it becomes released to the circulatory system (Kingsley *et al.,* 2025). Treatment associated with inflammation and pain disorders is one of the most demanding and challenging zones of contemporary drugs (Robb *et al.,* 2020). This induces the researcher for the continued explore for new biomedicines in this field. Though, anti-inflammatory agents have numerous uninvited side effects therefore earlier health reports endorsing that neutraceuticals have a major impact on inflammatory mechanism (Burayk *et al.,* 2022).

Notably, there are many instances of successful drug development from medicinal plants, as green medicines provide the safest way to obtain secondary metabolites. The *in silico* research identified quercetin, rutin, resveratrol, kempferol, and ellagic acid as potential inhibitors of COX-1, COX-2, 15-LOX1, and 15-LOX2 (Al-Nour *et al.,* 2019).

Recent study highlights the importance of bioactive secondary metabolites in suppressing the inflammatory factor like Cyclooxygenase (COX), Lipoxygenase (LOX) and cytokines responsible for pain transmission as well as inflammation. For example, earlier studies were carried out on ginger (*Zingiber officinale*) versus diclofenac (Boarescu *et al.,* 2024); garlic (*Allium sativum*) versus ibuprofen (Khan *et al.,* 2023); turmeric (*Curcuma longa*) versus COX- 2 (Sohilait *et al.,* 2017) and aloe (*Aloe vera*) versus naproxen (Yahya & Al-Rajhi 2022) for the treatment of inflammations and its associated illnesses like joint pain, gout and arthritis respectively (Vonkeman *et al.,* 2010). The significant therapeutic outcomes of these studies verified that ginger due to its phytoconstituent (6-gingerol and 6-shogaol), garlic (rhamnetin, alliin, allicin), turmeric (curcuminoids) and aloe (chlorogenic acid) can suppress the inflammation and overcome the pain of arthritis, rheumatoid arthritis, osteoarthritis respectively, In brief, our up-to-date literature survey confirmed that structures of secondary plants metabolites presented in Figure 4C, not only highlight the diverse applications of the phytoconstituents but also emphasize that almost every plant structure has a specific medicinal application.

Although the new analgesic and anti-inflammatory drugs candidate will offer several compensations over available contemporary medicines, the botanicals treatment is somewhat lengthy due to structures of phytochemical components. Structurally these phytoconstituents may possibly network with a number of amino acids responsible for a pharmacological mechanism, which creates an interest to find the robust therapeutic action of such bioactive components (Bharti, 2018). Currently computational docking exploration have seen to be a hot topic for the successful understanding of Drug-Disease-Gene (DDG) mechanism, which can support the researchers in scheming of new biomedicine for the human populations. Therefore, docking study can be a frontline tool to predict the Protein Binding (PB) with bioactive phytoconstituents responsible for pain and inflammation management (Sonkar *et al.,* 2024; Ogungbe & Setzer, 2016).

In this context, the aims of the current study were (1) to dock the isolated plant ligand quercetin and the synthetic ligands diclofenac and analgin with the protein to assess the analgesic and anti-inflammatory properties of *C. ariculata*, (2) to investigate the molecular mechanisms of *C. auriculata* secondary phytoconstituents linked to analgesic and anti- inflammatory effects through computational analysis alongside *in vivo* studies, (3) predicting the docking study as a primary method to anticipate protein receptor binding, (4) the phytopharmaceutical industry recognizes *C. auriculata* plant metabolites as a viable synergistic biomedicine for managing various pathogenic diseases.

MATERIALS AND METHODS

Collection of plant sample

The overland part (fresh leaves and flowers) of were collected from the region of Kadur, Karnatak during flowering session, additionally the plants taxonomic identification was done by botanist (KK-1845CA). The sample was clear from all unwanted foreign dirt by washing and shade dried under laboratory temperature (27°C). Subsequently plant material dried out on laboratory environment and scrappy into small pieces, by electronic grinder (Speed range: 3, 000 rpm; size of particles: 40

mesh; dimensions: w21xd29xh24cm, weight; 13 kg MRC-UK) up to powder and stored in air tight glass container to control the undesirable air and moisture and used for further analysis. The analytical extracts were prepared by Soxhlet extraction technique of *C. auriculata* by Analytical grade (AR) ethanol (96%). Extraction procedure was executed for 18 hr. In brief, the unwanted solvent from *C. auriculata* Leaves Methanolic Extract (CALME) and *C. auriculata* Flowers Methanolic Extract (CAFME) was vaporized (in a vacuum at 65°C) using rotary evaporator (Heidolph, Schwabach-Germany). Extraction procedure was completed in three syphoning cycle (n=3) for 18 hr. The obtained CALME and CAFME was green and brown color sticky mass represent the total percentage yield 4.3% w/w and 2.9% w/w respectively.

Phytochemical investigation

Standard methods were used for recognition of potential Bioactive Phytoconstituents (BPC) namely alkaloid, carbohydrate, flavonoid, glycosides, proteins, saponins, steroid and tannins (Siddique & Al-Samman, 2022).

HPTLC finger print analysis and estimation of secondary metabolite

The procured CALME and CAFME dissolved in methanol (99.85% v/v) and used for HPTLC analysis. The CALME and CAFME were simultaneously applied on aluminum HPTLC plate (Merck-Germany; L x W, 10 cm × 10 cm; 200-m layer thickness) with particle size (10-12 µm) and pore size (60 Å). Analytical grade (AR) quercetin {$C_{15}H_{10}O_7$ (≥95% w/w)} was sonicated (Fisher brand-Model 505-Sonicator- Pittsburgh) in methanol (99.85% v/v) and applied on same HPTLC plate (Merck-Germany) in a standardized concentration (1.0 mg mL^{-1}) by a Camag Linomat (IV) auto sampler. At the end the plate was dried by dryer to evaporate any imaginable solvent from the sample spots. The appropriate mobile phase was selected by various hit and trial method using different mobile phases, including; benzene: ethyl acetate: glacial acetic acid (BEG-2:4:1-v/v/v), toluene: ethyl acetate and hexane (THE-4:2:1 v/v/v), petroleum ether: methanol: chloroform (PMC-8:0.25:1.75 v/v/v) and hexane: dichloromethane: glacial acetic acid (HDG-5:3:2 v/v/v/)} respectively were the developed ternary solvents used to detect CALME and CAFME phytoconstituents. In the next step the HPTLC plate was placed in glass developing chamber that contains the mobile phase (25 mL v/v). The visual graphic of CALME, CAFME and quercetin were established up to the optimum height (80 mm), further the fingerprinting study completed under UV (Single Beam Benchtop T60 UV-vis Spectrophotometer, Deuterium Lamp; India) and quantification of the selected compounds were assured by chromatographic auto scanning (UV λ_{max} 254 and 366 nm) detection systems that configured with WinCat software (Koala *et al.,* 2021).

Docking analyses

Docking analyses were done to find the possible communications between proteins human COX-1 (PDB ID; 6y3c-COX1), TNF (PDB ID;1rj8-TNF) and ligand quercetin (ZINC Id- 3869685), diclofenac (ZINC Id-1281), analgin (ZINC Id-1782155) in.pdb format in Swiss-Dock web server (http://www.swissdock.ch/) (Robb *et al.,* 2020). The selected ligands preparations were completed by ChemDraw-12 software and these structures were scrutinized and submitted to Swissdock for ligand compatibility. In brief the PDB file was uploaded to allow successful docking of receptor with ligand and "Construct the PDB file for docking plug-ins" modules. Before to docking analysis, protein structure of COX1(https://doi.org/10.2210/pdb6Y3C/pdb) and TNF (https://doi.org/10.2210/pdb1RJ8/pdb) were retrieved by RCSB and these structure were finalized by eliminating water (H_2O) molecules through UCSF Chimera software. The binding of the ligand with receptors and calculation of hydrogen (H2) bond amongst the two fragments were studied by Discovery Studio Visualizer (https://discover.3ds.com/discovery-studio-visualizer-download) and UCSF Chimera (https://www.cgl.ucsf.edu/chimera/).

Lipinski rules and drug-likeness study of selected ligand

The drug-likeness was studied by Lipinski rules to predict the potency of selected ligands. The Lipinski's "rule of five (5)" were applied to confirmed whether the selected compound behaved similar to those of drugs and acquired less than five hydrogen donors. A compound is acknowledge as similar to drug by the Lipinsky rule of five if it fulfills two or other additional requirements: it require less hydrogen donors than five, while the Molecular Weight (MW) need to be less than 500 Daltons and lipophilicity (Log P) must be lower than five, and it must have lesser hydrogen bond acceptors than ten.

Biological evaluation Anti-inflammatory assay

Anti-inflammatory activity of CALME and CAFME was studied by mercury displacement method (Demsie *et al.,* 2019). Albino rats of either sex weighing between 150-200 g were selected in the design study and were accommodated under standardized laboratory conditions with an alternate day-night phase for at least seven days to familiarize with the workroom milieu. The rats were fasted for 24 hr before the experiment with water *ad libitum.* Subsequently the rats were randomly divided into three groups of six animals each. Group I received 1 mL Tween-80 (1% v/v p.o) orally, which served as control. Group II received diclofenac sodium at a dose of 30 mg/kg body weight orally (p.o), which served as a standard. Group III received CALME and CAFME (dissolve in DMSO) at a dose of 200 mg/kg body weight orally (p.o). 30 min later of the management of experimental doses (200 mg/kg b.w, p.o), formalin (0.1 mL, 1% v/v) was injected at the right hind paw to produce the edema. Latter on 4 hr after formalin injection the measurements of paw volume were mathematically

analyzed by mercury dislodgment technique with the help of plethysmometer. Finally, the result of percent inhibition of the inflammation was determined after administration of formalin at selected time line (4 hr) of the experiment. All of the animal procedures in this study were conducted in accordance with the with Institutional Animal Care guidelines of Department of Pharmaceutical Chemistry Kuvempu University, Post Graduate Centre Kadur-CPCSEA-12(577 548)-INDIA.

Analgesic assay

Analgesic activity was carried out by tail flick method as guided earlier (Katri & Dąbrowska, 2019). The pre trained healthy albino mice of either sex weighing range between 25- 30 g were selected to assess analgesic activity. Animals were divided into six groups and each group has six experimental animals. The area of the tail (3-4 cm) was stained by picric dye (no physical harm, skin allergy or itching observed) and it immersed in the warm water bath which thermo-statistically regulated at 51±0.5°C. The withdrawal time of the rat's tail from warm water (in seconds) was documented as the reaction time or tail flick time which was documented when the rats indicated any displays sensory activity. The pain signs involved of exciting, beating paw, and leaping. The reaction times were recorded at the interval of 0, 30 and 60 min after the administration of drug. Group I received Tween-80 (1mL, 1% p.o) orally, which served as control. Group II received analgin at a dose of 30 mg/kg body weight, intraperitoneally (i.p), which served as a standard; Group III received CALME and CAFME at a dose of 200 mg/kg body weight per oral (p.o), respectively. The maximum cut off time for immersion was 180 sec to avoid the injury of the tissues of tail. The tail flicking responses of the animals were observed at different reaction times. Tail flick variation or mean increase in latency after drug dosing protocol was used to indicate the analgesia produced by CALME and CAFME and selected standard drugs analgin.

Antimicrobial assay

Preparation of experimental sample

The selected extract namely CALME and CAFME were used as per the design experiment and the suspension of each extract (200 mg) was dissolved separately in 5.0 mL of Diethylformamide (DMF) in a analytical grade sterile glass Test Tubes (TT), to acquire the solution of each extract of 40 mg/mL (w/v) concentration (Shanmuganathan *et al.,* 2018).

Estimation of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) was determined as per the design experimental strategy (Shanmuganathan *et al.,* 2018). Total 8 Test Tubes (TT) were selected and characterized as L1, L2, L3, L4, L5, L6, L7 and L8 for CALME while F1, F2, F3, F4, F5, F6, F7 and F8 for CAFME respectively. Each title extract (200 mg w/w) was added in 5 mL of DMF in first test tube. In subsequent TT transfer 2 mL of previous mixture from 1st test tube and the volume was control up to 4 mL with 2 mL DMF. In the same order serial dilution was established up to eight test tubes to confirm serial dilution. Different sterile glass petri plates of optimum size were taken and marked each of them as, Standard (Std) and Control (C), after a time period of 24 hr the biological activity of bacteria was recorded.

Statistical analysis

The statistical analysis established through One-way ANOVA through Graph Pad prism and experimental values are documented as Mean ± SEM (Standard Error Means) with a p value of <0.05 subjecting as statistically significant.

RESULTS

Phytochemical screening

Potential phytoactive secondary metabolites were either present (✓) or absent (X) according to the results of phytochemical analyses for CALME (percentage yield10.53% w/w) and CAFME (percentage yield 7.53% w/w). Alkaloids, proteins, tannins, carbohydrates, flavonoids, glycosides, saponins, and steroids are among the many herbal elements found in CALME and CAFME. The presence of proteins, carbohydrates, flavonoids, glycosides, saponins, and steroids may be the cause of CALME restorative effects. It's interesting to note that the presence of steroids, proteins, carbohydrates, flavonoids, and glycosides supports CAFME therapeutic claim. The CALME showed negative results for alkaloids and tannins, while CAFME showed absence of phytochemical ingredients, including alkaloids, saponins, and tannins. The Table 1 displays the results of our lengthy phytochemical study which could positively suggesting that both CALME and CAFME are devoid of alkaloids and tannins and scientifically uncover the naturally occurring potential phytoconstituents of *C. auriculata*.

Thin Layer Chromatography (TLC) analysis

The results of chromatographic outcomes are covered in Table 1. The optimized mobile phase was selected by various hit and trial method using different mobile phases. The HPTLC method was augmented to forecast natural therapeutic phytoconstituents in *C. auriculata* extracts. Amongst all short listed mobile phase the HDG (5:3:2 v/v/v/) offered excellent resolution and gave distinct and conspicuous chromatographic spots of CALME and CAFME phytoconstituents respectively. The CALME showed five spots (R1-0.21, R2-0.29, R3-0.43, R4- 0.18, and R5-0.31) at 366 nm while CAFME showed a total of four spots [R1-0.51, R2-0.34, R3-0.24, and R4-0.18)] at 254 nm. Additionally, it has been seen from the HPTLC chromatogram that the target constituents quercetin having the distinct and largest spot at Rf=0.47 (Figure 1F1 and Figure 1F2). Further there was no overlapping and tailing of any phytoconstituents observed on CALME and CAFME

HPTLC chromatogram, this revealed the significant amount quercetin content in CALME (2.08% w/w) and CAFME (0.53% w/w) respectively. Moreover, in an earlier study, quercetin was chromatographically quantified and confirmed to mechanistically reduce the inflammation and pain threshold in experimental rats by correcting the release of the anti-inflammatory mediators.

Anti-inflammatory assay

The anti-inflammatory results of *C. auriculata* against formalin induced inflammation in experimental rats were presented in Table 2 and Figure 1F3. In contrast with control (1%, 1 mL Tween-80), the inhibition ($p<0.05$) doses of CALME and CAFME-treated groups were significantly reduced. Diclofenac sodium exhibited 36.45% of activity under the presented experimental conditions. The CALME showed 32.21% anti-inflammatory activity while CAFME showed 32.21% anti-inflammatory activity respectively. Anti-inflammatory activity is due to present of secondary metabolites namely polyphenolics compounds. The associated hydroxyl groups of polyphenolics compounds suppressed the inflammation due to their radical species scavenging properties (Fakhri *et al.*, 2022).

Analgesic assay

The potential analgesic results of *C. auriculata* were discussed in Table 2. The compiled tail writhes of treated groups (CALME, CAFME) and standard (analgin) were significantly fall down ($p<0.01$) in contrast to control (1%, 1 mL Tween-80). In tail flick method, the activity of inhibition was 56.35, 39.45, 33.89 and 30% of *C. auriculata* extract and 54.04, 46.77, 35.37 and 31.74% of standard drug (analgin) after 15, 30, 45 and 60 min respectively. The analgesic action can be subjected through diverse mechanisms such as, anti-inflammatory effect, prostaglandin-regulated effect, and free nitric oxide, mediation through catecholamine mechanism, binding to neuronal receptors and via serotoninergic system. Similarly to that of non-steroidal anti-inflammatory drugs *C. auriculata* usually do not increase the pain verge in the tissues architectures, as local anaesthetics and narcotics perform.

Antimicrobial activity

The result of antibacterial activity confirmed that, the leaves of *C. auriculata* were found to be active against different set of bacteria tested (*Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa,* and *Salmonella typhi*) at concentration 31.25 µg/0.1 mL (MIC) whereas below that concentration there was no inhibition of extract against microbial organism (Table 3).

Table 1: Phytochemical and chromatography analysis of *C. auriculata* leaves and flowers extracts.

Investigational tests for secondary metabolites			Designated *Cassia auriculata* methanolic extract			
			Leaves		Flowers	
Alkaloids			X		X	
Carbohydrates			✓		✓	
Flavonoid			✓		✓	
Glycosides			✓		✓	
Protein			✓		✓	
Saponnins			X		✓	
Steroids			✓		✓	
Tannins			X		X	
Chromatographic biography of quercetin						
Extraction Solvent (mL v/v)	Extract	Volume of mobile phase (mL /v)	Selected Mobile phase (v/v/v mL)*	Elution Time (minutes)	Rf at 254 nm	Rf at 366 nm
Methanol 5 mL	CALME	10	HDG	7	5(0.21, 0.29, 0.43, 0.18, 0.31)	4(0.51, 0.34, 0.24, 0.18)
	CAFME	10	HDG	7	4(0.21, 0.14, 0.20, 0.13)	4(0.11, 0.19, 0.13, 0.10)
Quantitative analysis of quercetin ($C_{15}H_{10}O_7$) at 254 nm (% w/w)						
Methanol 5 mL	CALME	10	HDG	7	2.08	-
	CAFME	10	HDG	7	0.53	-

Whereas, CALME: *Cassia auriculata* leaves methanolic extracts; CAFME: *Cassia auriculata* flowers methanolic extracts; (✓): Present and (X): Absent; *HDG: Hexane: dichloromethane: glacial acetic acid.

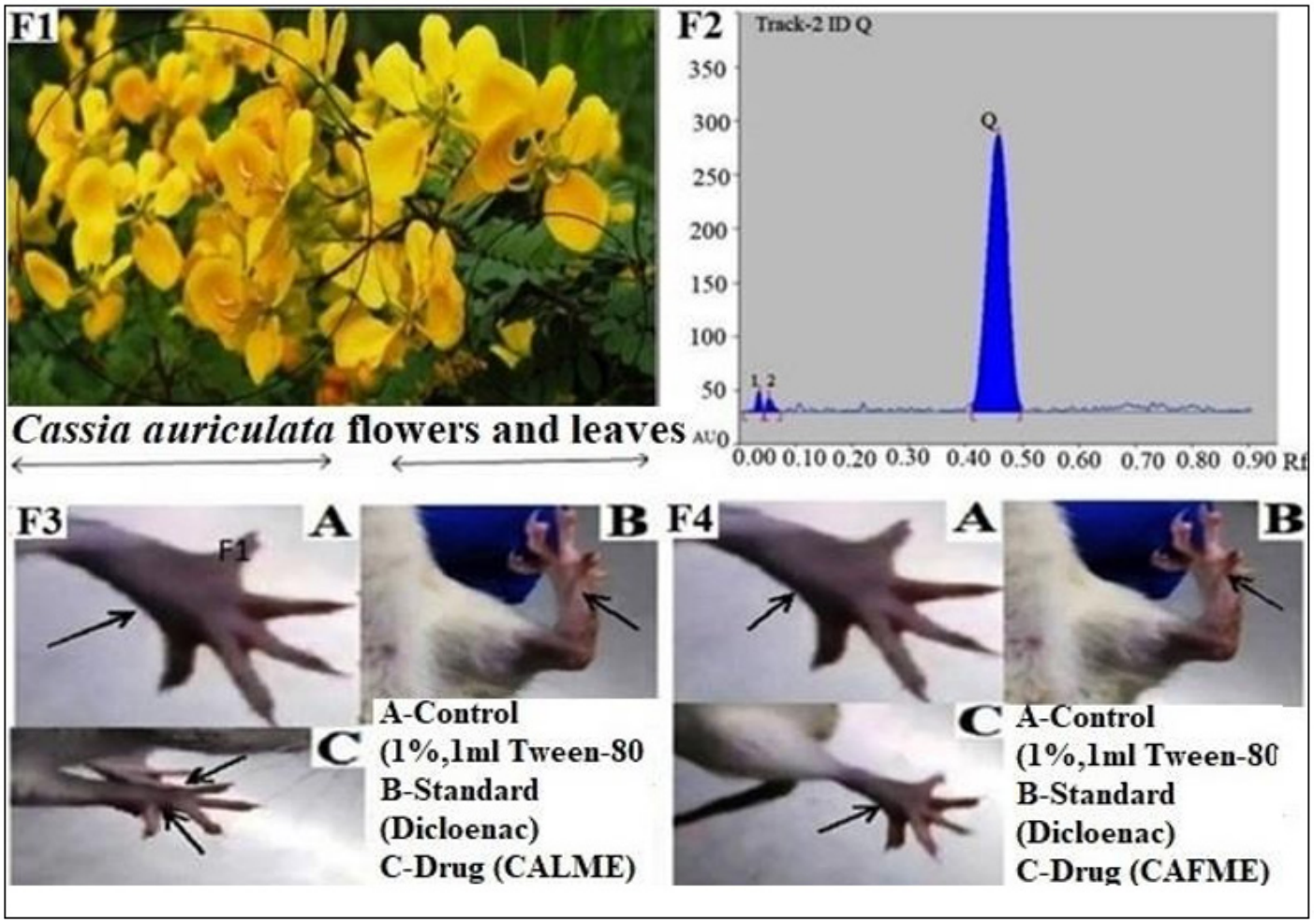


Figure 1: (F1) Handpicked flowers and leaves of *C. auriculata*; (F2) HPTLC chromatogram of standard quercetin; (F3 and F4) Anti-inflammatory activity of (I) CALME: *Cassia auriculata* leaves methanolic extract; (II) CAFME: *Cassia auriculata* flowers methanolic extract.

Molecular docking of inflammatory protein COX-1 (6y3c-COX1) and ligands (quercetin, diclofenac and analgin)

The selected protein COX-1 (6y3c-COX1) was belongs to Human COX-1 category and allied with inflammatory sensations and pain transmitters (Patrono, 2016). COX-1 receptor has a range of involvements in body physiological environment such as thermoregulation, flexibility, endogenous proteins drug interaction and hypo tonicity (Ghazanfari *et al.,* 2021). Similarly, qercetin is described by the researcher in diverse issues, including liver cirrhosis, jaundice, cancer, and as a natural antioxidant agent (Hou, 2024).

Molecular docking analysis

The docking analysis is a significant tool to forecast the compatibility amid accountable receptor and ligand and associated with diverse parameters to contribute better outcomes of Proteins and Ligand (PL) complexes (Agu *et al.,* 2023). The bioactive constituent of *C. auriculata* leaf is cast-off as ligand fragments to dock with COX-1 (6y3c-COX1) protein. The quercetin quantifies from CALME while diclofenac and analgin served as a standard ligand drug were allowed to dock with the designated protein COX-1 (6y3c-COX1). Results explore successful docking of ligand (quercetin, diclofenac and analgin) individually with protein COX-1 (6y3c- COX1). Quercetin scored the least binding energy value (-8.29 Kcal/mol) Figure 2F1, that was nearest to diclofenac binding affinity energy (-8.27 Kcal/mol) Figure 2F2, while record higher binding affinity energy (-7.77 Kcal/mol) then analgin (Figure 2F3). The potential binding sites of protein COX-1 (6y3c-COX1) for diclofenac were forecasted from protein residue that comprises of two amino acid ARG-433 and LEU-509 at a distance of 3.27Å and 3.54 Å respectively. Similarly, the binding sites of COX-1 (6y3c-COX1) for quercetin were predicted from protein residue that attached with four amino acid ARG-438 (3.25 Å), GLU-486 (3.31 Å), GLY-214 (3.35 Å) and LEU-508 (3.58 Å) respectively. Further the binding pockets of COX-1 (6y3c- COX1) for analgin were predicted from protein residue that attached with two amino acid CYS- 512 (3.51 Å) and HSD-513 (2.76Å) respectively (Table 4). Our study forecast the potential anti- inflammatory order of analgin>quercetin>diclofenac. Additionally, this prediction

plugs-in a virtuous relationship between the phenolic constituents (quercetin) and the anti-inflammation prospective of CALME.

Molecular docking of inflammatory protein TNf (1rj8-TNF) and ligands (quercetin, diclofenac and analgin)

The best fitted poses adopted by quercetin, was cast-off as CALME derived natural ligand fragments while diclofenac and analgin allowed docking with TNf (1rj8-TNF) protein as a reference ligand. As discussed in Figure 3F1, Figure 3F2 and Figure 3F3. Docking results gave a successful distinct PL interaction with protein TNf (1rj8-TNF). The result of lowest binding energy compound was recorded as good healing drug candidate as compared with binding energy of others PL complexes. Analgin scored the least binding energy value (-9.87 Kcal/mol), that was higher than diclofenac binding energy (-9.64 Kcal/mol) and quercetin binding energy (- 9.23 Kcal/mol) respectively. The potential binding sites of protein TNf (1rj8-TNF) for analgin were predicted from protein residue that contained one amino acid GLN-358 at a distance of

3.13Å. Likewise the binding sites of TNf (1rj8-TNF) for diclofenac were predicted from protein residue that linked with two amino acid residue GLN-358 (3.27 Å) and ARG- 298 (2.40 Å) respectively. Additionally, the binding pockets of titled protein

Table 2: Results of anti-inflammatory and analgesic activity of *C. auriculata* different extracts.

Anti-inflammatory activity of CALME.								
Groups	**Treatment**	**No. of animal (n)***	**Dose (mg/kg body weight)**	**Mean value of edema after 4hr**				
I	Control (1%, 1 mL Tween-80)	6	-	0.169±0.038#				
II	Standard (Diclofenac sodium)	6	30	0.122±0.026*				
III	CALME	6	200	0.139±0.091**				
Anti-inflammatory activity of CAFME								
I	Control (1%, 1 mL Tween-80)	6	-	0.235±0.050#				
II	Standard (Diclofenac sodium)	6	30	0.145±0.67*				
III	CAFME	6	200	0.160±0.090**				
Analgesic activity of CALME								
Groups	**Treatment**	**No. of Animal (n)***	**Dose (mg/kg body weigh)**	**Mean time (minutes) taken to withdraw the tail at different time intervals ± S.E**				
				0 min	**15 min**	**30min**	**45min**	**60min**
I	Control (1%, 1 mL Tween-80)	6	0	1.67± 0.173#	1.83± 0.141#	1.98± 0.129#	2.83± 0.071#	2.92± 0.132#
II	Standard (Analgin)	6	30	3.7± 0.119*	5.42± 0.138*	6.2± 0.108*	8.0± 0.168*	9.2± 0.138*
III	CALME	6	200	2.77± 0.141**	5.21± 0.221**	7.15± 0.20**	8.35± 0.112**	8.81± 0.334**
Analgesic activity of CAFME								
I	Control (1%, 1 mL Tween-80)	6	0	1.22± 0.11#	1.85± 0.18#	2.14± 0.15#	2.19± 0.03#	2.23± 0.17#
II	Standard (Analgin)	6	30	1.80± 0.02**	3.35± 0.18**	4.78± 0.05**	5.76± 0.31**	7.19± 0.74**
III	CALME	6	200	1.58± 0.19*	2.85± 0.02*	3.82± 0.12*	4.86± 0.10*	6.46± 0.18*

Values are Mean±S.E; One-way Analysis of Variance (ANOVA) followed by Dunnett test #$p<0.01$ versus group I; Control, *$p<0.05$, **$p<0.01$ versus standard-Diclofenac sodium and analgin (30 mg/kg b.wt.), CALME; *Cassia auriculata* leaves methanolic extracts; CAFME; *Cassia auriculata* flowers methanolic extracts; $n^*=6$.

for quercetin were expected from protein residue that involved with one amino acid LEU-353 at a distance of 3.56 Å. Our study forecast the potential analgesic and anti-inflammatory order of quercetin>diclofenac>analgin with TNf (1rj8-TNF). These outcomes were well aligned with *in vivo* results where quercetin significantly ameliorated the pain and inflammation comparable to analgin and diclofenac. These outcomes were also coordinated with recent findings (Khoswanto & Siswandono 2022; Liu *et al.*, 2022). Moreover, this forecasting established efficacious link amid the polyphenolic phytoconstituents, analgesic and the anti-inflammation approach of CALME. The relationships of PL binding energies are discussed in Figures 4 (A) and (B).

Drug-likeness analysis of selected compounds

The results analysis of Lipinski's five rules is discussed in Table 4, an orally active compound must have a Molecular Weight (MW)

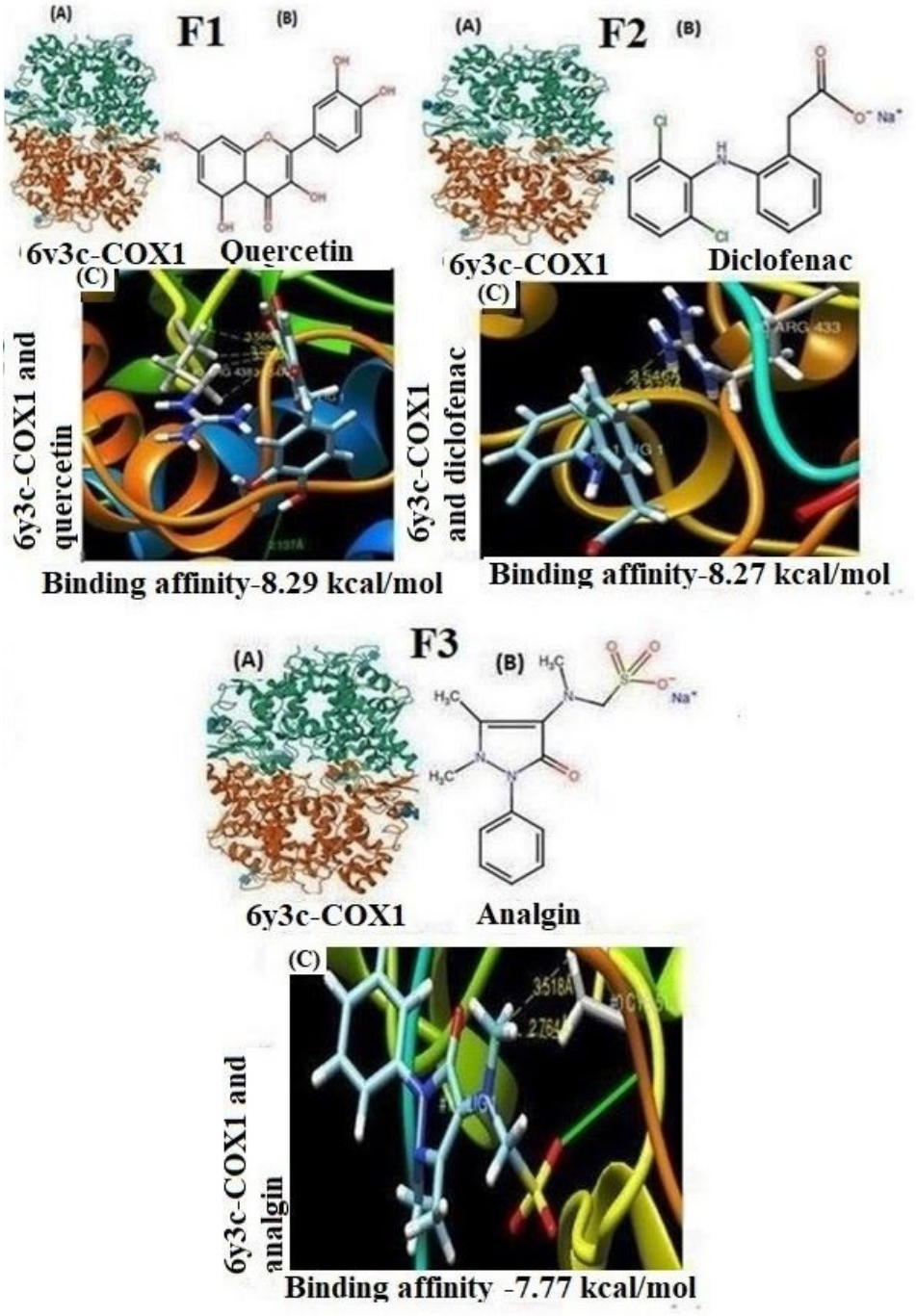


Figure 2: Computational representation and molecular docking analysis. F1: (A) Protein 6y3c- COX1, (B) Ligand quercetin (C) Docking between 6y3c-COX1 and quercetin; F2: (A) Protein 6y3c-COX1, (B) Diclofenac, (C) Interactions between 6y3c-COX1 and diclofenac; F3: (A) 6y3c-COX1, (B) Analgin, (C) 6y3c-COX1 and analgin interactions.

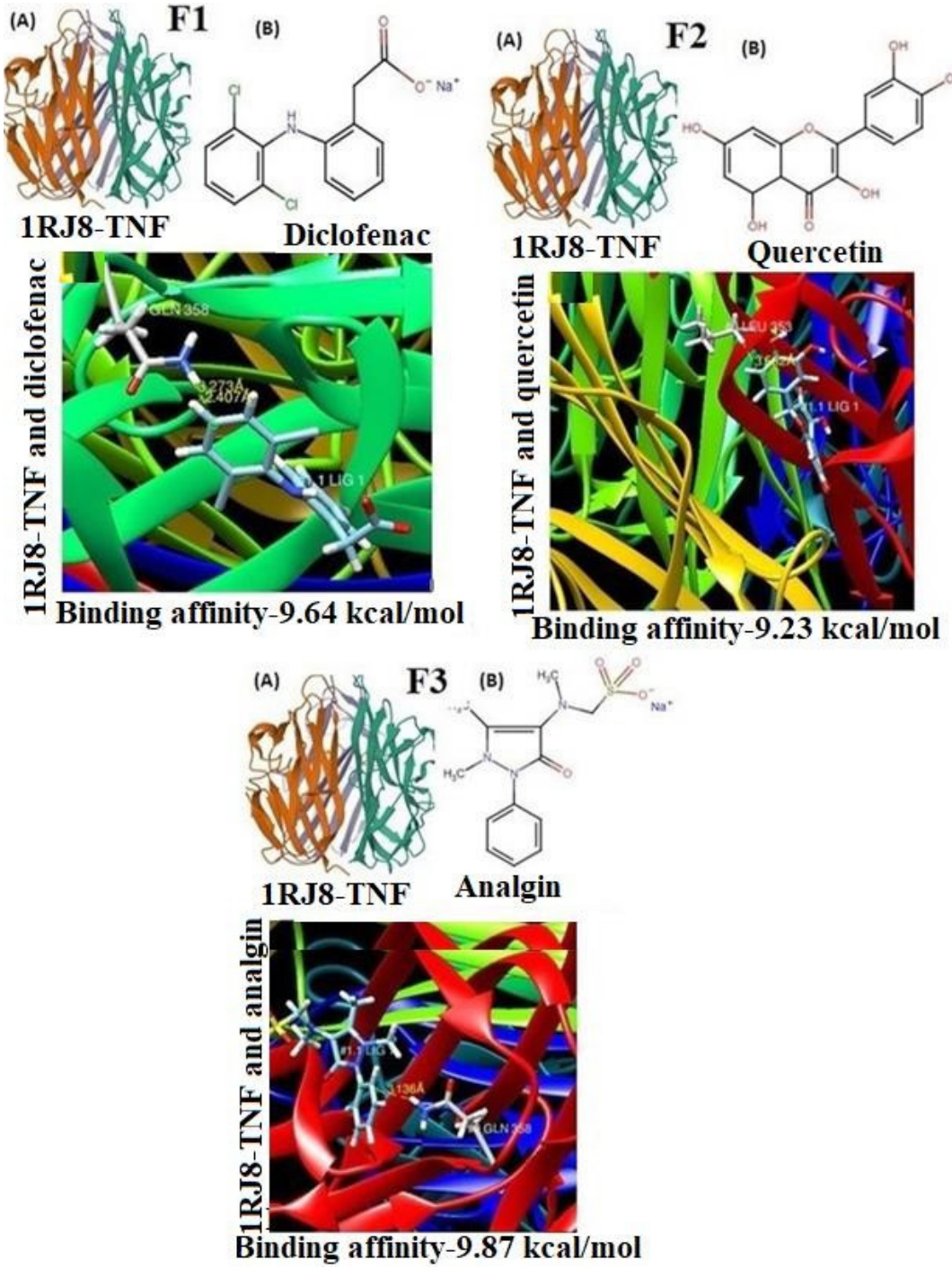


Figure 3: Molecular docking analysis. F1: (A) Protein1RJ8-TNF, (B) Ligand quercetin, (C) Docking between protein 1RJ8-TNF and quercetin; F2: (A) Protein 1RJ8-TNF, (B) Diclofenac, (C) Interactions between 1RJ8-TNF and diclofenac; F3: (A) 1RJ8-TNF, (B) Analgin, (C) Docking between 1RJ8-TNF and analgin.

not beyond 500 g/mol, Hydrogen Bond Acceptors (HBAs) not more than 10, Hydrogen Bond Donors (HBDs) not beyond 5, Log P value must be lower than 5, and the rotatable bonds not less than 10. Drug-likeness analysis suggests, a drug is said to be non-oral active if two or more of these rules are not considered. Quercetin has fulfilled the Lipinski's five rules and showed good drug-likeness factors similar to diclofenac and analgin.

DISCUSSION

As described above, in this finding, we acknowledge the mechanistic role of natural phytoconstituents born from *C. auriculata* as an analgesic and inflammatory agent and additionally proposed as a biomedicine against pathogenic impairment. The presence of plant secondary metabolites are scientifically defined as tannins, saponins, alkaloids, and flavonoids, was investigated

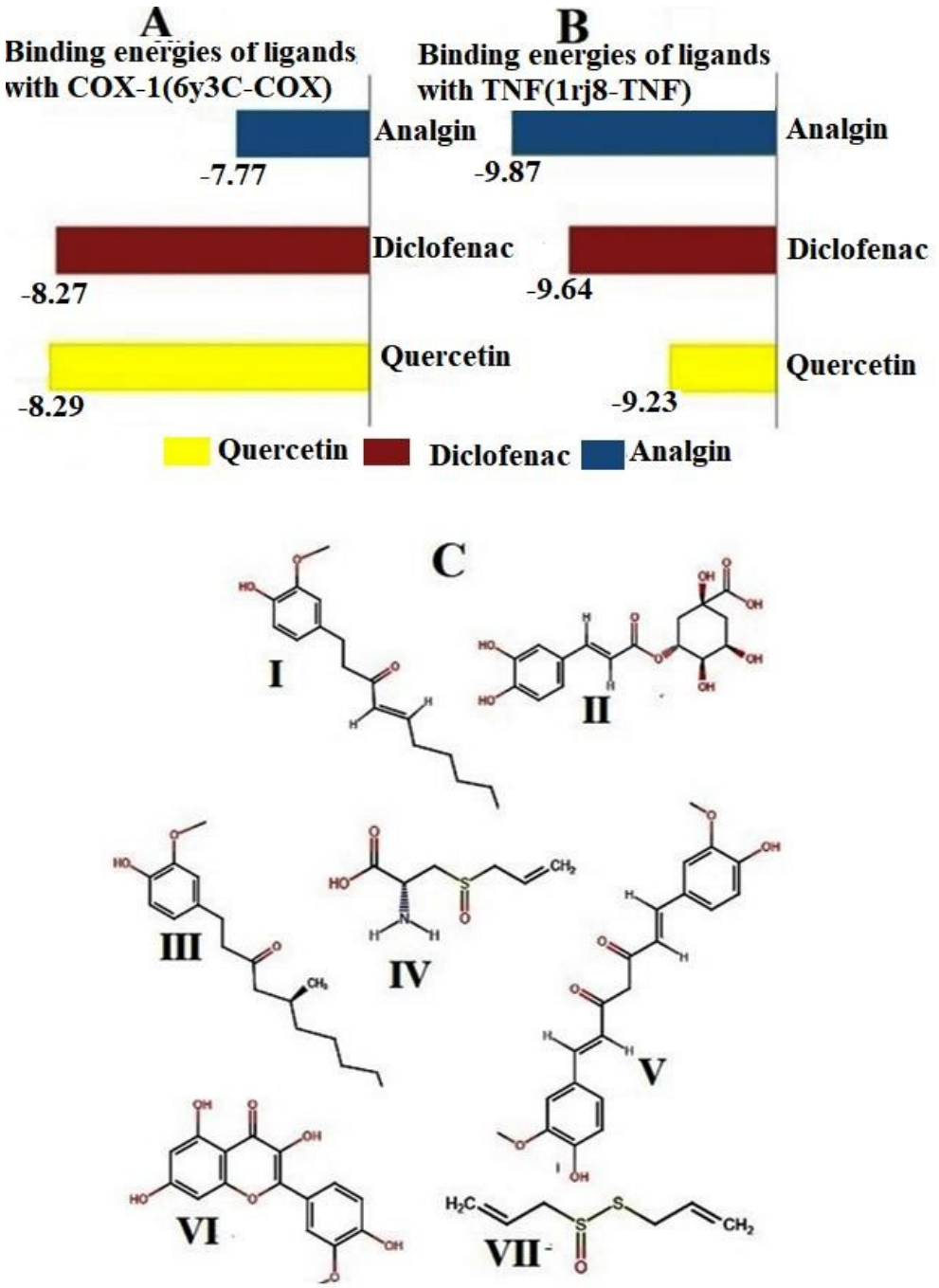


Figure 4: (A) Comparative explanation of binding frees energies of protein 6y3c-COX1 and (B) TNF (1rj8-TNF) with synthetic ligand diclofenac, analgin and compared with quercetin. (C) COX: Cyclooxygenase-inhibiting secondary metabolite (I) 6-shogaol, (II) Chlorogenic acid, (III) 6-gingerol, (IV) Alliin, (V) Curcumin, (VI) Rhamnetin, (VII) Allicin. Structures were obtained from ACD/ChemSketch-12.01 software and https://pubchem.ncbi.nlm.nih.gov.

in CALME and CAFME. It has been widely accepted in previous research that the presence of polyphenolics and tannins in the title plant's leaves and flowers are pharmacologically required for its anti-inflammatory, analgesic, and antibacterial properties (Rahman *et al.,* 2024; Burayk *et al.,* 2022; Sohilait *et al.,* 2017). Tannins and saponins are one of the trendiest secondary metabolites said to be useful in diabetes, arthritis, nerve problem, as a muscle relaxant, antioxidants, antibacterial, and nephroprotective agents (Das *et al.,* 2020). The throbbing effect of prostaglandins, which cause pain and inflammation, may be lessened by a high level of plant content. The plant components of CALME and CAFME are now thought to be anti-inflammatory and analgesic agents and they may be protective candidates for pain and inflammation.

Further, our study indicates that both CALME and CAFME exhibit anti-inflammatory effects against paw edema induced by

formalin, depending on the dosage used. The anti- inflammatory effect observed with a dosage of 200 mg/kg of *C. auriculata* extracts was slightly less effective compared to that of diclofenac. Just like carrageenan, the formalin-induced edema model is recognized as a scientifically valid method for studying inflammation, as it lacks systemic antigenic properties. Furthermore, the proposed model is highly precise, effective, and demonstrates a significant degree of reproducibility. From a biological perspective, the mechanism behind formalin-induced edema can be interpreted through a two-tiered response.

The earlier aspects of pharmacology conclude with the production of inflammatory mediators, specifically cellular serotonin, circulating histamine, and cytokines, while another aspect of inflammation involves the formation and release of prostaglandins thus, our research clearly indicates that *C. auriculata* can be effectively categorized as an anti-inflammatory agent (Hoffmann & Klemm 2022; Pandey *et al.,* 2021).

It is, however, interesting to note that in addition to other pain causing methods, the formalin assay is a widely accepted and accurate technique to understand pain mechanism by inducing and measuring pain responses in animals (Patil *et al.,* 2019). This method creates a mild to moderate pain sensation that the rats quickly respond to, making it susceptible to Over-the- Counter (OTC) pain relief (Mitra *et al.,* 2016). Administering formalin (0.1 mL, 1% v/v) led to noticeable licking reactions in the chosen animals. Hence, the current findings suggest that extracts from *C. auriculata* may have a decreasing effect on licking responses in rats treated with formalin, indicating their potential as pain relievers. However, the doses of CALME and CAFME used showed a lower pain-relieving effect compared to diclofenac. Similarly, CALME and CAFME effectively reduced swelling caused by formalin, as well as the number of tail movements due to heat stroke, and decreased the frequency of licking in rats given formalin (0.1 mL, 1% v/v).

Table 3: Results of antibacterial activity of *C. auriculata* extracts and standard drug.

Leaves methanolic extract									
Dose (µg/0.1 mL)			Zone inhibition (mm)						
			B.S		E. C		P.A	S.T	
Std. (Std1-Std8)	Extract (F1-F8)	Std.	Extract	Std.	Extract	Std.	Extract	Std.	Extract
1000	4000	30	19.5	29.5	17	35	20	32	21
500	2000	30	18	26.5	16.5	31	19	31.5	18
250	1000	29	16	25	16	31	16	29	16
125	500	27	15	24	15	23	14	25.5	15
62.5	250	21	14	17.5	13	24	13	23	12
31.25	125	23	13	19.5	12	21	13	19	11
15.63	62.5	20	11	16	11	20	12	18	9
7.8	31.25	13	10	15	9	17	10	16.5	5
Control	DMF-Zero Activity								
Flowers methanolic extract									
Std. (Std1-Std8)	Extract (F1-F8)	Std.	Extract	Std.	Extract	Std.	Extract	Std.	Extract
1000	4000	30	19.5	29.5	17	35	20	32	20
500	2000	30	18	26.5	16.5	31	19	31.5	16
250	1000	29	16	25	16	31	16	29	13.5
125	500	27	15	24	15	23	14	25.5	11
62.5	250	21	14	17.5	13	24	13	23	9
31.25	125	23	13	19.5	12	21	13	19	6.5
15.63	62.5	20	11	16	11	20	12	18	5
7.8	31.25	13	10	15	9	17	10	16.5	2.5
Control	DMF-Zero Activity								

(Std1-Std8): Gentamicin; Extract (L1-L8): *Cassia auriculata* leaves methanolic extracts; Extract (F1-F8)-*Cassia auriculata* flowers methanolic extracts; B.S: *Bacillus subtilis*, E.C: *Escherichia coli*; P.A: *Pseudomonas aeruginosa*, S.T: *Salmonella typhi*; DMF: Dimethyl formamide.

Table 4: *In silico* study and Lipinski rules analysis of bioactive ligand quercetin and synthetic ligand.

Comparative docking results of proteins COX1 and TNFα.								
Docking analysis with COX1								
Ligand	Docking score Kcal/mol	No of residue	No of hydrogen	Distance Å				
Quercetin	-8.29	ARG-438, GLU-486, GLY-214, LEU-508	4	3.25, 3.31, 3.35, 3.58				
Diclofenac	-8.27	ARG-433, LEU509	2	3.27, 3.54				
Analgin	-7.77	CYS-512, HSD-513	2	3.51, 2.76				
Docking analysis with TNFα								
Quercetin	-9.23	LEU-353	1	3.56				
Diclofenac	-9.64	GLN-358, ARG-298	2	3.27				
Analgin	-9.87	GLN-358	1	3.13				
Comparative Lipinski rules analysis								
Targeted ligand	Molecular formula	Molecular Weight (g/mol)	NHD	NHA	NRB	TPS A(Å^2)	cLogP	Follow Lipinski's Rule five*
Quercetin	$^{C}15^{H}10^{O}7$	302.238	3	7	1	127	1.988	0
Diclofenac	C14H11Cl2NO2	296.153	1	3	4	52	4.364	0
Analgin	$^{C}13^{H}17^{N}3^{O}4^{S}$	311.363	0	7	4	87	0.766	0

NHD: hydrogen-bond donors; NHA: hydrogen-bond acceptors; TPSA: Parametric polar surface area; Artistic explanation; NHD ≤ 5, NHA ≤ 10, MW ≤ 500 daltons, and clog $p \leq 5$.

We reported significant results of *in silico* anti-inflammatory and analgesic activity of bioactive ligand (quercetin), standard drug (diclofenac and analgin) docked with proteins COX-1 (6y3c-COX1), and TNf (1rj8-TNF). The COX-1 protein mechanistically augments the inflammation process by triggering inflammatory mediators namely interleukins, prostaglandin and cytokines (Loke *et al.,* 2008). We found significant amount of quercetin in CALME and CAFME which predicted as anti-inflammatory and analgesic compound comparable to diclofenac and analgin. In an earlier study *C. paradise* phytoconstituents (beta-pinene, alpha- pinene, and linalool) were reduced inflammation and pain threshold in experimental rats (Alazragi & Baeissa, 2023). In their study the researchers selected these phytoconstituents as ligand and allow docking with anti-inflammatory protein PTGS2 and they documented sufficient result as paralleled to diclofenac (Zahra *et al.,* 2023). Significant binding energy and h-bond cloud showed a potential reduction in inflammation by diclofenac in COX-1 (6y3c-COX1) docking analysis, whereas quercetin was next to diclofenac and can be used as anti-inflammatory agent that have comparable docking binding energy to diclofenac.

Correspondingly the computational analysis revealed encouraging results for analgesic activity. This phenomenon aligns with the binding energy of anlgin which was also greater than the quercetin (Figure 4). The *C. auriculata* derived quercetin and standard drug analgin was docked with TNf (1rj8-TNF). The binding energy of analgin was significantly comparable with quercetin which confirmed the medicinally importance of these phytoactive constituents contrary to inflammation and pain transmittance. Likewise, the selected ligand (analgin) was powerfully held by one h-bond (GLN-358) residues as well quercetin also held by one h-bonds with LEU- 353 while diclofenac held by two h-bond GLN-358 and ARG-298 respectively. Highest binding energy and h-bond cloud showed a significant reduction in pain by analgin in TNf (1rj8-TNF) docking, whereas quercetin and diclofenac was next to analgin can be utilized in pain treatment due to the free binding energy comparable to analgin (-9.87 kcal/mol).

To ascertain their drug-likeness properties, drug must confirm the basic physicochemical properties, the drug with molecular weight equal to or lower than 500 g/mol understand well for Absorption, Distribution, Metabolism and Excretion (ADME). Lipophilicity influences the solubility of drug across the cell membrane. In our study all the ligands were adheres to Lipinski's rule of five and expressed good bioavailable in Table 4. When a medication is engaged orally, its biological ability is the part of the medicament that circulates in the blood fluid. The frontline determination of the molecule's bioavailability is Gastrointestinal (GIT). Therefore, adequate hydrophillicity is compulsory for a drug claims be orally bioavailable. The acceptable compound should be greatly absorbed, metabolized, and released from the biological flow without expressing any hazardous effects.

The representative key for *C. auriculata* may be utilized in a synergistic manner to decrease the required dosages of

anti-inflammatory and analgesic pharmaceuticals, thereby minimizing potential harm to patients and promoting environmental sustainability. However, this study does not provide detailed insights into the mechanisms by which the analgesic and anti- inflammatory metabolite quercetin operates at the genetic level, nor does it clarify its relationship with the cellular assembly of protein structures. Conversely, our research suggests that quercetin can play a synergistic role in lowering the dosages of contemporary analgesics and anti-inflammatory medications, attributed to its abundant polyphenolic composition, which contributes to effective analgesic, anti-inflammatory, and antimicrobial properties. Furthermore, the studies presented support a potential correlation, indicating that docking analyses yield consistent data for drug design, comparable to other methodologies in botanical research. It will be essential in the future to evaluate the quantity of gene transcripts produced in *C. auriculata* prior to its application as a biomedicine.

CONCLUSION

The overall context of the current finding indicated that *C. auriculata* played a critical function in reducing inflammation and discomfort, which are controlled by TNf (1rj8-TNF) and COX-1 (6y3c-COX1). Beyond their benefits, the majorities of sophisticated analgesics and anti- inflammatory medications are highly costly and have a wide range of adverse effects. In light of this, we present a natural medication regimen as a multifaceted approach to regulating the TNF and COX-1 pathways. This study will be a versatile and effective drug discovery strategy that will yield evidence-based mechanisms of action of *C. auriculata.*

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ABBREVIATIONS

HPTLC: High-Performance Thin-Layer Chromatography; BUltraviolet spectroscopy; **OTC:** Over-the-counter; **NSAIDs:** Nonsteroidal anti-inflammatory drugs; **PL:** Proteins and ligand; **DMSO:** Dimethyl sulfoxide; **HBAs:** Hydrogen bond acceptors; HBDs**:** Hydrogen bond donors; **COX:** Cyclooxygenase; **LOX:** Lipoxygenase; **ANOVA:** Analysis of Variance. **CALME:** *Cassia auriculata* Leaves Methanolic Extract; **CAFME:** *Cassia auriculata* Flowers Methanolic Extract; **AR:** Analytical Grade; **BPC:** Bioactive Phytoconstituents; PDB: Protein Data Bank; **ROS:** Reactive Oxygen Species; **MW:** Molecular Weight; **DDG:** Drug-Disease-Gene; **PB:** Protein Binding; **DMF:** Diethylformamide; **MIC:** Minimum Inhibitory Concentration; **SEM:** Standard Error Means; **TLC:** Thin Layer Chromatography; **Rf:** Retention factor; **GIT:** Gastrointestinal tract; **NHD:** Hydrogen-bond donors; **NHA:** Hydrogen-bond acceptors; **NRB:** Number of rotatable bonds; **TPSA:** Parametric polar surface area.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization, *in silico* analysis, writing, Nadeem Ahmad Siddique; writing, Mohd Rasheeduddin,Imran; data collection, Sayeeda Anjum; data collection, Ali Mohammed Mohammed Ali AL-samman; writing, Abida Khan.

SUMMARY

This research investigates the analgesic and anti-inflammatory properties of *C. auriculata* and demonstrates the mechanism of action of bioactive quercetin. To manage pain and inflammation, we utilized quercetin extracted from CALME. The proteins COX-1 (6y3c-COX1) and TNf (1rj8-TNF) were subjected to docking studies with quercetin, diclofenac, and analgin. Among these, analgin exhibited the highest binding affinity energy value of -7.77 Kcal/mol, whereas quercetin showed the lowest at -8.29 Kcal/mol, which is closest to diclofenac's value of -8.27 Kcal/mol. The findings from the *in silico* study were promising, indicating that the binding energy values for anti-inflammatory agents were more efficient than those for analgesics, suggesting a potential alternative to current therapeutic options. Additionally, *C. auriculata* may be employed synergistically to minimize drug dosages, thereby reducing harm to patients and being more environmentally friendly. However, this study does not provide detailed insights into whether, or how, the analgesic and anti-inflammatory targeted metabolite quercetin operates at the gene level, or if it is related to the cellular assembly of protein structures. Conversely, our research supports the notion that quercetin can play a synergistic role in lowering the dosage of contemporary analgesics and anti-inflammatory medications, attributed to its rich polyphenolic structure, which contributes to its potent analgesic, anti-inflammatory, and antimicrobial properties. It will be crucial in the future to evaluate the quantity of gene transcripts produced in *C. auriculata* prior to their application as a biomedicine.

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