INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 24, No. 4, p. 24-36, 2024

RESEARCH PAPER OPEN ACCESS

Phytoconstituents of *Cassia auriculata Linn* inhibits the inflammatory enzymes (5-LOX, COX-1 and COX-2): An *insilico* study to identify anti-inflammatory drug candidates

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Key words: Pro-inflammatory cytokines, Interleukin (IL), Tumor necrosis factor-alpha (TNF-alpha), Ethnomedicine

http://dx.doi.org/10.12692/ijb/24.4.24-36 Article published on April 03, 2024

Abstract

Inflammation is the most essential part of body's immune system and responsible for diseases manifestation in asthma, rheumatoid arthritis, allergy, aging, autoimmune diseases and etc. During of these conditions, many cytokines were regulated. The pro-inflammatory cytokines perform an essential function in development of an effective defense against disease infections and progression. Along with cytokines many enzymes play a critical role in regulation of inflammation. The enzymes such as 5-LOX, COX-1 and COX-2 are vital targets. Objective of study is to comprehensive screening of highly potential and precise phytoconstituents from ethnomedicinally important *Cassia auriculata Linn* which may have potential inhibitors for pro-inflammatory targets. Methanol extract of *Cassia auriculata Linn* leaf was subjected to HR-LCMS analysis. The phytochemical signature was analyzed and 85 phytochemicals are taken for the molecular docking studies with pro-inflammatory markers. After, through screening 10 compounds were showing good binding energy with hydrogen bond interaction with all three targets. Further, these compounds were subjected to ADME property prediction, wherein 6 compounds were showing the parameter values within acceptable range. Toxicity prediction reveals the 5 compounds are non-toxic. *Insilico* investigations revealed that 5 phytoconstituents namely Gallic acid; 2,6-Dihydroxybenzoic acid, Kaempferol; N-(3-Benzooxazol-2-yl-4- hydroxy-phenyl)-2-ptolyloxyacetamide; and Glycophymoline of *Cassia auriculata Linn* methanol leaf extract are potentially inhibiting the inflammatory enzymes. The study identified the precise phytoconstituents from *Cassia auriculata Linn* for anti-inflammatory activity.

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Introduction

Inflammation is considered to be the body's defensive reaction to tissue damage and infection. Vasodilation and the migration of immune cells and plasma proteins to the site of inflammation are features of inflammation. Clinical signs including fever (heat), dolor (pain), rubor (redness), and tumor (swelling) can be found in the most well-known description of inflammation (Hurley 1964) When an innate immune system is stimulated by infections, dead cells, or irritants, it produces inflammation as a defensive immunological response (Guo, Callaway *et al.* 2015). Acute inflammatory responses might potentially lower the risk of infection or harm by creating cellular and molecular associations. Also, this mechanism prevents acute inflammation and supports tissue homeostasis. However, uncontrolled inflammation causes over time inflammatory disorders like Alzheimer's disease, osteoarthritis, Parkinson's disease, obesity, coronary heart disease, diabetes mellitus and cancer (Das and Buchholz 2019). The arachidonic acid pathway provides several anti-inflammatory targets and is responsible for producing inflammatory mediators. When treating inflammation, NSAIDs are the most often prescribed drugs because they inhibit the enzymes cyclooxygenase (COX) and lipoxygenase (5-LOX) (Mukhopadhyay, Shukla *et al.* 2023). The cause of inflammation begins and increases with the over expression of arachidonic acid (AA) cascade mediators, particularly those originating from the pathways of 5-LOX and COX (P, Manju *et al.* 2018).

Medicinal plants contribute an important component of the nation's natural resources. Those provide a major contribution to providing the rural population with basic healthcare services. They operate as essential raw materials for the manufacturing of traditional medicines as well as therapeutic drugs (Rupeshkumar, Kavitha *et al.* 2014). *Cassia auriculata Linn* is one such plant is well known for its ability to effectively treat a number of diseases. It is an evergreen shrub with beautiful yellow flowers. Various Asian locations, especially India, facilitate the growth of this plant (Salma, Janhavi *et al.* 2021). *Cassia auriculata Linn* belonging to Caesalpiniaceae (Fabaceae) family, In Indian ethnomedicine, this plant commonly known as 'Tanner's Cassia' or 'Avartaki' called in Ayurveda, 'Avaram', 'Avarike', 'Taravada', 'Aval' and 'Hemapushpam'. It is found throughout wasteland in Asia. This plant species have been used in traditional ayurvedic medicine to heal or relive of simple problems like gum and teeth pain, ulcer, fracture, soreness and snakebite pain. The different parts of the *cassia auriculata Linn* plant has been reported its use in management of several therapeutic purpose like anti-inflammatory, antimicrobial, wound healing, local anesthetic and smooth muscle relaxant activities etc.,(Gupta, Tandon *et al.* 2008, Surana, Gokhale *et al.* 2008, Rajagopal and Rajakannu 2022). Therefore, it is very important to know precise phytoconstituents to improve the therapeutic usage, as using crude extract or crude material for the treatment may have side effects or reduced efficacy. To address the limitation, our study attempts to determine the active phytoconstituents for anti-inflammatory property (Chanderraj 2023).

In our study, the leaf extract of *cassia auriculata Linn* is prepared using *methanol* and subjected it for LCMS analysis. Followed, by identified compounds were subjected for various *insilico* tools to understand the inhibitory effective enzymes called 5-LOX, COX-1 and COX-2. Our present study emphasized the importance of natural product derived dual COX-LOX inhibitors and summarized the bioactive natural products isolated from *cassia auriculata Linn* leaf extract showed potent dual COX-LOX inhibition properties. The study reveals the precise and potential bioactive compounds having dual target inhibitory property, such compounds may further subject to validation studies, results in alternative therapy for the management of inflammatory diseases.

Materials and methods

Plant sample collection

Cassia auriculata Linn plant is collected from the premises is of Karnataka State Akkamahadevi Women University, Vijayapura (KSAWU), Karnataka. Plant was authenticated by Department of Botany, KSAWU, Karnataka by referring to Madras flora. The

fresh and healthy plant of *Cassia auriculata Linn* leaves are separated, than washed with running tap water, followed by distilled water and dried properly in the shade for 1-2 weeks to remove the moisture. Further, leaves were powdered by a grinder and passed through a 40 mm size mesh sieve (Petrenko, Timofeev *et al.* 2022). The powdered leaves material were stored in an airtight container and used for further studies.

Preparation of extracts

About 50 gm of shade dried leaves powder of *Cassia auriculata Linn* was extracted in Soxhlet assembly with methanol solvent (polarity index: 5.1, boiling point: 64.7 ºC) not exceeding the boiling point. The 48 hours of extract was concentrated under reduced pressure in the rotary vacuum evaporator. The extract obtained with methanol solvent was weighed, and the percentage yield was calculated in terms of the dried weight of the leaves powder of plant. Percentage of yield of extract is 60% (Raja, Jeganathan *et al.* 2013).

Identification of Phytochemical profile by HR-LCMS

The phytochemical profile of the obtained crude methanolic extract from *Cassia auriculata Linn* leaves was analyzed using High Resolution-Liquid Chromatography Mass Spectroscopy method (Instrument- HRLCMS-qToF-agilent Technologies, USA). Data acquisition software is Agilent Mass Hunter. Data Processing Software: Agilent Mass Hunter Qualittive Analysis B.06. Column details are ZORBAX Eclipse Plus -C18 150 x 2.1 MM, 5 microns (Aglient). Solvent used are solvent A is 0.1% formic acid in Milli-Q water and solvent B used as acetonitrile. Ion mode is dual AJS ESI. MS-resolution power minimum range 200 m/z. Mass resolution threshold is 0.010 %. Column temperature is 40°C and Injection volume is 5 µL. The 85 phytoconstituents are resulted in HR-LCMS (Ramakrishnan, Kalakandan *et al.* 2018) (Performed at SAIF, IIT Mumbai, INDIA).

Ligand preparation

The 85 phytoconstituents of *Cassia auriculata Linn* were selected for virtual screening and molecular docking study against pro-inflammatory cytokines. The 3D structures of 85 phytoconstituents are retrieved from database called PubChem (https://pubchem.ncbi.nlm.nih.gov/) (Kim, Thiessen *et al.* 2016). Using the LigPrep of Schrödinger maestro the ligands were prepared. Ligands were converted 2D to 3D structures by including tautomeric variations, ionization, stereo chemical, also energy minimization and optimization for ligands geometry, desalted and corrected for ligands chiralities and missing hydrogen atoms. Ligands bonds orders were fixed, and neutralized of charged groups. The tautomeric and ionization states were generated between 6.8 to 7.2 pH using Epik module (Sastry, Adzhigirey *et al.* 2013). The Lipinski rule of 5 checked based on analysis of four consistent physicochemical properties of ligands. These properties are molecular weight (MW) is ≤500 Dalton (Da), the octanol/water partition coefficient (logP) is ≤ 10 (Mohd Amin, Md Idris *et al.* 2020).

Protein structure preparation

The critical pro-inflammatory enzymes COX-1 (PDB ID-3N8Y), COX-2 (PDB ID-3LN1) (Boukhatem and Belhadj 2023) and 5-LOX (PDB ID-3O8Y) (Rabiu, Hamzah *et al.* 2021) ware retrieved from the PDB database (Protein Data Bank) (Rose, Duarte *et al.* 2021). The proteins ware prepared by protein preparation wizard (standard method). In that, the bond ordering, formal charges, missing hydrogen atoms, topologies, incomplete and terminal amide groups of protein structures are refined. Beyond the hetero atoms 5 Å, the water molecules were eliminated. For the heteroatom found in the protein structure, potential ionization states were produced, and the most stable state was selected. The hydrogen bonds were allocated, and the retained water molecules orientations were adjusted. The protein structure was then minimized with caution using the OPLS2005 force field to reposition side-chain hydroxyl groups and prevent potential steric conflicts. A predetermined Root Mean Square Deviation (RMSD) tolerance of 0.3 Å limits the minimization to the supplied protein coordinates (Sastry, Adzhigirey *et al.* 2013).

Active site prediction

The SiteMap module from the Schrodinger package was used to identify and characterize the active sites and binding residues of proteins. In the first step of the SiteMap calculation, one or more sites on the protein surface that might be favorable for ligand binding to the receptor are found and described using grid position points. In order to aid in the molecular docking process with protein and ligand research, contour maps that produced hydrophilic and hydrophobic hydrogen bonding possibilities were created (Ge and Ganamet 2023).

Molecular docking analysis

After preparation of ligands and receptor, the glide docking analysis were carried out using the previously prepared receptor grid and the ligand molecules in Schrodinger maestro package. The approving interactions between ligand and receptor were scored by Glide ligand docking module. For GLIDE (Yadav, Imran *et al.* 2021), the three separate molecular docking modes were used sequentially, HTVS (high-throughput virtual screening) docking and scoring, SP (standard precision) docking and scoring, and XP (extra precision) docking and scoring. Using XP mode, all docking calculations concluded. A flexible docking method that automatically creates conformations for each ligand was used to progress the docking. The poses are produced by a series of hierarchical filters that assess how ligands interact with proteins or receptors. The majority of comparable docking conformations contained the lowest-energy docked complexes (Pandi, Kulanthaivel *et al.* 2022).

ADME/T properties prediction

The in-silico ADME properties of the proposed ligands were determined by using QikProp module of Schrödinger software Maestro. The pharmacokinetics and pharmacodynamics of the ligands are studied using QikProp guidance to ascertain the drug-like properties. Predicted important ADME characteristics like Molecular weight: (acceptable range: ≤ 500), *aqueous solubility:* QPlogS (−6.5 < x < 0.5), *apparent Caco-2 cell permeability in nm/sec:* QPPCaco (nm per sec; 500 great), *Conformation-independent predicted aqueous solubility*, log S. S in mol dm^{-3}:CIQPlogS (−6.5 < x < 0.5), metabolism: #metab (1–8), rule of three: ro3 (0), *Central Nervous System* permeability: CNS (−2 = completely inactive, −1 = very low activity, 0 = low activity, 1 = medium activity, 2 = completely active), QPlogKp: (−8 < x<−5), and *brain/blood partition coefficient*: QPlogBB (−3.0- 1.2) (Mohd Amin, Md Idris *et al.* 2020). The toxicity of selected compounds was predicted using ProTox-II online server (Banerjee, Eckert *et al.* 2018). Different toxicity endpoints, including acute toxicity, hepatotoxicity, carcinogenicity, mutagenicity, and others, are predicted using the ProTox-II tool. SDF (structural data file) and SMILES (simplified molecular-input line entry system) were used in the creation method throughout generation. Computing the toxicity dosages has become relatively simple in compared to estimates based on animal models because it can save the time required for experiments on animals (Murad, Alqurashi *et al.* 2022).

Binding Free Energy Calculation by Using Prime/MM-GBSA Approach

MM-GBSA (Molecular Mechanics-Generalized Born Surface Area) processes the binding free energies of the protein ligand complex using Schrödinger Suite 2018-4 Prime module. The complexes were refined with Prime under the OPLS 3e force field adopting the Variable Dielectric Surface Generalized Born (VSGB) continuum solvation model (Kalirajan, Pandiselvi *et al.* 2019). The top compounds that were retrieved from the docking procedure then underwent the G scores. $\Delta G_{binding}$ was calculated for the protein-ligand complexes using MM-GBSA analysis available in the Prime module of GLIDE.

$\Delta G_{binding}$ was calculated based on the following formula:

$\Delta G_{binding} = G_{complex} - (G_{receptor} + G_{ligand})$

$\Delta G_{binding} = \Delta E_{MM} + \Delta G_{GB} + \Delta G_{SA}$

$\Delta G_{binding}$ = Energy of the minimized complex – (Energy of the minimized receptor + Energy of the minimized ligand) (Ongaro, Oselladore *et al.* 2021).

Results and discussion

Plant extraction

About 50 gm of shade dried leaves powder of *Cassia auriculata Linn* was extracted in Soxhlet assembly with 250ml of methanol solvent. The extract obtained with methanol solvent was weighed. The yield of plant extract is found to be 6gms.

Table 1. Compounds are detected in HR-LCMS.

	Compounds are detected in ESI +ve ionization		Compounds are detected in ESI -ve ionization
1	beta-D-Galactopyranosyl-(1- >4)-beta-D-galactopyranosyl- (1->4)-D-galactose	1	2-Hydroxy-3-chloropenta-2,4- dienoate; 2,6-dihydroxybenzoic acid
2	Pirbuterol	2	Mecarbinzid
3	2,3-Butanediol glucoside	3	Crosatoside B
4	Miserotoxin	4	Gallic acid
5	Epigallocatechin	5	Glycophymoline
6	Methyl N-methylanthranilate	6	(+)-Gallocatechin
7	Americanin B	7	Resorcinol
8	3alpha,4,7,7alpha-Tetrahydro4-hydroxy-1H-isoindole1,3(2H)-dione	8	Glyceryl lactopalmitate
9	Dulxanthone B	9	Saphenamycin
10	(5alpha,8beta,9beta)-5,9- Epoxy-3,6-megastigmadien-8- ol	10	N-(3-Benzooxazol-2-yl-4- hydroxy-phenyl)-2-ptolyloxyacetamide
11	1,3,5,8-Tetrahydroxy-6- methoxy-2- methylanthraquinone 8-O-b-Dglucoside	11	p-Hydroxyphenylbutazone
12	Rehmaionoside C	12	Sulfadoxine
13	Jasmolone glucoside	13	Vanillic acid
14	6-Hydroxykaempferol 6,7- diglucoside	14	Methotrimeprazine
15	Quercetin	15	Sulfadoxine
16	Isoorientin 7-glucoside	16	Pedaliin
17	Hyperoside	17	Gambiriin A3
18	Fabianine	18	Albanol A
19	Maritimetin	19	Protoaphin aglucone;
20	Ethyl 7-epi-12- hydroxyjasmonate glucoside	20	Myricitrin
21	Xanthoaphin	21	Cefoselis
22	4,7-Didehydroneophysalin B	22	Protoaphin aglucone
23	Polysorbate 20	23	Orcein
24	Belladonnine	24	7-Methyl-1,4,5- naphthalenetriol 4-[xylosyl-(1- >6)-glucoside]
25	Auriculine	25	Isonocardicin A
26	Retapamulin	26	Gladiatoside C2
27	19-Noretiocholanolone	27	5,7,8,3',4'- Pentahydroxyisoflavone
28	17-Hydroxylinolenic acid	28	Kaempferol
29	1,3-Diacetoxy-4,6,12- tetradecatriene-8,10-diyne	29	Atractyloside
30	D-Urobilin	30	Aurasperone D
31	Melongoside G	31	Hordatine A
32	Androsterone	32	Cytochalasin Npho
33	Flavidulol C	33	all-trans-heptaprenyl diphosphate
34	Avermectin B2a monosaccharide	34	Triamcinolone hexacetonide
35	Erythromycin E	35	(2S,2'S)-Oscillol 2,2'-di(α-Lfucoside)
36	Phaeophorbide b		
37	Asparanin B		
38	9Z-Octadecen-12-ynoic acid		
39	23-Acetoxysoladulcidine		
40	3-(5,6,6- Trimethylbicyclo[2.2.1]hept-1- yl)cyclohexanol		
41	Harderoporphyrin		
42	Campesteryl p-coumarate		
43	Harderoporphyrin		
44	Leukotriene F4		
45	DG(18:4(6Z,9Z,12Z,15Z)/18:4 (6Z,9Z,12Z,15Z)/0:0)		
46	Pheophorbide a		
47	16beta-Hydroxysteroid		
48	Allosamidine		
49	Euphornin		
50	3-cis-Hydroxy-b,e-Caroten-3'- one		

Phytochemical profile by HR-LCMS

The HR-LCMS studies of *Cassia auriculata Linn* leaf methanol extract revealed the presence of 85 compounds are listed in Table 1. And the structures of 85 compounds are retrieved from PubChem database. The analysis of compounds performed in ESI positive and negative mode ionizations (Rafiq, Wagay *et al.* 2022). Compounds are detected in ESI +ve shown in Fig.1, also compounds detected in ESI -ve shown in Fig. 2.

Ligand preparation

The 85 phytochemicals were optimized using LigPrep. As a result of total 1020 conformations were obtained.

The evaluation of drug-likeness was performed on the basis of 'Lipinski's rule of five' (ro5). 486 compounds were obeying the 'Lipinski's rule of five' filtration. The filtered compounds are proceeded to perform protein-ligand docking (Chen, Leung *et al.* 2010).

Table 2. Docking results of *Cassia auriculata Linn* phytochemicals with proinflammatory cytokines.

Sl. No	Compound name(XP)	Targets	Docking score	Glide g-score	Glide e-model
1	Maritimetin	COX-1	-11.090	-11.090	19.743
		COX-2	-10.265	-10.265	-33.309
		5-LOX	-6.170	-6.170	-33.774
2	Gallic acid	COX-1	-7.865	-7.865	-38.152
		COX-2	-8.535	-8.535	-30.071
		5-LOX	-6.886	-6.886	-40.780
3	kaempferol	COX-2	-10.242	-10.242	-12.468
		5-LOX	-6.378	-6.378	-47.840
4	Glycophymoline	COX-1	-8.751	-8.766	-4.488
		5-LOX	-5.267	-5.281	-25.272
5	2,6-dihydroxybenzoic acid	COX-1	-8.387	-8.387	-38.703
		5-LOX	-6.077	-6.077	-30.629
6	Methyl N- methylanthranilate	COX-1	-7.234	-7.235	-21.535
		5-LOX	-4.447	-4.447	-25.107
7	Vanillic acid	COX-1	-6.734	-6.734	-24.803
		5-LOX	-6.826	-6.826	-26.135
8	Mecarbinzid	5-LOX	-5.361	-6.239	-46.371
9	Resorcinol	5-LOX	-3.558	-3.558	-22.751
10	N-(3-Benzooxazol-2-yl-4- hydroxy-phenyl)-2-Ptolyloxyacetamide	5-LOX	-5.284	-5.593	-59.969

Table 3. ADME property prediction of 6 shortlisted compounds.

Compounds name	QPlogS	CIQPlogS	QPPCaco	#metab	ro3	CNS	QPlogBB	QPlogKp	Jm	QPlogKhsa	QPlogHERG
Maritimetin	-2.107	-1.576	181.729	6	0	-2	-1.442	-4.222	0.142	-0.713	-3.508
Gallic acid	-0.894	-0.473	106.298	3	0	-2	-1.395	-4.771	0.386	-0.867	-2.647
kaempferol	-2.345	-1.571	115.574	6	0	-2	-1.487	-4.796	0.022	-0.645	-3.449
Glycophymoline	-1.813	-0.707	415.260	0	0	2	1.014	-5.690	0.008	0.217	-5.384
2,6-dihydroxybenzoic acid	-0.744	-0.568	285.425	2	0	-1	-0.965	-4.708	2.708	-0.842	-2.515
N-(3-Benzooxazol-2-yl-4- hydroxy-phenyl)-2-Ptolyloxyacetamide	-2.578	-1.269	59.842	4	0	0	-0.171	-6.941	0.000	0.050	-6.207

Protein preparation

An X-ray crystallography structure of enzymes COX-1 (PDB ID-3N8Y), COX-2 (PDB ID-3LN1) and 5-LOX (PDB ID-3O8Y) are obtained by PDB database. Protein Preparation Wizard was used to prepare the protein structure for docking. The protein was prepared by removing water molecules, generating states using Epik, assigning bond order; allocate hydrogen bonds (Shelley, Cholleti *et al.* 2007). The energy minimization of structure using force field OPLS3. The Receptor Grid Generation used for structure binding site, to set a grid box (Sastry, Adzhigirey *et al.* 2013). After protein preparation the protein-ligand docking is performed.

Table 4. Predicted MM-GBSA free binding energy score.

Sl. No	Compound name	Targeted proteins	MMGBSA-dG-binding energy kcal/mol	MMGBSA-dG-binding coulomb kcal/mol	MMGBSA-dG-bind(NS) kcal/mol	MMGBSA-dG-bind(NS) coulomb kcal/mol
1	Maritimetin	COX-1	-32.26	-27.59	-57.79	-24.72
		COX-2	-67.60	-25.66	-72.80	-15.15
		5-LOX	-59.52	-2.63	-63.28	-8.74
2	Gallic acid	COX-1	-33.32	-19.03	-37.65	-12.78
		COX-2	-31.17	-0.80	-38.52	-8.49
		5-LOX	-46.03	-19.29	-48.93	-11.89
3	kaempferol	COX-2	-53.12	-14.52	-67.98	-13.29
		5-LOX	-60.68	-17.58	-74.25	-19.19
4	Glycophymoline	COX-1	-58.42	33.24	-79.45	33.72
		5-LOX	-52.98	-19.91	-58.03	-19.39
5	2,6-dihydroxybenzoic acid	COX-1	-42.84	-10.31	-47.25	-11.08
		5-LOX	-40.10	-8.79	-48.45	-14.26
6	N-(3-Benzooxazol-2-yl-4-hydroxy-phenyl)-2-Ptolyloxyacetamide	5-LOX	-65.41	-10.81	-80.26	-14.73

Molecular docking

Virtual screening can now have a positive impact on the discovery process, because of advances in computational technique. The binding mechanisms of compounds to the amino acids found in the protein active pocket were examined using a grid-based docking analysis.

To evaluate and identify the potential palliative lead molecule, subjected to glide docking analysis by Schrodinger suite, the active 85 phytochemicals of *Cassia auriculata Linn* leaves with three main pro-inflammatory enzymes such as COX-1, COX-2 and 5-LOX. The results of docking analysis were described in Table 2.

Table 5. Toxicity prediction of shortlisted 6 compounds.

Compounds	Predicted LD50 MG/kg	Toxicity class	Hepatotoxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity
Maritimetin	500	4	Inactive	Active	Active	Active	Inactive
Gallic acid	2000	5	Inactive	Mild active	Inactive	Inactive	Inactive
Kaempferol	3919	5	Inactive	Inactive	Inactive	Inactive	Inactive
Glycophymoline	388	4	Mild active	Inactive	Inactive	Inactive	Inactive
2,6-dihydroxybenzoic acid	1250	4	Inactive	Inactive	Inactive	Inactive	Inactive
N-(3-Benzooxazol-2-yl-4- hydroxy-phenyl)-2-ptolyloxyacetamide	1600	4	Mild active	Inactive	Inactive	Inactive	Inactive

Table 6. Hydrogen bond interactions with selected targets.

Sl. No	Name of compound	Targeted pro-inflammatory cytokines	Number of hydrogen bond	Residue concerned with hydrogen bond
1	Gallic acid	COX-1	2	MET522,SER530
		COX-2	2	MET508
		5-LOX	5	TYR234,GLN656,LEU657
2	Kaempferol	COX-2	3	LEU338,ARG499,PHE504
		5-LOX	2	GLU228,GLN656
3	Glycophymoline	COX-1	1	SER530
		5-LOX	2	GLU228,GLN656
4	2,6-dihydroxybenzoic acid	COX-1	2	MET522,SER530
		5-LOX	3	GLU228,GLN656
5	N-(3-Benzooxazol-2-yl-4- hydroxy-phenyl)-2-Ptolyloxyacetamide	5-LOX	2	GLU228,GLN656

ADME prediction

The 10 phytochemicals were finalized from docking results, which are proceeded for ADME property prediction. The ADME properties are assessed to determine their safety profile using the QikProp 4.4 tool of Maestro software (Bharadwaj, Ahmad *et al.* 2023). Further, 6 compounds were selected from ADME property prediction.

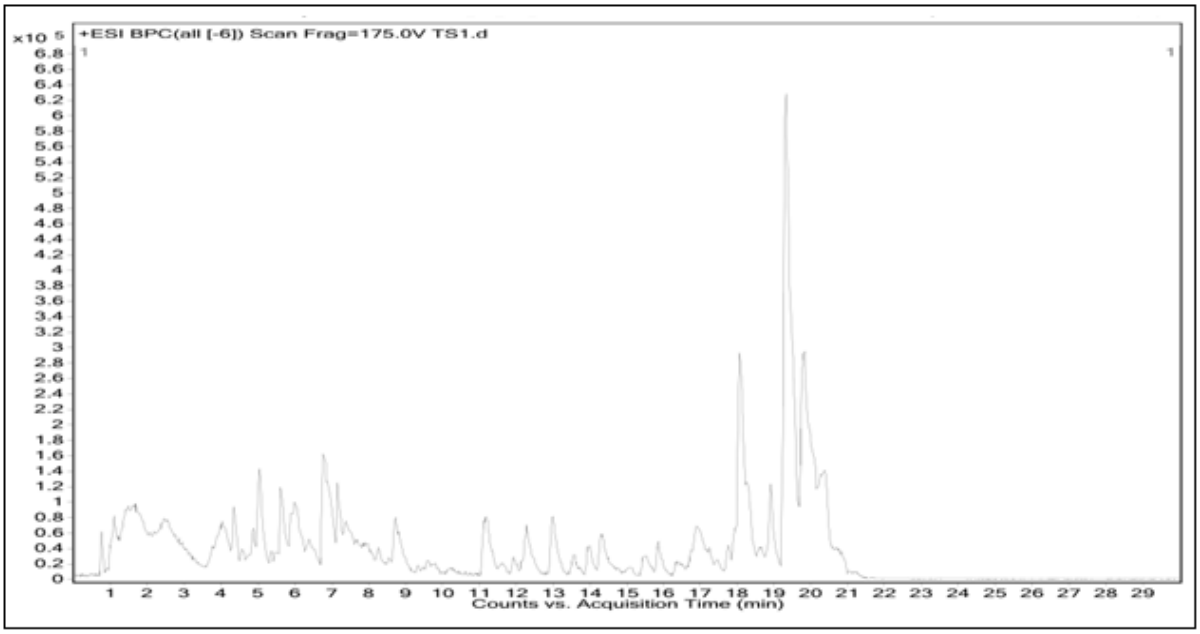

Fig. 1. LC-MS chromatogram of methanolic Leaves extract of *Cassia auriculata Linn* on +ve ionization.

The QikProp parameters for the compounds are within the permitted range which shown in Table 3. Thus, these 6 compounds were preceded for further analysis.

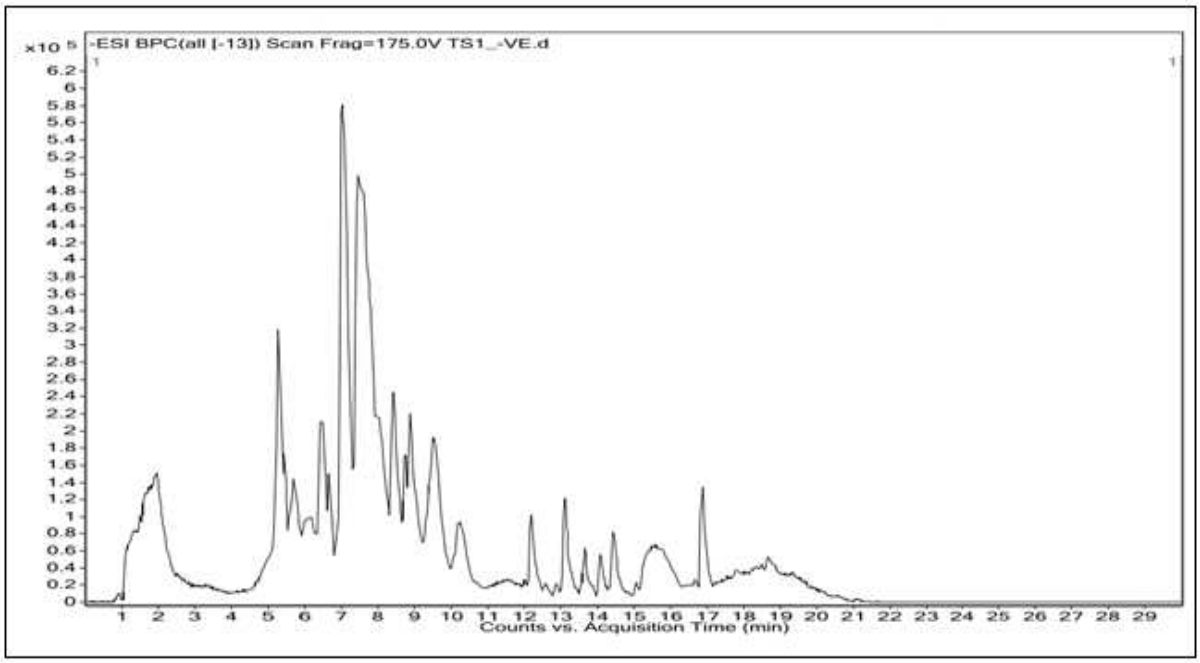

Fig. 2. LC-MS chromatogram of methanolic Leaves extract of *Cassia auriculata Linn* on -ve ionization.

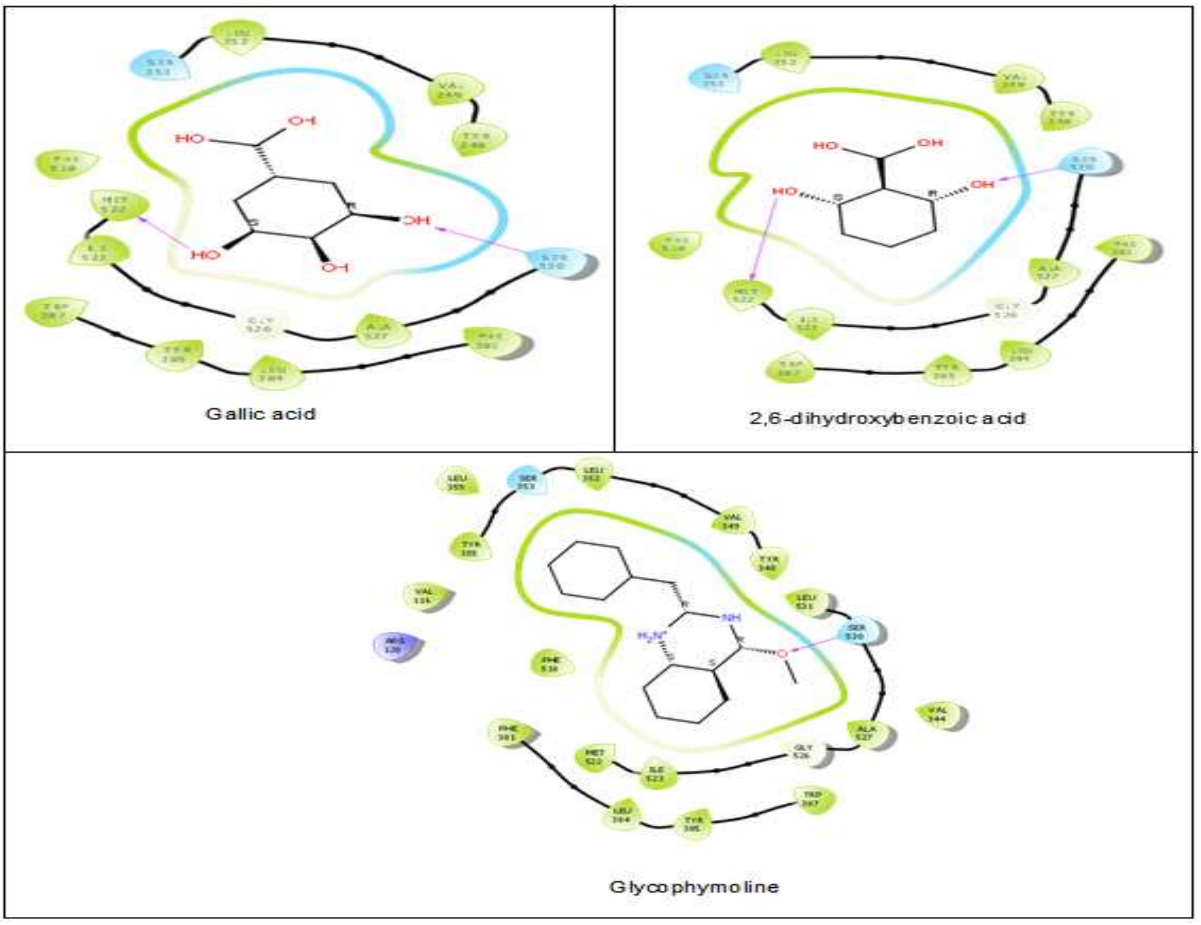

Fig. 3. 2D structure of hydrogen bond interaction of bioactive compounds with pro-inflammatory receptor COX-1.

Binding free energy calculation

MM-GBSA is very popular method for predict binding affinity of compounds to protein. The 6 compounds are evaluated for binding affinity with protein. The MM-GBSA result illustrated that all the 6 compounds are showing highest negative values which implies strongest binding affinity between protein and ligand molecule. The relative binding free energies (G bind) of each ligand molecule were shown using the prime MMGBSA (Molecular mechanics-generalized Born surface area) technique (Petrenko, Timofeev *et al.* 2022), and the findings are shown in Table 4.

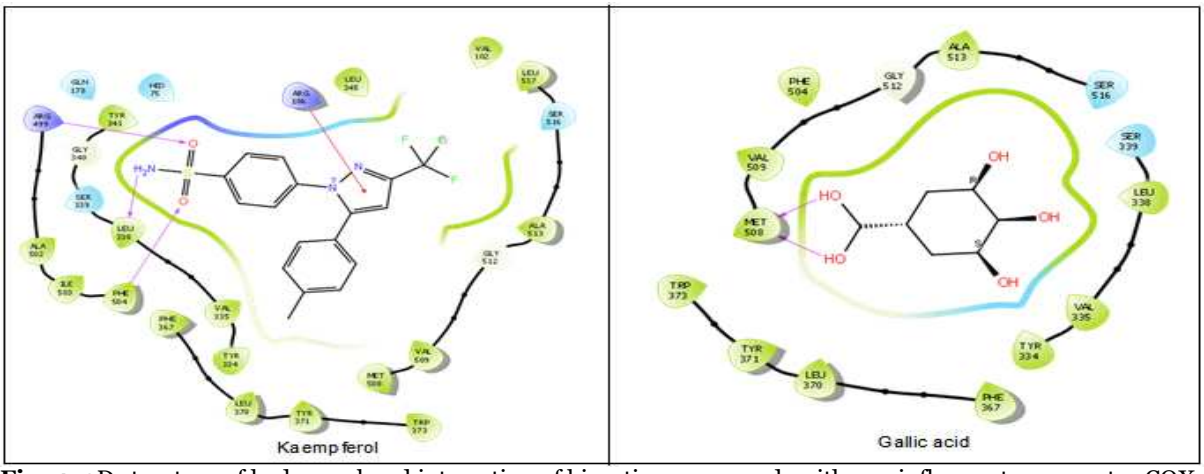

Fig. 4. 2D structure of hydrogen bond interaction of bioactive compounds with pro-inflammatory receptor COX-2.

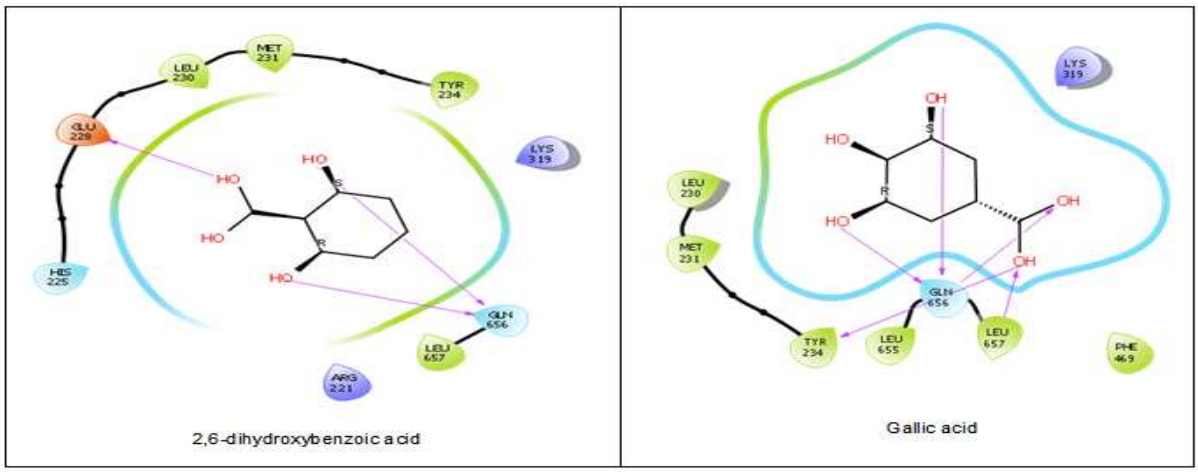

Fig. 5. 2D structure of hydrogen bond interaction of bioactive compounds with pro-inflammatory receptor 5-LOX.

Toxicity prediction

The toxicity prediction was performed to nine short listed compounds by using Protox-II online tool. Toxicology investigation was carried out in order to forecast the safety features of compounds (Divya Rajaselvi, Jida *et al.* 2023). The main toxicity endpoints were considered, and the medications that did not adhere to the safety guidelines for toxicity endpoints were not taken into further consideration in our priority list. In 6 compounds 5 compounds are non-toxic because which are in 4 and 5 class of toxicity also inactive in hepatotoxicity, carcinogenicity, immunotoxicity, mutagenicity and cytotoxicity class shown in Table 5.

The finalized 5 compounds are Kaempferol, Gallic acid, Glycophymoline, 2,6-dihydroxybenzoic acid and N-(3-Benzooxazol-2-yl-4- hydroxy-phenyl)-2-ptolyloxyacetamide are showing best compounds for further studies.

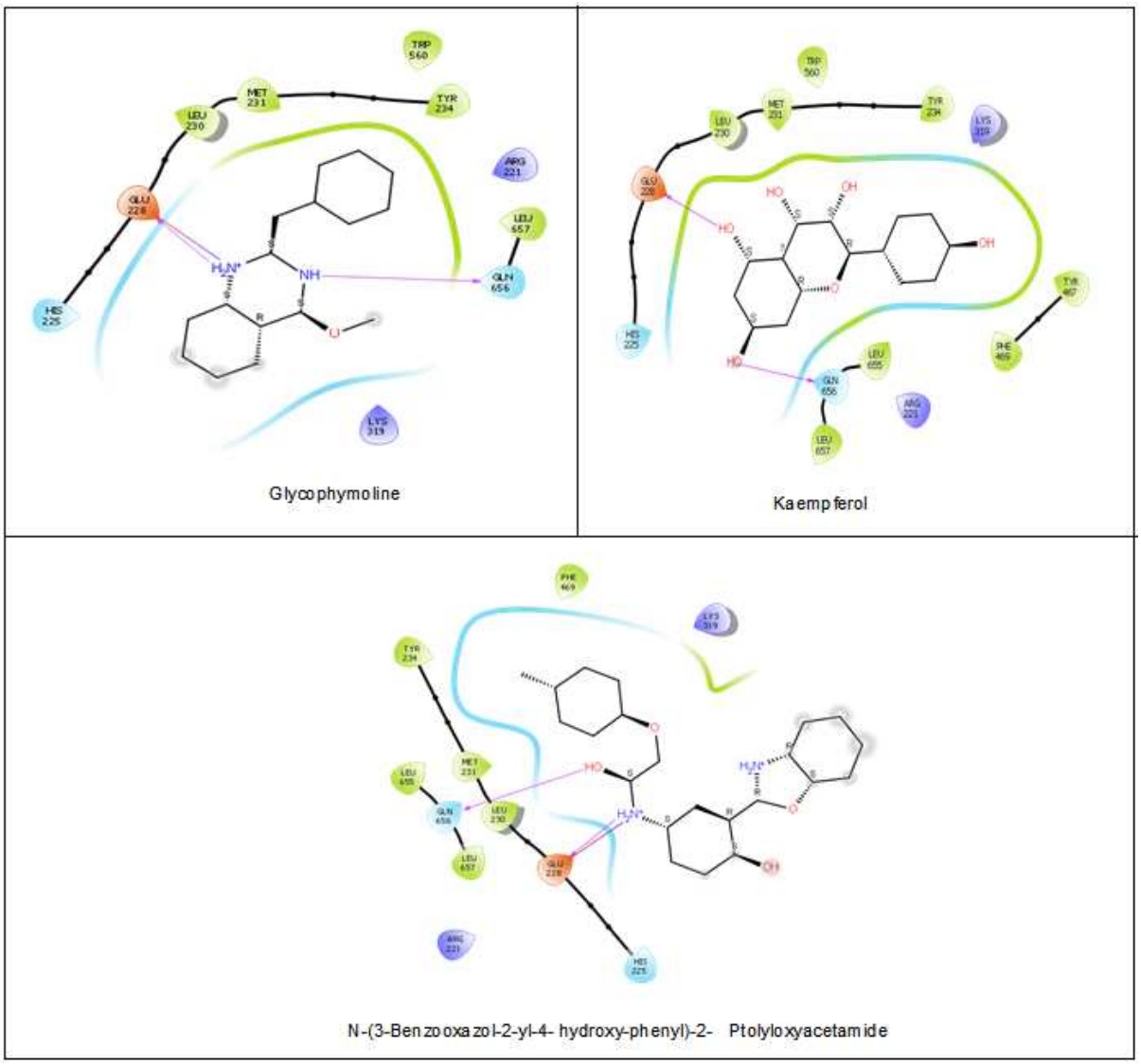

Fig. 6.

Hydrogen bond interaction

According to the study, 5 compounds namely Kaempferol; Gallic acid; Glycophymoline; 2,6-dihydroxybenzoic acid; and N-(3-Benzooxazol-2-yl-4-hydroxy-phenyl)-2-p-tolyloxyacetamide are considered as targeted drugs because of good binding interaction with targeted proteins. Kaempferol (Bangar, Chaudhary *et al.* 2023) is exhibited 3 and 2 hydrogen bond interaction with COX-2 and 5-LOX respectively. N-(3-Benzooxazol-2-yl-4- hydroxy-phenyl)-2-p-tolyloxyacetamide (Kondratov, Komarov *et al.* 2001) is showing 2 hydrogen bind interaction with 5-LOX.

The ligands interacted with the various residues surrounding the active pocket through hydrophobic, hydrogen-bonding, and other interactions. Compound 2,6-Dihydroxybenzoic acid (Düwel and Metzger 1973) exhibited 2 and 3 hydrogen bond interaction with COX-1 and 5-LOX respectively. Glycophymoline (Sarkar and Chakraborty 1979) exhibited 1 and 2 hydrogen bond interaction with COX-1 and 5-LOX respectively.

Gallic acid (Bai, Zhang *et al.* 2021) compound exhibited 2, 2 and 5 hydrogen bond interactions with COX-1, COX-2 and 5-LOX. The ligands interacted with the various residues surrounding the active pocket through hydrophobic, hydrogen-bonding, and other interactions. Residues concerned with hydrogen bond shown in Table 6. And protein ligand interaction images shown Fig. 3, 4 and 5.

Conclusions

Present research work, virtual screening, insilico ADMET and molecular docking studies were carried out to identify the possible bioactive phytoconstituents against pro-inflammatory enzymes using HR-LCMS analysis and *insilico* techniques. Target of the early responsive pro-inflammatory enzymes such as COX-1, COX-2 and 5-LOX were selected for the study. Phytochemical signature of *cassia auriculata Linn* leaves ware analyzed based on the results of HR-LCMS, 85 phytochemical constituents obey Lipinski rule of five, and these compounds were subjected to molecular docking with pro-inflammatory targets. The 10 compounds are showing good binding energy with 1,2,3 and 5 hydrogen bond interaction with three targets, and the 6 compounds have values for each parameter that are within the acceptable rang based on the ADME prediction and 5 compounds are considered as non-toxic based on toxicity prediction. This work suggest that, the 5 compounds namely Gallic acid; 2,6-Dihydroxybenzoic acid, Kaempferol; N-(3-Benzooxazol-2-yl-4- hydroxy-phenyl)-2-ptolyloxyacetamide; and Glycophymoline are found as potential bioactive compounds inhibiting pro-inflammatory targets COX-1, COX-2 and 5-LOX. Also Gallic acid is inhibiting all the three main responsive enzymes, hence this compound is possibly considered as multi-targeted drug for inflammatory diseases. Further, *Invitro and Invivo* validation of these compounds may explore treatment regime for the management of inflammatory diseases.

Recommendation (s)

This work may help the researchers to find a direction for the development of safe, efficacious dual COX-LOX inhibitors by further isolation of similar classes of phytochemicals from the unexplored bioactive fractions and by investigating structurally similar phytoconstituents.

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