Bioassay-guided isolation, identification and molecular ligand-target insight of lipoxygenase inhibitors from leaves of *Anisomeles malabarica* R.Br.

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ABSTRACT

Background: *Anisomeles malabarica* R. Br. (*Lamiaceae*) is extensively used in traditional medicine in major parts of India for several medicinal purposes, including their use in rheumatism. **Materials and Methods:** The air-dried leaves of *A. malabarica* were extracted with ethanol, defatted with n-hexane and then successively partitioned into chloroform and n-butanol fractions. Bioassay-guided fractionation and purification of chloroform fraction from *A. malabarica* lead to the isolation of lipoxygenase (LOX) inhibitors. The structures of isolated compounds were elucidated by ultraviolet, infrared, ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR and mass spectrometry spectroscopic techniques and assessed further by *in vitro* soybean lipoxygenase (sLOX) assay. In addition, the enzyme type inhibition was evaluated through molecular docking technique as a part of computational study. **Results:** The bioactive compounds 3, 4 dihydroxy benzoic acid (1) and 4′, 5, 7-trihydroxyflavone (2) were isolated from chloroform fraction of *A. malabarica*, whose bioactivity was observed to be dose-dependent compared to n-butanol fraction. Among the compounds, 3, 4 dihydroxy benzoic acid showed significant sLOX inhibitory activity with 74.04% ±2.6% followed by 4′, 5, 7-trihydroxyflavone (34.68% ±1.9%). The computational analysis of compounds showed their molecular interaction with important amino acid residues and nonheme iron atom in the catalytic site of LOX by enlightening their potential binding mode at molecular level. **Conclusions:** The LOX inhibitory constituents were identified from *A. malabarica* by means of bioassay-guided fractionation process. The results derived from *in vitro* and computational experiments confirm the potential of the isolated compounds and provide additional evidence for its traditional use in inflammatory disorders.

Key words: 3, 4 dihydroxy benzoic acid, *Anisomeles malabarica,* anti-inflammatory, lipoxygenase, molecular docking

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INTRODUCTION

Lipoxygenases (LOX; EC 1.13.11.12) are a family of nonheme iron containing dioxygenases enzymes that are involved in the generation of lipid hydroperoxide products viz., lipoxins, hydroxyeicosatetraenoic acids and leukotrienes (LTs) [Figure 1]. Leukotrienes in turn have been proposed to play an important role in hypersensitive inflammatory responses such as asthma, ulcerative colitis, rheumatoid arthritis, psoriasis, glomerular nephritis, and cancer.[1] The three different LOXs such as 5-LOX, 12-LOX, and 15-LOX are found in the neutrophils, platelets and endothelial cells and they give rise to hydroperoxides of eicosatetraenoic acids by inserting oxygen into the 5, 12, and 15 positions of arachidonic acid.[2] LOX has been considered as a peroxidizing enzyme which metabolizes dietary and membrane lipids through a series of free radical reactions.[3]

The soybean lipoxygenase (sLOX) exhibit catalytic mechanism similar to that of human lipoxygenases such as 5-LOX by catalyzing oxidation of linoleic acid, arachidonic acid and other unsaturated fatty acids.[4] As a result, the sLOX inhibition assay was used for the recognition of substances, which might also work as inhibitors of mammalian LOXs, such as 5-LOX. LOXs, and LT represents the major therapeutic targets in the field of anti-inflammatory drug discovery process, over the last decades.[5,6] Many LOX inhibitors have been consequently developed and some have presented effectiveness in asthma models, such as allergen-induced bronchoconstriction.[7]

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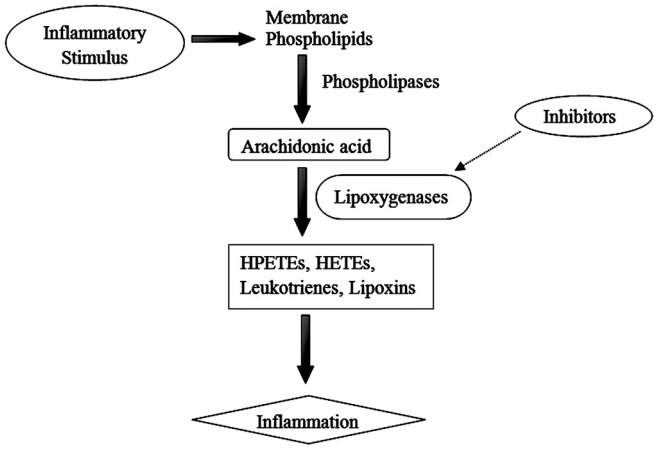

Figure 1: Conversion of fatty acid substrate (arachidonic acid) into eicosanoids by lipoxygenase pathway

The medicinal plants are considered to be the potential sources to discover new drugs. The use of medicinal plants in the treatment of various diseases is well-associated with traditional medicine from different parts of the world. Therefore, searching for natural and selective LOX inhibitors as alternatives to synthetic drug molecules is of great interest. In search of plants with potential anti-inflammatory activity, the ethanol extract of *A. malabarica* was screened for inhibition of soybean 15-lipoxygenase (15-sLOX) activity. *A. malabarica* R. Br. is an aromatic, perennial herb, belonging to the family *Lamiaceae* and is widely distributed in major parts of India and especially in South India. It is commonly called as Peymarutti (Tamil), Gouzaban (Hindi), Chodhara (Marathi), Karithumbi (Kannada) and Malabar catmint (English).[8] The infusions of leaf are used in dyspepsia, catarrhal afflictions, intermittent fever, bowel disorder, boils, and tetanus from ancient period.[9] The essential oil and decoction obtained from the leaf is externally used in the treatment of rheumatism. The plant has been documented to possess antispasmodic, diaphoretic, emmenagogue, and antiperiodic properties.[8,10] The ethanol extract of the plant has been revealed to acquire significant antipyretic and anti-inflammatory activity.[11] Ethnobotanically, the anticonvulsant activity of the plant leaves has been recognized in folklore medicines.[8,10] The anticancer effect of ethanol extract of the plant has been reported.[12,13] Recently, the flavonoid fraction from the leaves of *A. malabarica* has been proved to possess antiepileptic activity.[14] The family *Lamiaceae* is reported to possess numerous secondary metabolites such as steroids, triterpenoids, phenolic compounds and flavonoids.[15] Accordingly, the various phytoconstituents such as anisomelic acid, ovatodiolide, geranic acid, citral, betulinic acid, beta-sitosterol, and apigenin glycosides have been reported earlier in *A. malabarica*.[16-18] However, until date, bioassay-guided isolation of *A. malabarica* using *in vitro* LOX activity was not determined. Hence, this study was undertaken to evaluate the sLOX inhibitory activity of *A. malabarica* and to identify anti-inflammatory lead compounds through *in vitro* and computational approach thereby validating its folkloric medicinal properties.

MATERIALS AND METHODS

General instrumentation and reagents

Nuclear magnetic resonance (NMR) spectra were recorded on a BRUKER, Avance 400 MHz (Switzerland) NMR instrument operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C nuclei at room temperature and referenced to the residual solvent signal. Aluminium sheets precoated with Silica gel 60 F_{254} plates (20 × 20 cm, 0.2 mm thick; E-Merck, Germany) were used for thin-layer chromatography (TLC) analysis. The ultraviolet (UV) spectra were recorded using Varian Cary 500 scan/UV-Vis-NIR spectrophotometer (Varian, Australia). λ_{max} (log ε) in nm; whereas, the Fourier transform infrared (IR) spectrum was recorded using a Nicolet 380 (Thermo Scientific, USA). The functional group was identified using potassium bromide (KBr) and scanned in the range of 4000-400/cm. ESI mass spectra were recorded on Finnigan MAT 8230 Mass Spectrometer (Finnigan, San Jose, California, USA) and Agilent 1100 LC-MSD-Trap-SL (Agilent Technologies, USA) using positive-ion modes.

For enzyme inhibition assay, linoleic acid, LOX (1.13.11.12) Type I-B (source: Soybean) and Nordihydroguairetic acid (NDGA) were purchased from Sigma (St. Louis, MO, USA). HPLC grade solvents and reagents used for extraction and silica gel (60-120 mesh) for column chromatography were obtained from Sisco Research Laboratories (Mumbai, India). All other chemicals and reagents used in this study were of analytical grade.

Plant materials

The leaves of *A. malabarica* were freshly collected between August and September 2010, from Karaikudi, Sivagangai District, Tamil Nadu. The plant was taxonomically identified and authenticated by Dr. G.V.S. Murthy, Joint Director, Botanical Survey of India, Tamil Nadu Agricultural University Campus, Coimbatore. A voucher specimen has been deposited (BSI/SRC/5/23/2012-13/Tech-19) at the Botanical Survey of India, Tamil Nadu Agricultural University Campus, Coimbatore.

Extraction and fractionation

The leaves of *A. malabarica* were washed, sliced, dried under shade and mechanically powdered by using blender, passed through 60 mm mesh sieve and then stored in an airtight container for further use. The air-dried powdered leaves (2.0 kg) of *A. malabarica* were extracted with ethanol (7 L × 2) at room temperature for 15 days with continuous stirring

by simple maceration process. After 15 days, the combined extracts were concentrated under reduced pressure to give dark brown syrupy residue of approximately 62.5 g (3.12% yield). The crude ethanolic extract obtained, was then suspended in distilled water, defatted with n-hexane, and then partitioned successively with solvents (chloroform and n-butanol) to obtain chloroform and n-butanol fractions, respectively. The crude extract and solvent fractions were examined for s15-LOX inhibition.

Membrane-stabilizing activity

The membrane-stabilizing activity of ethanol extract of *A. malabarica* was assessed by the modified method of Sadique *et al.*[19] Human blood was obtained from a healthy volunteer and transferred to heparinized centrifuge tube and was mixed with equal volume of sterilized Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% NaCl in water) and centrifuged at 3,000 rpm for 10 min. The supernatant (plasma and leukocytes) was carefully removed, while the packed cells were washed thrice with isosaline (0.85%, pH 7.2) and 10% v/v suspension was made with isosaline. The assay mixtures consist of 2 ml of hyposaline (0.25% w/v), 1 mL of phosphate buffer saline (0.15 M, pH 7.4), erythrocyte (human red blood cell [HRBC]) suspension (0.5 mL) and various concentrations of extracts and standard (50-1000 µg/mL). Diclofenac sodium was used as the reference standard. The control was prepared with saline by omitting the extracts. The reaction mixtures were incubated for 30 min at 56°C on a water bath, cooled and centrifuged for 10 min at 5000 rpm. The absorbance of the released hemoglobin was measured at 560 nm. The percentage inhibition of hemolysis or membrane-stabilization was calculated by the following formula.[20]

$$\text{Inhibition}(\%) = 100 - \frac{\left(\begin{array}{l}\text{optical density of control}\\ -\text{optical density of test}\end{array}\right)}{\text{optical density of control}} \times 100$$

Soybean 15-lipoxygenase inhibition assay

In vitro 15-sLOX inhibitory activity was measured using spectrophotometric method.[21] Briefly, 160 µL of sodium phosphate buffer (100 mM, pH 8.0), 10 µl of test sample and 20 µL of sLOX (1.13.11.12) Type I-B solution were mixed and incubated for 10 min at 25°C. The reaction was then initiated by the addition of the linoleic acid substrate (10 µL, 300 mM) solution. With the formation of (9*Z*, 11*E*)-(13*S*)-13-hydroperoxyoctadeca-9, 11-dienoate, the changes in absorbance at 234 nm were measured for 6 min. NDGA was used as positive control for LOX inhibition.[22] Stock solutions were prepared at concentration of 25 mg/mL for extracts, 5 mg/mL for solvent fractions and 1 mg/mL for pure compounds. Pure compounds were tested at a final concentration of 25 µg/mL. All the reactions were performed in triplicate and the percentage inhibitory concentration was calculated by the formula:

$$\text{Inhibition}(\%) = \frac{\left(\begin{array}{l}\text{absorbance of control}\\ -\text{absorbance of sample}\end{array}\right)}{\text{absorbance of control}} \times 100$$

Bioassay-guided fractionation and isolation of compounds

The bioactive chloroform fraction (26.3 g) was purified by column chromatography over silica gel column using gradient hexane-ethyl acetate system, yielding 42 fractions of 100 mL each. TLC analysis of collected fractions was carried out using chloroform: methanol: water (8:4:1) as the mobile phase and the separated bands were visualized using vanillin-sulfuric acid reagent. The fractions were pooled according to their similarity in R_f values on TLC to yield five major fractions (F_1-F_5), which were also evaluated for bioactivity using 15-sLOX assay. Bioassay determined fraction F_2 (2.8 g) obtained from hexane/ethyl acetate (80:20) eluate was resoluted to contain the active compound (s). Subsequently, this fraction was further purified over silica gel column using gradient hexane/EtOAc system to afford the active compounds, Compound (1) (95:5 v/v, 43 mg) and Compound (2) (75:35 v/v, 27 mg), respectively. Further, the purity of two compounds was determined by TLC analysis and the structures of these two compounds were identified on the basis of spectroscopic methods [Figures 2-7] and

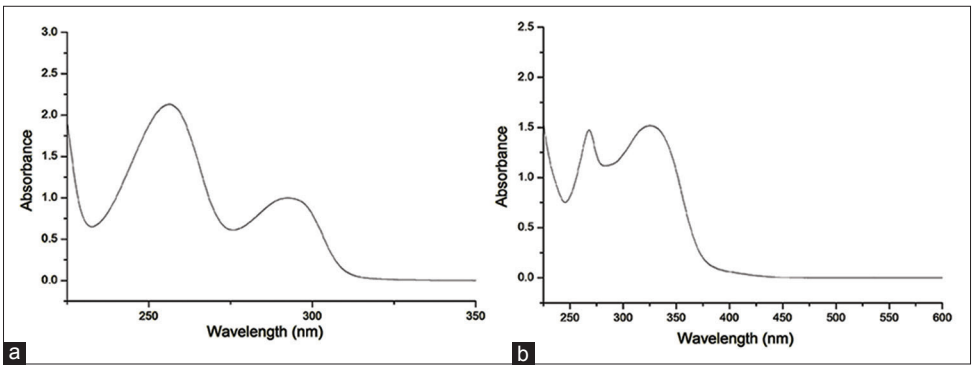

Figure 2: Ultraviolet-visible spectrum of purified Compound 1 (a) and 2 (b) from *Anisomeles malabarica* leaves

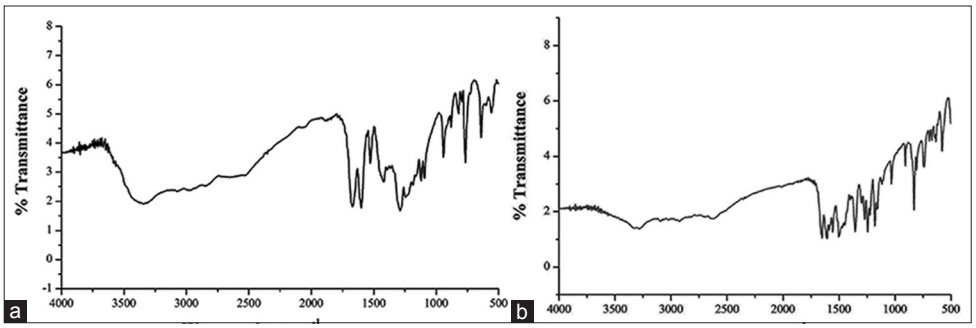

Figure 3: Fourier transform infrared spectrum of purified Compound 1 (a) and 2 (b) from *Anisomeles malabarica* leaves

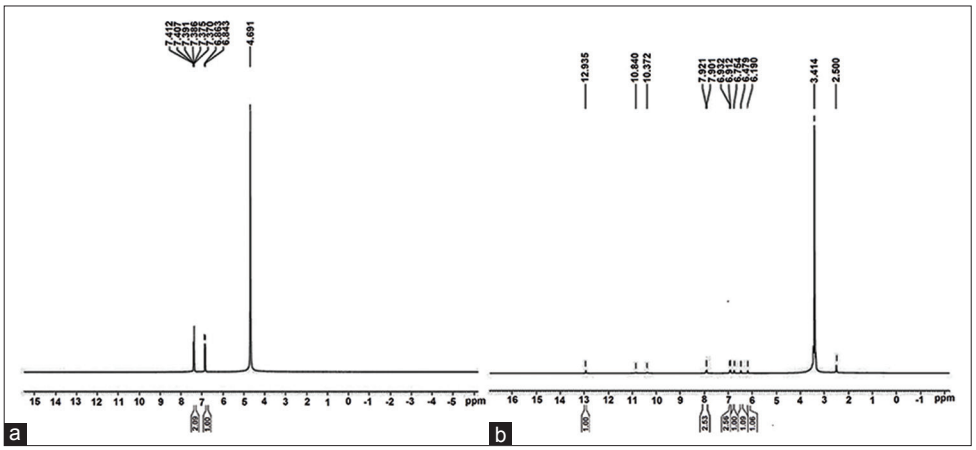

Figure 4: Proton nuclear magnetic resonance profile of purified Compound 1 (a) and 2 (b) from *Anisomeles malabarica* leaves

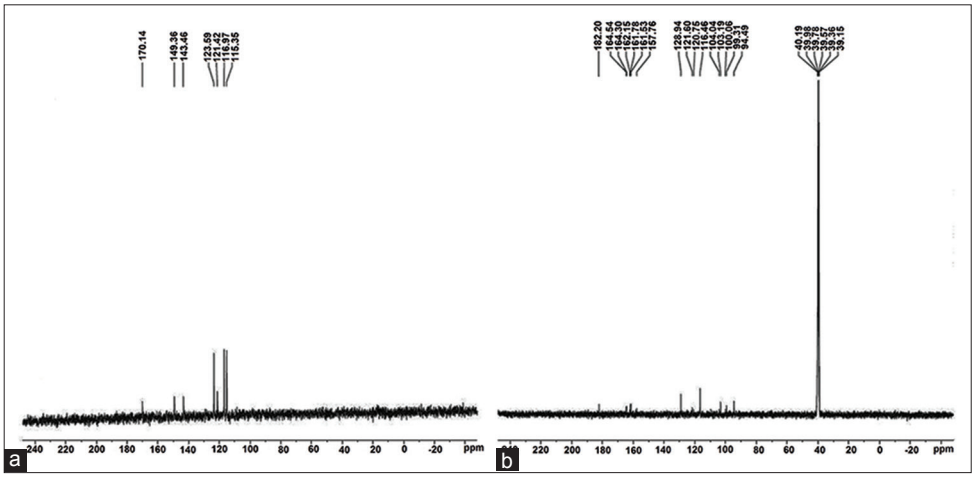

Figure 5: Carbon nuclear magnetic resonance profile of purified Compound 1 (a) and 2 (b) from *Anisomeles malabarica* leaves

comparison with literature data. The isolated compounds were further tested against 15-sLOX inhibition to establish their anti-inflammatory activity.

Molecular docking simulations

The bioactive compounds were imported into Maestro and they were cleaned and optimized using Optimized Potential for Liquid Simulations (OPLS)-2005 force field with steepest descent followed by truncated Newton conjugate gradient protocol in "LigPrep" module. Conformers were generated for prepared molecules using ConfGen by applying OPLS-2005 force field. All the conformations of the ligands were examined after the generation of conformers and to avoid strange conformation of complex structure in lead compounds, original state was retained for docking studies. The typical Protein Data Bank (PDB) co-ordinates are not suitable for direct use in molecular modeling calculations.[23] The X-ray structure of sLOX complex with epigallocathechin (PDB ID: 1JNQ)[24] was

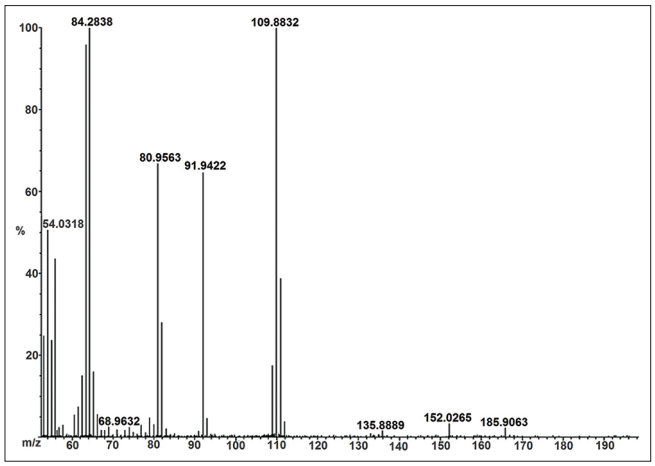

Figure 6: Mass spectrum of purified Compound 1 from *Anisomeles malabarica* leaves

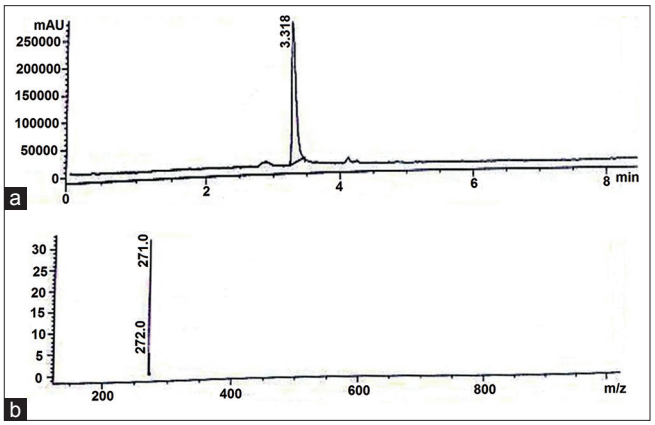

Figure 7: (a) Liquid chromatography and mass spectrometry maximum chromatogram and (b) Molecular mass of purified Compound 2 from *Anisomeles malabarica* leaves

retrieved from Brookhaven PDB and Prepared through Protein Preparation Wizard tool implemented in Maestro 9.1 (Schrodinger, LLC, New York). Molecular docking was carried out using Glide module[25] to find out the possible binding conformations and it also provides information to understand the interactions of LOX receptor-antagonist.

The protein was minimized by applying an OPLS-2005 force field and progressively weaker restraints were applied only to nonhydrogen atoms, for Glide calculations. This refinement procedure was done based on the recommendations by Schrodinger software, because Glide uses the full OPLS-2005 force field at an intermediate docking stage and is claimed to be more sensitive to geometrical details than other docking tools. Water molecules, which are 5Å away from the active site were removed and H atoms were added to the structure. The most likely positions of hydroxyl and thiol hydrogen atoms, protonation states, and tautomers of His residues, and Chi 'flip' assignments for Asn, Gln and His residues were selected. After ensuring that the protein and ligands were in the correct form for docking, the receptor-grid files were generated using a grid-receptor generation program. The grid-enclosing box was generated at the centroid of the co-crystallized ligand and the choice of ligands to be docked was selected from the workspace. The ligands were docked with the protein by using Glide module in extra precision mode (XP), which uses Monte Carlo based simulated algorithm based minimization. Glide generates conformations internally and passes these through a series of filters. The final energy evaluation is done with Glide score and a single best pose is generated as the output for a particular ligand. All calculations were carried out on a Red hat 5.1 Linux platform (Red Hat Inc., USA) running on a Lenovo Intel core 2 duo processor and 2 GB of RAM using the molecular modeling software package Schrodinger, LLC, New York, 2010.

Absorption, distribution, metabolism and excretion predictions

The absorption, distribution, metabolism, and excretion properties were calculated by Qikprop 3.2 (Schrodinger, LLC, New York)[26] which predicts physically significant and physiochemical descriptors of potential drug compounds.[27] The bioactive compounds were neutralized before being used by Qikprop. This step is crucial, as QikProp is incapable to neutralize a structure and no properties will be generated in the normal mode. The program predicts principle descriptors and physiochemical properties along with detailed analysis of log P (octanol/water), QP% (human oral absorption), and log HERG (HERG K channel blockage). It also estimates the acceptability of the compounds based on the Lipinski's rule of five, which are necessary for rational drug design.[28]

RESULTS AND DISCUSSION

Human red blood cell membrane-stabilizing activity

The HRBC membrane-stabilizing study was undertaken to confirm the stability of HRBC membrane by plant extract. The prevention of hypotonicity-induced HRBC membrane lysis was considered as a quantification of anti-inflammatory activity of drugs, since HRBC membranes are analogous to lysosomal membrane components. The ethanolic extract of *A. malabarica* at various concentrations (50-1000 μg/mL) was incubated separately with HRBC solution and the percentage protection was compared with standard drug Diclofenac sodium at the same concentrations. The extract exhibited maximum percentage protection of 83.43 ± 1.67%, at a concentration of 1000 μg/mL [Table 1]. From the results, it was noted that the ethanol extract inhibits red blood cell (RBC) hemolysis, significantly and dose-dependently with better stabilizing effect, similar to control. This result may be featured due to the presence of high phenolic content

in *A. malabarica* has revealed through phytochemical screening (unpublished data). It is also demonstrated that the vitality of cells are mainly depends on the reliability of their membranes.[29] The excessive accumulation of fluid due to rupturing of membrane within the cells is mainly associated with the hemolytic effect of hypotonic solution. The injured RBC membrane causes the cell more vulnerable to secondary damage during free radical induced lipid peroxidation. On the whole, it was observed that the formation of free radicals due to bimolecular breakdown in turn develop cellular damage as seen in rheumatoid arthritis.[30,31] The membrane-stabilizing properties of extract are well-documented to interfere with the release of early phase inflammatory mediators, namely the prevention of phospholipases release and other intracellular components.[32]

Bioassay-guided isolation of the lipoxygenase inhibitors from *Anisomeles malabarica*

Lipoxygenases are a family of enzymes and they catalyze the first step in the arachidonic acid cascade in mammals.[33] A very good correlation exists between the inhibitory activity of mammalian 5-LOX's and sLOX's. Hence, s15-LOX assay was used to screen several medicinal plant extracts for their anti-inflammatory activity. In this study, the ethanolic extract of *A. malabarica* was evaluated to determine their 15-sLOX inhibitory activity and it showed strongest inhibition of 78.36% ±0.57% at the test concentration of 100 μg/mL [Table 2]. Hence, in quest of their bioactive constituents, the extract was further subjected to bioassay-guided fractionation and purification through silica gel chromatographic method. The bioassay-guided fractionation, examined through the same assay, signified that the active substances were confined in the chloroform fraction relatively at three different concentrations (25, 50 and 100 μg/mL). The results demonstrated that, the ethanol extract and chloroform fraction showed better inhibition in an *in vitro* conditions and the inhibition was found to be dose-dependent in case of fraction, while NDGA (25 μg/mL), a reference standard has offered potent and dose-dependent inhibition of LOX's activity than the extract, n-butanol and chloroform fractions [Table 2]. The anti-inflammatory activity of *A. malabarica* extract and its fraction may perhaps be elucidated by the persuasive inhibitory outcomes of their phenolic components on arachidonic acid metabolism through the LOX pathway and they also act as a scavenger of free radicals formed during arachidonic acid metabolism.[34,35]

Table 1: *In vitro* anti-inflammatory activity of *A. malabarica* extract by HRBC membrane-stabilization method

Concentration (μg/mL)	Percentage protection[a]	
	A. malabarica	Diclofenac sodium
50	23.48±2.34	25.22±1.99
100	28.28±3.28	30.69±2.34
125	36.22±2.97	37.93±0.71
250	49.69±2.57	54.32±2.33
500	66.78±2.09	68.77±1.22
1000	83.43±1.67	88.08±1.10

[a]Values are means±SD of three independent experiments. *A. malabarica: Anisomeles malabarica*; HRBC: Human red blood cell; SD: Standard deviation

Table 2: *In vitro* 15-sLOX inhibitory profile of the crude extract and fractions of *A. malabarica* in terms of % inhibition

Sample	Test concentration (μg/ml)	% inhibition[a]
Crude EtOH	100	78.36±0.57
n-butanol	100	28.08±0.63
	50	13.65±1.01
	25	9.73±0.21
Chloroform	100	58.16±1.08
	50	52.12±0.72
	25	45.02±0.77
NDGA	25	78.66±0.28

[a]Values are means±SD of three independent experiments. sLOX: Soybean lipoxygenase; NDGA: Nordihydroguairetic acid; SD: Standard deviation; *A. malabarica: Anisomeles malabarica*; EtOH: Ethanol

The bioactive chloroform fraction was therefore further selected and fractionated using column chromatography as described. The bio-assay revealed that the fraction F_2 possess maximum inhibition at all the tested concentrations (25, 50 and100 μg/mL) [Figure 8]. The purification of most active fraction F_2 has resulted in the isolation of phenolic constituents. The purified compounds were identified as 3, 4 dihydroxy benzoic acid (Compound 1) and 4', 5, 7-trihydroxyflavone (Compound 2) by comparison of spectral data's with reported literature.[36,37] The two known compounds were isolated for the first time from

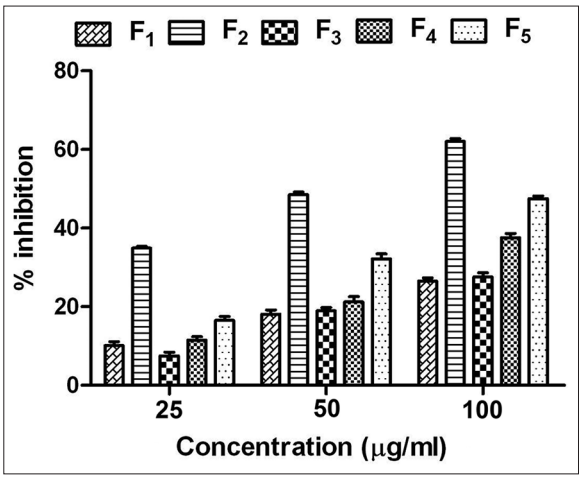

Figure 8: I*n vitro* 15-soybean lipoxygenase inhibitory activity of each column fraction (F1–F5) of *Anisomeles malabarica* in terms of % inhibition

this plant *Anisomeles malabarica*. The anti-inflammatory mechanisms of 3, 4 dihydroxy benzoic acid and 4', 5, 7-trihydroxyflavone was evaluated in different models of inflammation and was reported earlier.[38-41] The chemical structures of the bioactive compounds are shown in Figure 9.

3, 4 dihydroxy benzoic acid

The compound was obtained as a pale white powder. Its molecular formula was established as $C_7H_6O_4$ from EIMS and NMR data. Melting point was found to be 197-198°C. EIMS m/z: 152 $[M-2H^+]^+$, 135, 109, 80, 54, 91; UV (MeOH) λ_{max} (log ε) 256 (2.12) nm, 292 (1.01) nm; IR (KBr) ν_{max}: 3336.21 (OH group), 1668.91, 1600.32, 1528.45 (aromatic carbon-carbon double bond), 1422.47 (O-H bend of carboxylic acid), 1288.72 (O-H bending), 1094.97 (C-O stretch), 942.50, 881.11 (C-H bend), 765, 641.47/cm; ^{1}H NMR (D_2O, 400 MHz): δ 6.84-6.86 (1H, d, J = 8.0 Hz, H-5), 7.41-7.40 (1H, d, J = 4.0 Hz, H-6), 7.39-7.37 (1H, s, H-2); ^{13}C NMR (D_2O, 100 MHz): δ C-1 = 123.59; C-2 = 116.97; C-3 = 149.36; C-4 = 143.36; C-5 = 121.42; C-6 = 115.35 and C-7 carry the carboxylic acid at 170.14.

4', 5, 7-trihydroxyflavone

The compound was obtained as a yellow powder and was determined to have the molecular formula $C_{15}H_{10}O_5$ by liquid chromatography-mass spectrometry (LC-MS) and NMR data. Melting point was found to be 312°C. The LC chromatogram shows a single peak corresponding to the retention time of 3.3 min and the molecular weight was found to be 271 $[M + H]^+$, as depicted by LC-MS analysis. UV (MeOH) λ_{max} (log ε): 268 (1.46), 332 (1.50) nm; IR (KBr) ν_{max}: 3298, 3100 (OH), 2931, 1649 (C = O), 1606 (Ar), 1241, 1176, 827/cm; ^{1}H NMR (DMSo-d_6, 400 MHz): δ 7.91 (2H, *d*, *J* = 8.0 Hz, H-2' and H-6'), 6.92 (2H, *d*, *J* = 8.0 Hz, H-3' and H-5'), 6.19 (1H, s, H-6), 6.48 (1H, s, H-8), 6.75 (1H, *s*, H-3), 12.93 (1H, s, 5-OH), 10.84 and 10.37 (each 1H, s, 7-OH and 4'-OH); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 182.20 (C-4), 164.54 (C-5), 164.30 (C-2), 162.15 (C-4'), 161.78 (C-9), 161.53 (C-7), 128.94 (C-2' and C-6'), 121.60 (C-1'), 116.46 (C-3' and C-5'), 104.04 (C-10), 103.19 (C-3), 100.06 (C-6), 94.49 (C-8).

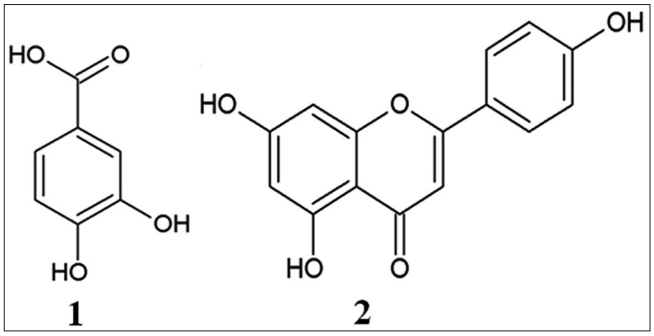

Figure 9: Soybean lipoxygenase inhibitory Compounds 1 and 2 isolated from the leaves of *Anisomeles malabarica*

Inhibitory effect of 3, 4 dihydroxy benzoic acid and 4', 5, 7-trihydroxyflavone on 15-soybean lipoxygenase

The isolated compounds were subsequently assessed for s15-LOX inhibitory activity and they were able to inhibit 15-sLOX. From the results, it was noted that the compound 3, 4 dihydroxy benzoic acid exhibited strongest inhibition of sLOX with 74.04% ± 2.606% at 25 μg/mL followed by 4', 5, 7-trihydroxyflavone, which exhibits moderate activity of 34.68% ± 1.950%. Our findings are in agreement with those of Gutierrez-Lugo *et al.*,[42] who reported that 4', 5, 7-trihydroxyflavone does not exhibit significant 15-sLOX inhibitory activity, but it was found to inhibit the activity of 15-hLOX selectively at a dose of 200 μM. NDGA was used as positive control and exhibit significant (83.55% ±2.126%) inhibition of sLOX's activity and found to be more potent than the compounds. The inhibitory activity of 3, 4 dihydroxy benzoic acid on LOX enzyme, has presented its strong potential to be developed as anti-inflammatory drug.

Molecular docking studies

With the intend of receiving insights into the structural basis and to examine the interaction between sLOX and identified compounds, a comparative docking experiment compounds was carried out along with known LOX inhibitor, NDGA. According to the crystal structure analysis, the catalytic site of LOX comprised of Fe-binding site adjacent to the substrate binding cleft. The substrate binding pocket consists of both polar and hydrophobic aminoacid residues within 5Å sphere. The structural geometry of the iron-binding site of sLOX enzyme consist of three amino acid ligands including the imidazole N-atoms of two histidine residues His 513, His 518 and the carboxylate oxygen of the C-terminal Ile857 which chelate with Fe. The compounds 3, 4-dihydroxy benzoic acid, 4', 5, 7-trihydroxyflavone and NDGA were docked into the active site of sLOX enzyme and the obtained results were evaluated in terms of docking energy and binding confirmation into the catalytic site of sLOX [Table 3, Figure 10a and b]. The compounds and NDGA exhibits

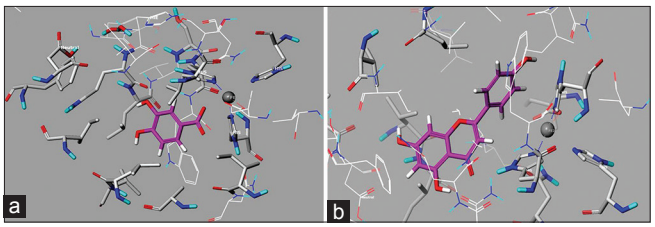

Figure 10: Binding mode of (a) 3, 4-dihydroxy benzoic acid (b) 4', 5, 7-trihydroxyflavone in the hydrophobic binding pocket adjacent to nonheme iron center of soybean lipoxygenase. Ligand is represented in magenta color as tubes. Amino acid residues within 5Å around the active site are denoted as tubes and sticks

π−π stacking interactions with Phe 576, His 518 and His 523 residues other than hydrogen bonding interactions. The compound 3, 4-dihydroxy benzoic acid does not show any hydrogen bond interaction whereas hydrogen bonding was found between His 518 (2.187Å) and carbonyl oxygen of pyranone moiety of 4', 5, 7-trihydroxyflavone [Figure 11a and b]. The most important residue in the hydrogen bond interaction of NDGA is Ile857, contributing a significant docking energy value of -10.63 kcal/mol. From the visual inspection, it was noted that the hydroxyl group forms a hydrogen bond (1.729Å) with the side chain of the carboxylate group of Ile857 [Figure 11c]. Moreover the docking results demonstrated that the potent compounds 3, 4-dihydroxy benzoic acid and NDGA, chelated the nonheme iron atom (Fe-858) through their hydroxyl group in a similar way to that of epigallocatechin, a co-crystallized ligand of 1JNQ. From the results, it was suggested that the enzyme inhibition shown by 3, 4-dihydroxy benzoic acid was almost certainly due to its capability of blocking the entry of the substrate in the active site through covalent binding with iron atom.[43]

Table 3: Docking results of isolated compounds along with the reference compound using Glide XP in Schrödinger 9.1

Compounds	Glide score[a] (kcal/mol)	Glide energy[b] (kcal/mol)	Active site residues interaction[c] (D…H-A)	H-bond length (Å)
1	−8.401	−28.062	-	-
2	−7.387	−20.911	NH (His 518)…O	2.187
NDGA*	−10.634	−18.364	OH…O (Ile 857)	1.729
			OH…N (Hie 513)	2.098
			NH (His 518)…O	2.299

[a]Glide score; [b]Glide energy; [c]Number of hydrogen bonds formed (D: Donor; H: Hydrogen; A: Acceptor); *Reference compound. NDGA: Nordihydroguairetic acid

Pharmacokinetic prediction of compounds

The drug-likeness of the two isolated compounds were assessed by calculating their physicochemical properties that includes stars (number of property or descriptor values that fall outside the 95% range of similar values for known drugs) (<5), molecular weight (<500 Daltons) and rule of five. All these properties were in acceptable range. Water solubility (QPlogS) calculations were done to figure out the absorption and distribution of drugs within the body. The values of QPlogS was found to be − 3.37 and − 0.79 for 4', 5, 7-trihydroxyflavone and 3, 4-dihydroxy benzoic acid, respectively. The cell permeability method (QPPCaco2) was used to calculate the drug metabolism and its access to biological membranes and the IC_{50} values for blockage of HERG K + channels were predicted using QPlogHERG. The QPPCaco2 and QPlogHERG properties were noted to be moderate (27.35 and − 1.51) for 3, 4-dihydroxy benzoic acid whereas 4', 5, 7-trihydroxyflavone exhibits better QPPCaco2 and QPlogHERG properties. The compound 4', 5, 7-trihydroxyflavone (73.61%) shows best human oral absorption percent than compound 3, 4-dihydroxy benzoic acid (52.83) [Table 4]. Overall the

Table 4: ADME properties of the isolated compounds obtained using QikProp module in Schrödinger 9.1

Compounds	Stars[a]	Molecular weight[b]	QPlogPo/w[c]	QPlogS[d]	QPPCaco[e]	QPlog HERG[f]	Percent human oral absorption[g]	Rule of five[h]
1	0	154.12	0.029	−0.799	27.35	−1.51	52.83	0
2	0	270.24	1.644	−3.374	117.45	−5.15	73.61	0

[a]Number of property or descriptor values that fall outside the 95% range of similar values for known drugs (0-5 acceptable); [b]Molecular weight of the molecule (130.0-725.0 acceptable); [c]Predicted octanol/water partition co-efficient log P (acceptable range: −2.0-6.5); [d]Predicted aqueous solubility, log S. S in moldm−3 is the concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid (−6.5-0.5); [e]Predicted apparent Caco-2 cell permeability in nm/s. Caco-2 cells are a model for the gut-blood barrier. QikProp predictions are for nonactive transport (<25 poor, >500 great); [f]Predicted IC_{50} value for blockage of HERG K^+ channels (concern below −5); [g]Predicted human oral absorption on 0-100% scale. The prediction is based on a quantitative multiple linear regression model. This property usually correlates well with human-oral-absorption, as both measures the same property (>80% is high <25% is poor); [h]Number of violations of Lipinski's rule of five. The rules are: mol_MW <500, QPlogPo/w <5, donorHB ≤5, accptHB ≤10. Compounds that satisfy these rules are considered drug like (maximum is 4). ADME: Absorption, distribution, metabolism and excretion

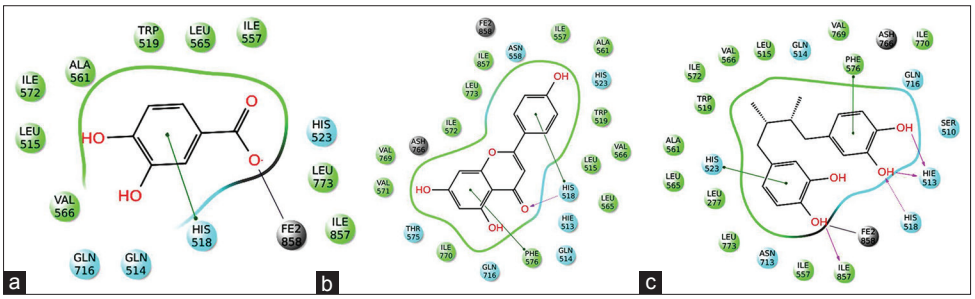

Figure 11: Two-dimensional representation of (a) 3, 4-dihydroxy benzoic acid, (b) 4', 5, 7-trihydroxyflavone and (c) Nordihydroguairetic acid with interacting residues of soybean lipoxygenase. Green line indicates π−π stacking, magenta line indicates hydrogen bond interactions and black line indicates chelation with iron atom

human usages of these two compounds are within the acceptable range.

CONCLUSIONS

This is the first report which demonstrates the bioassay-guided isolation of LOX inhibitors from leaves of *A. malabarica*. Among the fractions, the chloroform fraction showed best sLOX inhibition, which may be due to the presence of phenolic constituents. The result of the present study revealed that the compound 3, 4 dihydroxy benzoic acid has maximum inhibitory ability toward sLOX, even though it was weaker than NDGA. *In vitro* biological assay accompanied by molecular docking calculations also highlighted the anti-inflammatory potential of isolated compounds by acting through a sLOX inhibition mechanism. The results provide evidence that the studied plant might be a potential source of anti-inflammatory agents by justifying its use in traditional medicine.

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