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Original Article

Anti-Inflammatory Activity of Gallic Acid by Suppression of Cyclooxygenase, Lipoxygenase and Nitric Oxide

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ABSTRACT

Background: Gallic acid, trihydroxy benzoic acid is a phenolic compound abundant in various fruits and plants with potent antioxidant and anti-inflammatory properties. This study aims to investigate dual COX LOX inhibition *in silico* and *in vitro* to exhibit safer anti-inflammatory potential sparing gastrointestinal toxicity observed with selective COX inhibitors. **Materials and Methods:** Anti-inflammatory activity of gallic acid was evaluated *in vitro* by measuring cyclooxygenase, lipoxygenase, and nitric oxide activity and gene expression in RAW macrophage cells. *In silico* molecular docking and simulation was performed for gallic acid and diclofenac standard against cyclooxygenase and lipoxygenase. **Results:** Gallic showed inhibition of cyclooxygenase and lipoxygenase with an IC_{50} of 38.94 µg/mL and 17.85 µg/mL against diclofenac standard with a 34.93 µg/mL and 26.54, 18.58 µg/mL. Gallic acid also reduced nitric oxide production in raw macrophages with an IC_{50} of 22.96 µg/mL against diclofenac showing µg/mL. Gallic acid suppressed expression of iNOS, COX2 and LOX mRNA in RAW 264.7 macrophages. Gallic acid showed *in silico* binding energy of -6 kcal/mol and -6.30 kcal/mol against cyclooxygenase and lipoxygenase. Diclofenac showed *in silico* binding energy of -7.21 and -7.53 kcal/mol against cyclooxygenase and lipoxygenase. Binding modes and molecular dynamics simulation analysis of gallic acid and diclofenac were studied in cyclooxygenase and lipoxygenase active site pockets. **Conclusion:** Gallic acid exerts its anti-inflammatory potential without shunting arachidonic acid pathway and can be used in place non-steroidal and steroidal anti-inflammatory drugs. Hence, our studies reinforce that gallic acid act through COX-2/5-LOX pathway sparing mucosal damage and effective for chronic inflammatory diseases.

Keywords: Gallic acid, Diclofenac, Cyclooxygenase, Lipoxygenase, Nitric oxide, iNOS, Anti-inflammatory.

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INTRODUCTION

Gallic Acid (GA) is a natural phenolic compound commonly found in nuts, tea leaves, medicinal plants, and various fruits with applications in inflammatory diseases, cancer, infections, and cardiovascular disease.[1-4] Gallic acid content was screened in Ayurvedic formulations like antiarthritic simhanada guggul others which can be mapped to its biological activities.[5,6] Gallic acid has been shown as a potential candidate for the treatment of various inflammation-related diseases exerting its effect through MAPK and NF-κB signaling pathways.[7]

Cyclooxygenase and lipoxygenase are two main enzymes in catalyzing the metabolic conversion of arachidonic acid to eicosanoids which mediates inflammation and homeostasis.[8] Phospholipase A2 releases arachidonic acid from cell membrane which used as substrate by cyclooxygenase and lipoxygenase to produce prostaglandins and leukotrienes which causes inflammation.[9,10]

Studies indicate that LPS stimulates RAW macrophages to activate intracellular inflammatory cascades involving arachidonic acid pathway and NFkB pathway.[11]

Nitric oxide is synthesized by iNOS enzyme in chronic inflammation excessively. Upregulation of iNOS and increased NO release triggers various pro-inflammatory mediators like interleukins, cytokines etc., in inflammatory disorders leading to pain and edema.

The inhibition of Cyclooxygenase (COX) alone leads to increased production of leukotrienes thereby making arachidonic acid availability for 5 LOX pathway. Another reason is production of vasodilators prostaglandins PGI2 and PGE2 is reduced. Nonselective inhibition of Cyclooxygenase causes Upper Gastrointestinal Bleeding (UGIB) in gouty arthritis due to NSAIDS[12] and allergic reactions accompanied by bronchospasm due to increase in leukotrienes. Therefore, dual inhibitors

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suggested a promising approach that should moderate negative effects connected with COX inhibition. The current study focuses on Gallic acid, a predominant polyphenol of ayurvedic plants and inflammatory markers that it inhibits and regulates *in silico* and *in vitro*.

MATERIALS AND METHODS

Cell Culture

RAW 264.7 cell line was purchased from NCCS, Pune and was cultured in Dulbecco's modified eagles' media (Himedia, India) supplemented with 10% foetal bovine serum (Himedia, India) and grown to confluence at 37°C at 5% CO_2 in a CO_2 incubator. The COX activity was assayed by the method of Walker and Gierse with slight modifications. The cell lysate in Tris-HCl buffer (pH 8) was incubated with glutathione 5 mM/L and haemoglobin 20 µg/L for 1 min at 25°C. The reaction was initiated by the addition of arachidonic acid 200 mM/L and terminated after 20 min of incubation at 37°C, by the addition of 10% trichloroacetic acid in 1 N hydrochloric acid. After the centrifugal separation and the addition of 1% thiobarbiturate, COX activity was determined by reading absorbance at 632 nm.

Determination of Cell Viability of RAW 264.7 Macrophages

Cytotoxic effect of gallic acid was assessed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay.[13] RAW 264.7 cells in logarithmic phase were treated with different concentrations of Gallic Acid (6.25, 12.5, 25 and 50 µg/mL) at 37°C for 24 hr followed by addition of 10 µL of MTT (5 mg/mL) for another 4 hr. The reaction is stopped by addition of 150 µL of DMSO and absorbance was measured at 570 nm. The viability of RAW 264.7 macrophage is calculated as percentage of control cells.

Effect of Gallic acid on cyclooxygenase in RAW Macrophages

RAW 264.7 cells were grown to 70% confluence followed by activation with 1 µL Lipopolysaccharide (LPS) (1 µg/mL). LPS stimulated RAW cells were exposed with different concentration of gallic acid and diclofenac. The cell lysate in Tris-HCl buffer (pH 8) was incubated with 5 mM/L glutathione, and 20 µg/L haemoglobin for 1 min at 25°C. The reaction was initiated by the addition of 200 mM/L arachidonic acid and terminated by the addition of 10% trichloroacetic acid after 20 min. After addition of 1% thiobarbiturate, COX activity was determined by reading absorbance at 632 nm with slight modifications of Gierse Walker method.[14]

Effect of Gallic acid on lipoxygenase in RAW Macrophages

RAW 264.7 cells were grown to 70% confluence followed by activation with 1 µL Lipopolysaccharide (LPS) (1 µg/mL). LPS stimulated RAW cells were exposed with different concentration of gallic acid and diclofenac. The reaction mixture contained 50 µL of cell lysate, and 10 mg/mL sodium linoleate (200 µL) and Tris-HCl buffer (pH 7.4). The LOX activity was monitored by absorbance at 234 nm which reflects the formation of 5-hydroxyeicosatetraenoic acid from linoleate. Percentage inhibition of the enzyme will be calculated.[15]

Determination of NO production

RAW 264.5 cells are seeded at a cell density of $5X10^4$ cells/well and induced with LPS (1 µg/mL). LPS Induced RAW 264.7 cells were treated with different concentrations of Gallic Acid (6.25, 12.5, 25 and 50 µg/mL) at 37°C for 24 hr followed by addition of Griess reagent to the culture supernatant and absorbance at 550nm is an index of NO in the form of nitrite.[16]

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6.0. Results were expressed as the Mean±SD. Data were analysed using Analysis of Variance (ANOVA) followed by multiple comparisons. Values of $p \leq 0.05$ were considered significant.

Molecular Docking Study and Molecular Dynamics simulation

Natural Substrate Arachidonic Acid (CID 4448999) and ligands: Gallic acid (PubChem CID: 370) and Diclofenac were employed for molecular docking. X-ray Crystal structures of proteins COX 2 (PDB ID: 5IKR) and 5LOX (PDB ID: 3O8Y) were employed in this study. Prior to docking, the proteins were pre-processed using Accelrys Viewer lite during which all the ligands and water molecules were deleted. Further the proteins were energy minimized using Schrodinger's Protein Preparation Wizard.[17,18] The residues forming the binding pockets, oxygenase, and peroxidase were identified based on extensive literature search and CASTp 3.0.[19,20] Molecular docking studies were carried out using AutoDock.[21] Hydrogen bonds and hydrophobic interactions were computed using LigPlus. 3D ligand interaction visualized using Discovery Biovia, PyMOL and Chimera were used for graphical representation of the protein-ligand binding.[22-24] The grid box was created around COX2 protein with the following parameters: Centre: X=37.862 Å, Y=0.74 Å, Z=62.153 Å; Size: X=40 Å, Y=40 Å, Z=40 Å) and LOX protein with the following parameters: Centre: X=10.423 Å, Y=66.956 Å, Z=4.194 Å; Size: X=40 Å, Y=40 Å, Z=40 Å). Docking Validation was performed using Root Mean Square Deviation (RMSD) calculations using web based Dincdocking.[25] Molecular Dynamics was performed using SIBIOLEAD.[26]

Effect of Gallic acid on iNOS, COX2 and LOX gene expression

The burst of NO upon LPS-stimulation in RAW 264.7 macrophages result from the upregulation of iNOS, COX 2 and LOX genes. Therefore, we examined the effect of Gallic Acid on the expression levels of iNOS, COX2 and LOX mRNA. RAW 264.7 cells were pretreated with various concentrations of gallic acid for 15 min and further incubated with LPS (1 µg/mL) for either 18 hr or 5 hr, to evaluate and mRNA expression, respectively with housekeeping genes, β-actin. Primers used in gene expression study is shown in Table 1.

RESULTS

Effect of Gallic acid on RAW 264.7 macrophage cell viability

The effect of gallic acid and diclofenac on RAW 264.7 Cell viability is shown in Figure 1a. The cell viability of RAW 264.7 cells exposed to gallic acid at concentration of 6.25-100 µg/mL were above 95% compared diclofenac were above 80%. The results indicate that gallic acid had no cytotoxic effect on RAW 264.7 macrophages at concentration up to 100 µg/mL. Cells were observed under 10X magnification Labomed TCM-400 inverted phase contrast tissue culture microscope and Figure 1b shows the observations.

Effect of Gallic acid on Cyclooxygenase in LPS induced RAW 264.7 macrophage

Studies have shown that COX2 inhibition studies of plant extracts in RAW macrophages is an important marker for anti-inflammatory potential.[27] Results showed in Figure 2a depicts gallic acid inhibited cyclooxygenase in LPS induced RAW 264.7 macrophage in a dose dependent manner with an IC_{50} of 38.94±1.1754 µg/mL against diclofenac standard 34.83±1.1580 µg/mL.

Effect of Gallic acid on lipoxygenase in LPS induced RAW 264.7 macrophage

Results showed in Figure 2b depicts gallic acid inhibited lipoxygenase in LPS induced RAW 264.7 macrophage in a dose dependent manner with an IC_{50} of 17.85±1.3896 µg/mL against diclofenac standard 26.54±1.0676 µg/mL. Validation studies for the anti-inflammatory use of *L. caustica* in Mapuche ethnomedicine, a phytochemical screening of the leaves and stem bark of *L. caustica* were analysed for inhibition of lipoxygenases.[28]

Effect of Gallic acid on NO release in LPS induced RAW 264.7 macrophage

The effect of gallic acid on the release of NO in RAW 264.7 macrophage is shown in Figure 2c. The results showed that gallic acid nitric oxide inhibition effect with an IC_{50} of 22.96±1.1873 µg/mL against diclofenac showing 18.58±1.1879 µg/mL respectively. Gallic acid is shown to attenuate the levels of NO, PGE2, and IL-6 in LPS-induced activation of RAW264.7 cells.[29]

Molecular Docking and Interaction analyses

Cyclooxygenase active site consists of a hydrophobic channel extending to the catalytic site at the centre of protein opposite of peroxidase active site. Docking of diclofenac showed H-bond interactions with the cyclooxygenase active site pocket residues Tyr 385, Ser530 and alkyl/π-alkyl bonds with Val 523, Val 349, Ala 527, Leu352 through π electron cloud of aromatic ring thus blocking hydrophobic channel thereby access of arachidonic acid substrate to the active site through molecule esoteric hindrance depicted in Figure 3 (iii) b. Docking of diclofenac showed H-bond interactions with the lipoxygenase active site pocket residues Val 243 and Arg 457 and and alkyl, π-alkyl bonds with Arg 370, Val 243, Ala 453 through π electron cloud of aromatic ring in a T shaped conformation depicted in Figure 3 (iv) b. Gallic acid showed H-bond interactions with the cyclooxygenase active site pocket residues Tyr 385, Ser 530, Met 522, Val 523 and π-alkyl bond with Ala 527, Leu 352 depicted in Figure 3(i) b. Docking of gallic showed H-bond interactions with the lipoxygenase active site pocket residues Ser 447, Leu 448 and Thr 545 and Arg 457 and and vander waals interaction with Arg 370, Val 243, Ala 453 depicted in Figure 3(ii) b. Docking interation analysis of ligands gallic acid and diclofenac with target proteins are shown in Figure 3 with (a) 3D surface representation shows the positioning of the ligand (in yellow) in the binding pocket of the protein (in cyan) (b) 2D representation shows the hydrogen bonds (in green dashed lines) and hydrophobic interactions (in red) between the ligand (in blue) and the protein (c) 3D visualization of hydrogen bond interaction with active site residues (d) visualization of Solvent Accessible Surface, hydrophobic interaction and hydrogen bond surface. Solvent Accessible Surface (SAS) Blue indicates highly solvent accessible surface and green indicates less solvent accessible surface. Hydrophobicity around the binding cavity (blue represents hydrophilicity and brown represents hydrophobicity) Hydrogen bond donors and acceptors around the binding cavity (green represents the receptor and purple represents the donor.) Comparative binding poses of gallic acid and diclofenac in COX and LOX active site is shown in Figure 3v(a-b). Figure 3(vi) is

Table 1: Gene expression study Primers.

Oligo Name	Sequence
iNOSF	5'-GGAGCGAGTTGTGGATTGTC- 3'
iNOSR	5'-GTGAGGGCTTGGCTGAGTGAG-3'
COX2F	5'-GAAGTCTTTGGTCTGGTGCCTG-3'
COX2R	5'-GTCTGCTGGTTTGGAATAGTTGC-3'
LOXF	5'-GCTTCGCCAGTAAGATCCAG-3'
LOXR	5'-TTGCGCATTTTCTGTTTCAG-3'
B-ActF	5'-CTGACCGAGCTGGCTAC-3'
B-ActR	5'-CCTGCTTGCTGATCCACA-3'

graphical representation of binding energy (kcal/mol) vs RMSD. Table 2 shows gallic acid docking pocket binding energies and inhibition constants with COX2 and 5LOX target proteins.

Molecular Dynamics

Simulation of molecular dynamics between ligands gallic acid and diclofenac with cyclooxygenase and lipoxygenase including protein stability is depicted in Figure 4. The radius of Gyration (rGyr) property was also examined to illustrate the stability of the ligands in COX-2 and 5-LOX binding pockets during the simulation of 100 ns (Figure 4). The rGyr parameter measures ligand flexibility with respect to principal moment of inertia. The diclofenac and gallic acid in complexes with COX-2 and 5-LOX exhibited an average rGyr value of 2.095±0.0035Å (diclofenac), 2.094±0.0035Å (gallic acid) and 2.3866±0.0061 (diclofenac), 2.398±0.0058Å (gallic acid), respectively. RMSD Graph in Figure 4 shows gallic acid and diclofenac is stable in cyclooxygenase with minor fluctuations, an average backbone RMSD of 0.1169 nm and 0.1088 nm respectively. Also, RMSD Graph in Figure 4 shows gallic acid and diclofenac is stable in lipoxygenase with minor fluctuations, an average backbone RMSD of 0.1067 nm and 0.1047 nm respectively. The RMSF figure also demonstrates that the loop's amino acid residues do not significantly vary, with an average RMSF of 0.2 nm. No major fluctuation was observed in the rGyr and showed steady behaviour. The protein's stable folding is demonstrated by the minimal variation in its radius of gyration.

Effect of Gallic Acid on COX2 mRNA expression

The mRNA expression of COX2, 5 LOX and iNOS were determined to relate the inhibitory effect of gallic acid on Nitric oxide release, COX2 and 5LOX inhibition to expression levels of iNOS, COX2 and 5LOX. The results shown in Figure 5 confirm gallic acid attenuated the elevated levels of iNOS, COX2 and 5LOX mRNA expression in LPS stimulated RAW macrophages. The results suggested that anti-inflammatory activity effect of gallic acid was exerted through downregulation of iNOS, COX2 and 5LOX gene expression. cDNA levels were observed using agarose gel electrophoresis and DNA concentration determined using nanodrop spectrophotometer. The mRNA expression

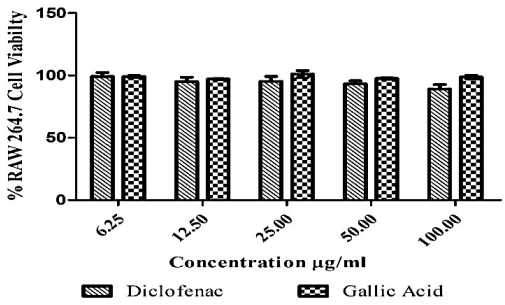

Figure 1a.

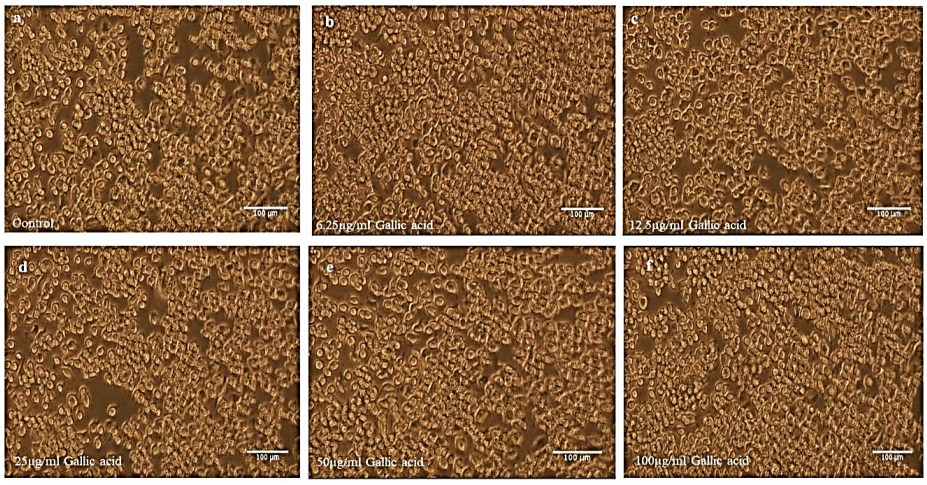

Figure 1b.

Figure 1: a) Effect of Gallic acid on RAW 264.7 macrophage cell viability. (Values are presented as mean±SD (*n*=3)). b) Viability of RAW 264.7 Macrophage cells at different concentrations of the gallic acid treatment.

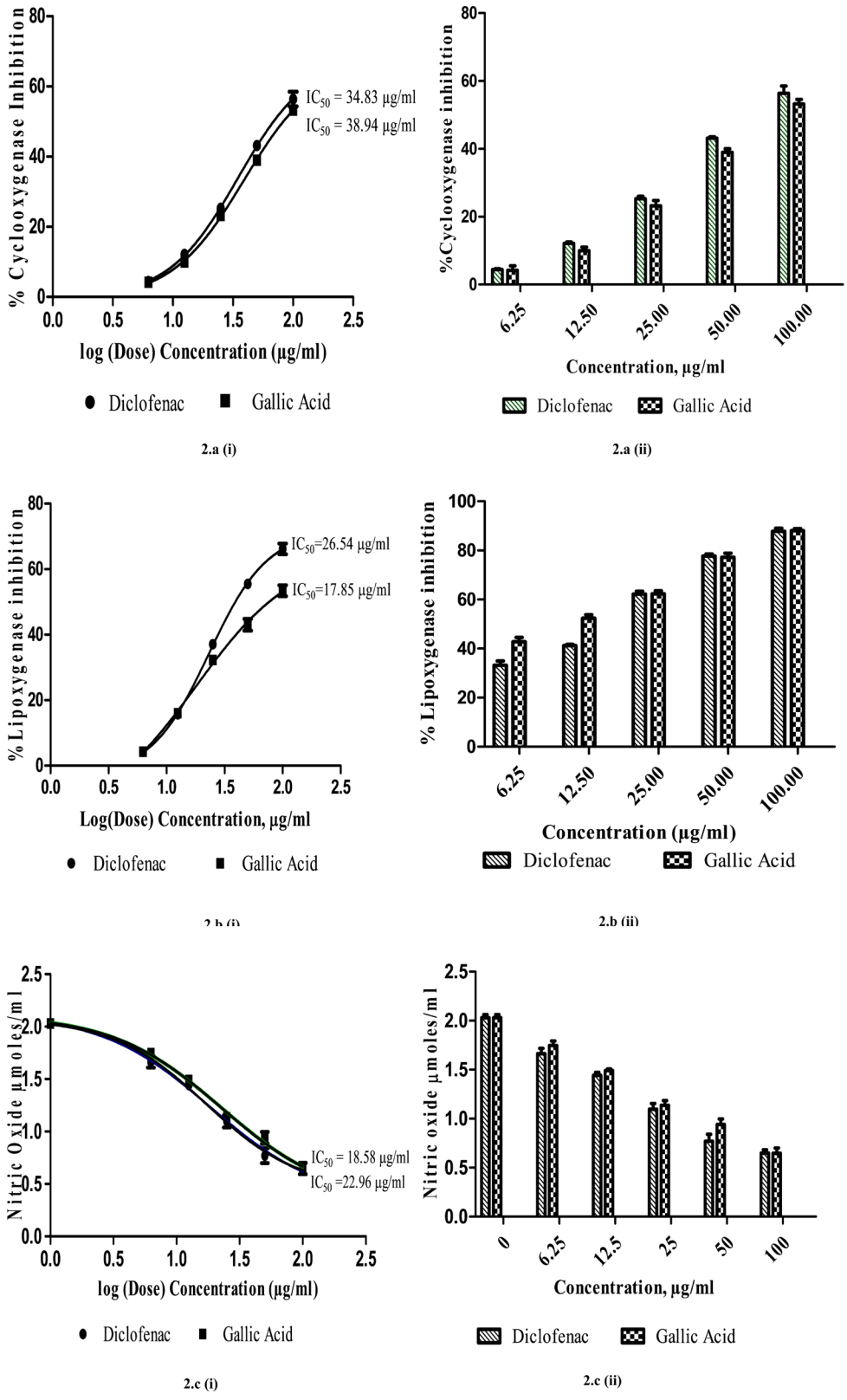

Figure 2: a (i) Dose response curve of gallic acid and diclofenac against cyclooxygenase. a (ii) Effect of gallic acid and diclofenac on cyclooxygenase inhibition. b (i) Dose response curve of gallic acid and diclofenac against lipoxygenase. b (ii) Effect of gallic acid and diclofenac on lipoxygenase inhibition. c (i) Nitric oxide release dose response curve of gallic acid and diclofenac. c (ii) Effect of gallic acid and diclofenac on nitric oxide release from LPS induced RAW macrophages. Values are presented as mean±SD (*n*=3).

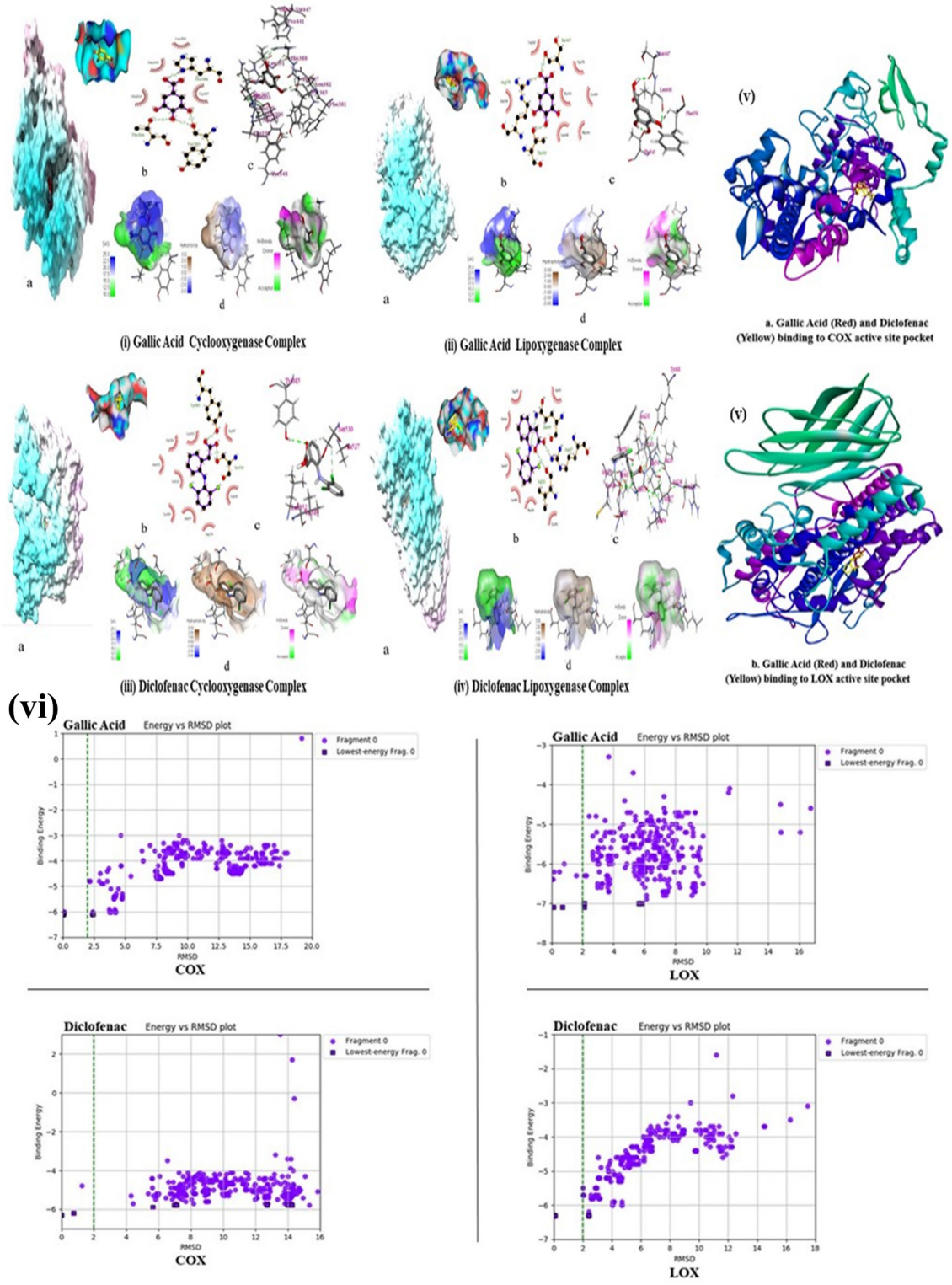

Figure 3: Visualization of docking analysis of gallic acid and diclofenac with cyclooxygenase and lipoxygenase.

levels of cyclooxygenase-2, and matrix metalloproteinase-9 from fibroblast-like synoviocytes of rheumatoid arthritis patients were shown to be suppressed by the gallic acid treatment. Gallic acid, ellagic acid and punicalagin from P*unica granatum* was reported to have Cyclooxygenase 2 (COX-2) and Nitric Oxide Synthase (iNOS) attenuation properties in LPS-induced RAW 264.7 cells.[29] A study showed multitarget approach for gouty arthritis by attenuation of COX-2, iNOS, CD86 and 5-LOX in macrophages by leonurine ameliorated monosodium urate crystal-induced inflammation.[30]

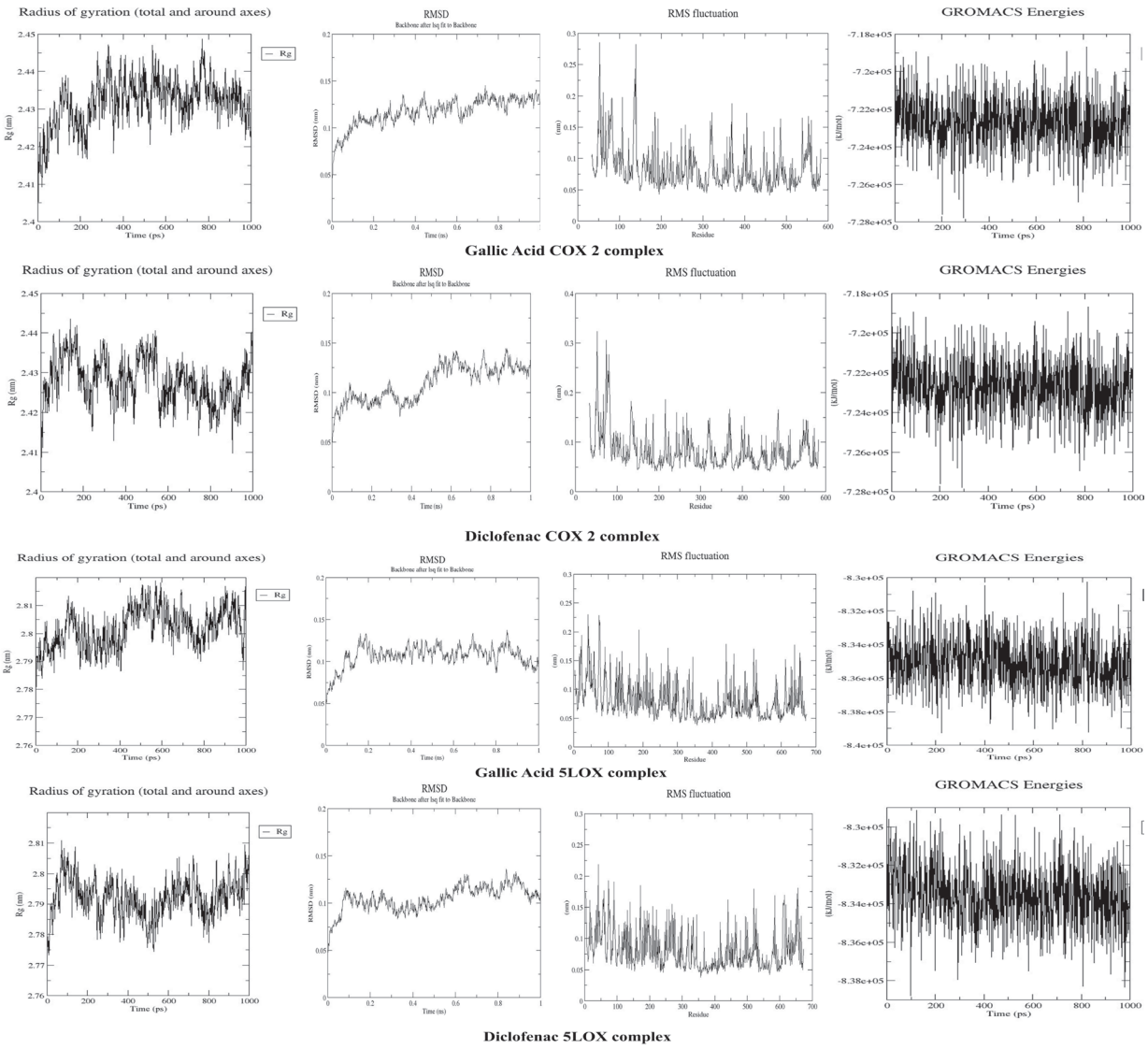

Figure 4: Molecular Dynamics simulation analysis of gallic acid cyclooxygenase complex, diclofenac cyclooxygenase complex, gallic acid lipoxygenase complex and diclofenac lipoxygenase complex.

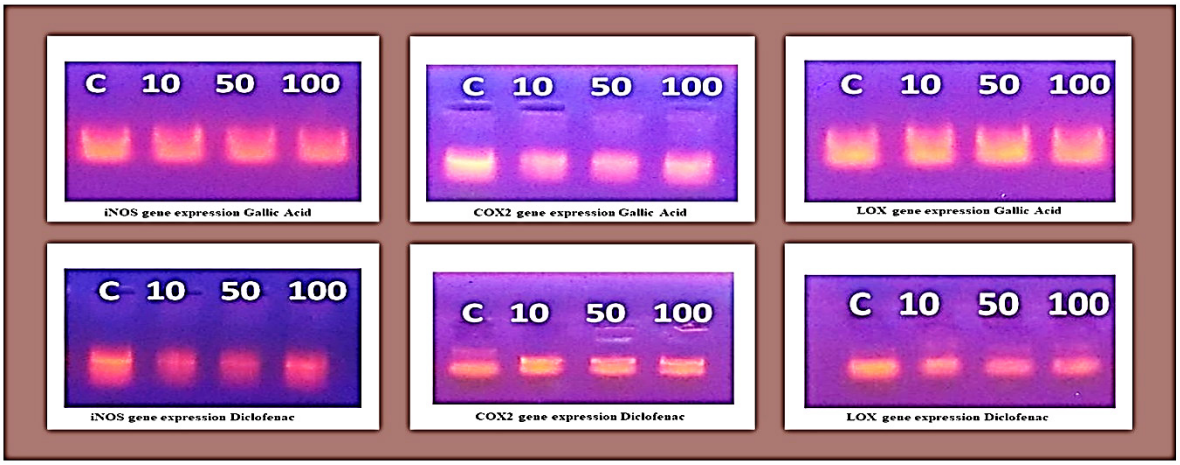

Figure 5: Effect of gallic acid on mRNA expression of iNOS, COX2 and 5LOX in LPS timulated RAW 264.7 cells.

Table 2: Molecular Docking analysis of gallic acid and diclofenac with cyclooxygenase and lipoxygenase.

Protein	Type of interaction	Diclofenac		Gallic Acid	
		ΔG (kcal/mol)	K_i (μM)	ΔG (kcal/mol)	K_i (μM)
COX2 5IKR	Van der Waals, H-bond, π-alkyl	-7.21	5.15	-6.0	38.54
5 LOX 3O8Y	Van der Waals, H-bond, π-alkyl	-7.53	3.02	-6.3	23.19

DISCUSSION

It has been reported that gallic acid demonstrate a variety of biological activities and due to this, have been progressively included as alternative to non-steroidal and steroidal anti-inflammatory drugs prescribed for chronic inflammation. We have studied mechanism of action of gallic acid which is not fully understood at the arachidonic acid pathway level earlier. Therefore, some authors have proposed experimental studies that indicate the potential role of dual inhibition of COX and LOX and dual inhibitors as future molecules for the treatment of chronic inflammatory disorders. Therefore, before empirical approaches, *in silico* studies have been implemented to reveal the binding capacity and affinity of gallic acid and diclofenac as reference standard. Gallic acid and diclofenac binding poses stay within the hydrophobic channel of COX2 and 5LOX active site. COX-2 and 5-LOX inhibitor drugs are stabilized by hydrogen bonds, van der waals and alkyl interactions inside the hydrophobic channel of active site pockets. Previous studies have shown gallic acid rich in ayurvedic plant *Emblica officinalis* and its analogues can inhibit COX isoforms.[31]

The data from *in vitro* cyclooxygenase and lipoxygenase inhibition in LPS stimulated RAW macrophages is supported by molecular docking and simulation analysis. Mithun Rudrapal *et al.*, findings reiterate potential bioactive from Indian spices apparent role as anti-inflammatory agents by dual synergistic inhibition of COX and LOX.[32]

CONCLUSION

The present study provides indication that gallic acid exerted its anti-inflammatory effect through suppression of iNOS, COX2 and 5LOX and is a potent dual COX/LOX inhibitor. *In silico* studies highlighted the structure and biological activity correlation of gallic acid through high binding energy and lower inhibition constant. Gallic acid showed strong binding to active site pocket residues of cyclooxygenase and lipoxygenase with reference to diclofenac standard. Ayurvedic formulations with high gallic acid content have the potential to be further screened for effectiveness in chronic inflammatory disorders like arthritis, gout, pyoderma gangrenosum and psoriasis. Further, *in vivo* studies will help us determine the effective concentration required in different inflammatory conditions and resolution time.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

COX2: Gallic Acid; **GA:** Cyclooxygenase 2; **5LOX:** 5 Lipoxygenase; **NO:** Nitric Oxide; **iNOS:** Inducible nitric oxide synthase; **FTIR:** Fourier Transform infrared; **NSAID:** Non-Steroidal Anti-Inflammatory Drug.

SUMMARY

Gallic acid is a phenolic acid, a natural secondary metabolite found in fruits, nuts and leaves of plants. Our study shows that the anti-inflammatory mechanisms of GA mainly involved arachidonic acid pathway attenuation with no obvious toxicity. Gallic acid demonstrated dual cyclooxygenase and lipoxygenase inhibition *in vitro* in LPS induced RAW macrophage cells. Gallic acid is showing concentration dependant reduction in NO level of LPS stimulated RAW macrophage cells. Gallic acid showed down regulation of COX-2 and 5-LOX gene expression in LPS induced RAW 264.7 cells. This study suggests gallic acid can be used as a potent anti-inflammatory agent for acute inflammation sparing gastric mucosal toxicity and preventing arachidonic acid metabolism shunt.

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