## RESEARCH

# Molecular docking prediction and in vitro studies elucidate anti-inflammatory effect of *Garcinia* extract against inducible nitric oxide synthase and cyclooxygenase-2 targets

Anuradha Kalita, Manas Das[*], Bhabajyoti Das and Momita Rani Baro

## Abstract

**Background:** *Garcinia* is a tropical plant that has been traditionally used in medicinal folklore for its potential antioxidant, antibacterial, anti-hyperlipidemic, anti-diabetic, hepatoprotective, etc. In this study, methanolic extract of *Garcinia* herbal supplement (GME) and its important phytoconstituents (Garcinol and hydroxycitric acid) were evaluated for their inhibitory action against important inflammatory markers iNOS and COX-2 in lipopolysaccharide-induced RAW 264.7 cells. iNOS and COX-2 play a major role in the process of inflammation, and inhibition of these molecules will help to alleviate the inflammatory process. The cells were pre-treated with two doses of GME (115 μg/ml and 230 μg/ml); Ggarcinol (6 μM and 12 μM); hydroxycitric acid (17.5 μg/ml and 35 μg/ml) followed by stimulation with 1 μg/ml of LPS for 24 h.

**Results:** The results of the study demonstrated that *Garcinia* and its active components Garcinol and HCA play an important role in suppressing LPS-induced relative mRNA expression of iNOS, COX-2, and subsequent reduction in the levels of total nitric oxide and prostaglandinE2. Molecular docking analysis of Ggarcinol and HCA with iNOS and COX-2 proteins showed potent interactions with negative binding energies.

**Conclusions:** This study suggests that *Garcinia* possess anti-inflammatory activity thus providing a possibility for drug designing as iNOS and COX-2 inhibitor.

**Keywords:** *Garcinia*, Lipopolysaccharide, Inducible nitric oxide synthase, Cyclooxygenase-2, Garcinol, Hydroxycitric acid

*Correspondence: manasdasne@gauhati.ac.in
Department of Zoology, Animal Physiology and Biochemistry Laboratory,
Gauhati University, Guwahati, Assam 781014, India

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**Graphical Abstract**
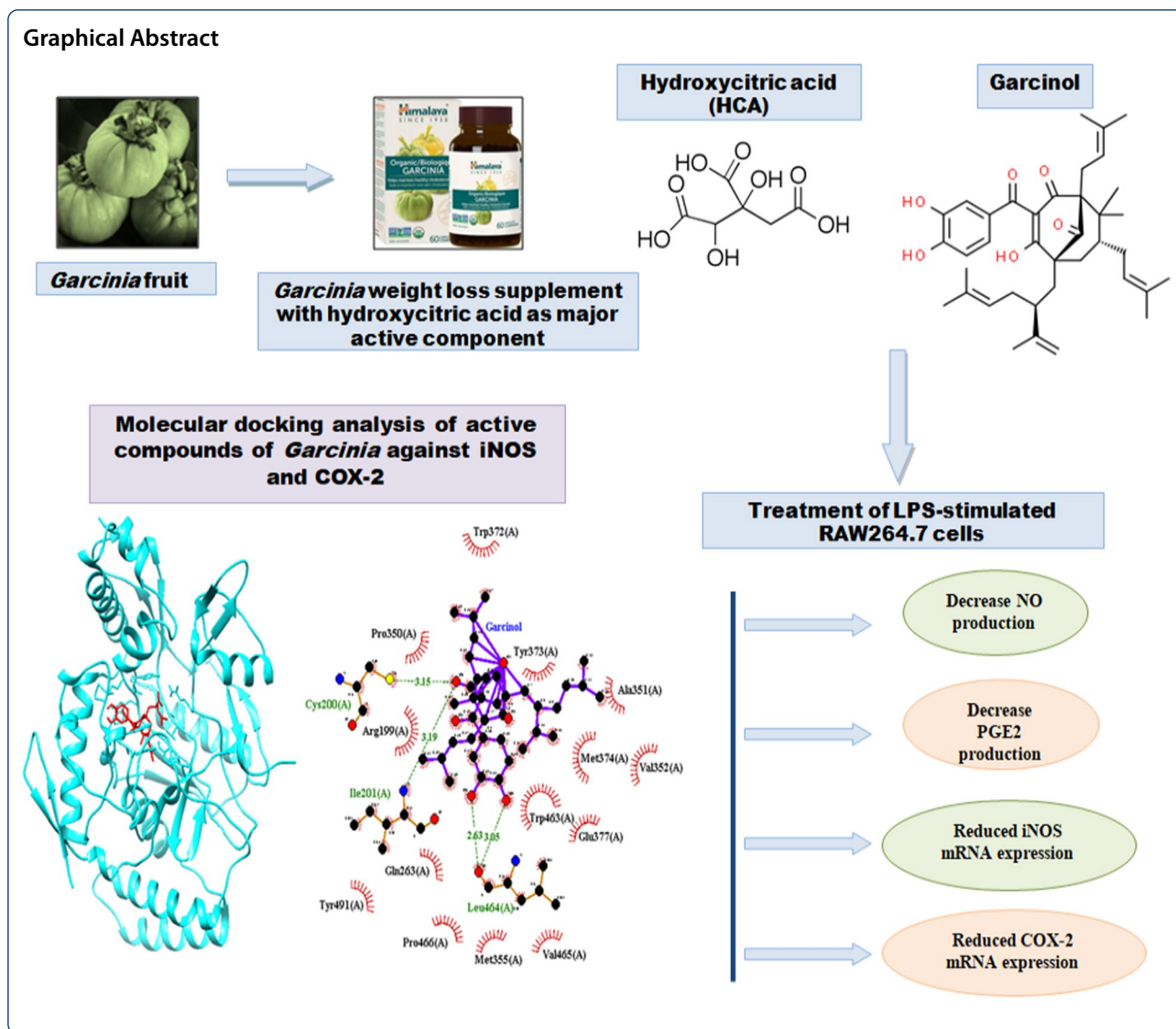


## 1 Background

The process of inflammation is mediated by several important mediators, out of which the inducible nitric oxide synthase (iNOS or NOS2) and cyclooxygenase-2 (COX-2) have been widely studied as markers of inflammation. iNOS is an enzyme that catalyzes the reaction leading to the production of nitric oxide (NO) from L-Arginine [1]. iNOS is mainly produced by macrophages in response to inflammatory stimuli. During the process of inflammation, macrophages increase the production of both NO and other free radicals to a great extent [2]. NO is a radical effector of the innate immune system [3]. NO can form other reactive nitrogen species (RNS) such as nitrogen dioxide ($NO_2$), dinitrogen trioxide ($N_2O_3$), peroxynitrite anion ($ONOO^-$), nitrosothiols, and other nitrosating species. Generally, the immune cells are

deficient in this enzyme. However, several of extracellular stimuli can trigger different signaling pathways causing the expression of iNOS. Lipopolysaccharide (LPS) is one of the most potent stimuli that activates the Toll-like receptor 4 (TLR4) and downstream signaling cascade [4]. Cyclooxygenases (COX) are enzymes that have three isoforms, namely COX-1, COX-2, and COX-3. Out of these three, COX-2 is mainly responsible for prostaglandin-mediated inflammation [5]. COX converts arachidonic acid to prostaglandins [6]. Inhibition of these molecules will open up new avenues in the treatment of inflammation. Currently, synthetic anti-inflammatory drugs are being used to treat inflammation. However, these drugs possess many side effects, and thereby research has been going on to find out natural anti-inflammatory agents. From earlier times, herbal medicines have been

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regarded to be the best remedies for many disorders. The requirement of herbal products is therefore increasing exponentially for pharmacological applications for their important medicinal properties. *Garcinia* has been traditionally used in Indian medicine for its medicinal properties. The whole plant including the fruits, leaves, bark, and roots has been used for making remedies against several diseases [7]. *Garcinia* belongs to the family Clusiaceae and is widely distributed throughout the tropical regions. The genus has more than 200 species worldwide. However, some of the well-studied species of Garcinia include *G.cambogia*, *G. mangostana*, *G. indica*, *G. kola*, *G. pedunculata*, *G. lanceifolia*, *G. xanthochymus*, and *G. cowa* [8, 9]. *Garcinia* is an important medicinal plant that possesses many important phytoconstituents. It has been traditionally used in tropical regions for its diverse medicinal properties. Evidence suggests that many important phytocompounds with anti-oxidant, anti-diabetic, anti-hyperlipidemic, anti-allergic, hepatoprotective, antibacterial, anti-inflammatory, neuroprotective as well as anti-cancer effects have been isolated from the fruit rind of *Garcinia* [10–18]. *Garcinia* plant is loaded with phenolic components including flavonoids, xanthones, benzophenones. The major therapeutic phytocompounds present in *Garcinia* include polyisoprenylated benzophenones such as garcinol, isogarcinol, camboginol, xanthochymol, and isoxanthochymol [19, 20]. The major active components of *Garcinia* are known to be extracted from the fruit rinds. Also, the fruits are rich in organic acids such as citric acid, acetic acid, malic acid, ascorbic acid, hydroxycitric acid [20, 21]. *Garcinia* herbal extract with high HCA content is available in the form of oral supplements well-known for weight loss and is marketed by different brand names. Garcinol, a polyisoprenylated benzophenone derivative, is one of the phytoconstituents that has been isolated from the fruit rind of *Garcinia* [20, 22–25]. Studies suggest that Ggarcinol could be a positive anti-inflammatory agent [25, 26]. In vitro study using IL-1β-Induced chondrocyte inflammation has reported the protective effect of Garcinol against osteoarthritis by reducing the secretion of pro-inflammatory cytokines such as IL-6 and TNF-α; iNOS and COX-2 expression [27]. Also, apart from Garcinol, the *Garcinia* acid or hydroxycitric acid (HCA) is one of the active components that attribute to the weight loss properties of *Garcinia* extract [22, 28]. The dried fruit of the *Garcinia* consists of 30 to 50% HCA. HCA has been generally used for its anti-inflammatory activities [29]. It has been known to reduce oxidative stress in some experimental models of inflammation [30–32]. The exact mechanism of the anti-inflammatory effects of *Garcinia* remains unclear. However, there is a possibility that phytocompounds exhibit their anti-inflammatory effects by inhibiting inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression.

In our previous study using the LPS-challenged rat model, it was observed that *Garcinia* herbal extract significantly inhibited the iNOS and COX-2 expression and also led to a subsequent decrease in the enzyme activities and serum NO and PGE2 levels [33]. The LC/MS analysis of GME has shown the presence of many important phytocompounds. The major active constituents found to be present in GME were hydroxycitric acid (HCA) and Garcinol [33]. Therefore, in this study, we aim to investigate the inhibitory potential of the GME and the active constituents present, namely Garcinol and HCA against iNOS and COX-2 in LPS stimulated RAW 264.7 cells. In addition, we evaluate the plausible mechanism of extracts' inhibitory role through in silico analysis using molecular docking studies to find out the potential interactions of HCA and Garcinol with the target proteins iNOS and COX-2.

## 2 Methods

### 2.1 Maintenance of cell line
The murine macrophage cell line (RAW264.7) was purchased from National Centre for Cell Science (NCCS), Pune, India. The cell line was maintained in the cell culture laboratory of the Department of Zoology, Gauhati University. The cell line was grown in Dulbecco's modified eagle's medium or DMEM (HiMedia). The media was supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml Penicillin, and 100 μg/ml Streptomycin. The cell line was maintained at 37 °C and 5% $CO_2$ in a $CO_2$ incubator. The cells were tested for any kind of *Mycoplasma* contamination before the experiments following the PCR-based method [34, 35].

### 2.2 Chemicals
LPS (E. coli serotype O111: B4, Sigma-Aldrich, USA) stock solution of 1 mg/ml was prepared in 1X PBS and stored in aliquots at −20 °C until the experiment. The methanolic extract of commercially available *Garcinia* herbal supplement (Himalaya, Lot No.: 11702264) was prepared for in vitro treatment. (-)-Hydroxycitric acid lactone **(**Sigma-Aldrich, USA, CAS No. 27750–13-6) stock solution of 5 mg/ml was prepared in miliQ water, and further dilution was done in DMEM. Similarly, Garcinol (Sigma-Aldrich, USA, CAS No.78824–30-3) stock solution was prepared by dissolving in 0.25%DMSO in miliQ water to make 20-mM stock solution, and further dilution was done in DMEM [36].

### 2.3 Determination of LPS dose
The LPS dose for stimulation of RAW264.7 cells was determined using the nitric oxide assay. The production

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of nitric oxide in the culture medium of control and LPS treated RAW 264.7 cells was measured using the nitric oxide estimation kit (HiMedia) following the manufacturer's protocol. The RAW 264.7 cells were seeded in a 6-well culture plate at a density of $2 \times 10^5$ cells/well and allowed to settle for 16 h. After 16 h of incubation, the medium was discarded and the cells were treated with fresh medium containing different doses of LPS (0, 0.5, 1, 1.5, 2, and 3 µg/ml). 24-h post-treatment, 100 µl of cell culture supernatant was used for TNO estimation. A triplicate set was taken for the measurement of each sample.

### 2.4  Cell viability assay and IC$_{50}$ determination
To ascertain the IC$_{50}$ dose for GME, Garcinol, and HCA cell viability assay was performed using the MTT reagent (HiMedia). The effect of GME, Garcinol, and HCA on cell viability was investigated using the MTT assay. RAW264.7cells were seeded in 96-well plates at a density of $1 \times 10^4$ cells/well and allowed to settle for 12 h at 37 °C. The culture medium was then discarded, and fresh media containing different concentrations of GME (0, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 µg/ml); Garcinol (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µM); and HCA (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/ml) was added and then incubated for 48 h at 37 °C. Post-incubation, 10 µl of MTT was added to each well and further incubated for 3–4 h at 37 °C. Next, the media containing MTT was discarded, and 100 µl/well of solubilizing reagent (dimethyl sulfoxide) was added and then incubated overnight. The absorbance of the plate was read at 570 nm and 630 nm with a microplate reader. The IC$_{50}$ value of GME, Garcinol, and HCA was evaluated from the MTT assay results.

### 2.5  Cell treatment
The cell experiments were divided into eight groups: control group, LPS treatment group, GME(115 µg/ml)+LPS treatment group, GME(230 µg/ml)+LPS treatment group, Garcinol(6 µM)+LPS treatment group, Garcinol(12 µM)+LPS treatment group, HCA(17.5 µg/ml)+LPS treatment group, HCA(35 µg/ml)+LPS treatment group. RAW 264.7 cells were cultured in 100-mm dishes at a density of $10^6$cells/cm$^2$ and allowed to settle for 16 h. After 16 h of incubation, the medium was discarded and the cells were pre-treated with medium containing 115 µg/ml and 230 µg/ml of GME; 6 µM and 12 µM of Garcinol; 17.5 µg/ml and 35 µg/ml of HCA for 12 h except for the control and LPS treated groups and then the medium was discarded. The cells were then stimulated with 1 µg/ml LPS for 24 h except for the control group.

### 2.6  Determination of total nitric oxide (TNO) level
The production of TNO in the culture medium of treated cells was measured using the NO estimation kit (HiMedia) following the manufacturer's protocol. 24-h post-LPS treatment, 100 µl of cell culture supernatant was used for total nitric oxide (TNO) estimation. A triplicate set was taken for the measurement of each sample. This assay is based on the reduction of nitrate (NO3$^−$) to nitrite (NO2$^−$) by a reducing agent at 37 °C. Converted nitrite and endogenous nitrite are collectively converted by Griess reagent to a blue-colored azo compound. This compound can be measured spectrophotometrically between 580–630 nm, and absorbance is directly proportional to the TNO concentration in the sample which is calculated from the standard plot of NaNO3.

### 2.7  Prostaglandin E2 assay
The cells were washed with pre-cooled PBS and dissociated the cells by scraping. The cell suspension was collected and centrifuged for 5 min at $1000 \times g$. The medium was discarded and cells were washed 3 times with pre-cooled PBS. For every $1 \times 10^6$ cells, 150–250 µl of pre-cooled PBS was added to keep the cells suspended. The freeze–thaw process was repeated several times until the cells were fully lysed followed by centrifugation for 10 min at $1500 \times g$ at 2–8 ℃. The resultant supernatant was used to carry out the assay using the PGE2 ELISA kit (Elabscience®, USA) following the manufacturer's instructions. The kit uses the Sandwich-ELISA principle.

### 2.8  RNA isolation and cDNA synthesis
For total RNA isolation, the cells were washed with ice-cold PBS 24-h post-LPS treatment. RNA was extracted with spin column followed by NucleoSpin RNA Plus, Mini kit for RNA purification with DNA removal column (Macherey–Nagel) according to the manufacturer's instructions. RNA (0.5 µg per sample) was reverse transcribed to cDNA using a first-strand cDNA synthesis kit (High Capacity, ThermoFisher Scientific) following the manufacturer's protocol. For real-time qPCR studies, the cDNA samples were diluted 50 times with sterile miliQ water.

### 2.9  Designing of primers
For real-time quantitative PCR amplification, the gene-specific primers for inos, cox-2, and gapdh were designed from species-specific cDNA sequences available in the NCBI database (accession numbers given in Table 1 with the help of primer-BLAST (NCBI), and the properties were checked using Oligo Calculator [37].

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**Table 1** Primer sequences used for real-time qPCR analysis

| Gene | Forward primer (5′→3′) | Reverse primer (5′→3′) | Accession number | Product size (bp) |
|------|------------------------|------------------------|------------------|-------------------|
| inos | CTATGGCCGCTTTGATGTGC | TTGGGATGCTCCATGGTCAC | U43428.1 | 111 |
| cox-2 | TCACGTGGAGTCCGCTTTAC | CCTTCGTGAGCAGAGTCCTG | NM_011198.4 | 112 |
| gapdh | ATGTGTCCGTCGTGGATCTG | GTGTAGCCCAAGATGCCCTT | GU214026.1 | 115 |

The specificity of primers was checked by inspecting the PCR amplicons on 1.2% agarose gel. The details of primer for real-time qPCR studies are shown in Table 1.

## 2.10 Real-time qPCR analysis

Real-time qPCR analysis was performed with the cDNA using different sets of primers used in the study (Table 1) with the help of Qiagen (Rotor-GeneQ). The relative abundance of the mRNA transcripts, in each sample, was normalized to the amount of an endogenous gene, gapdh. 10 µl TB Green PCR Master mix (TAKARA), 2.8 µl of each forward and reverse primers (10 µM), and 2 µl of cDNA template were used; and qPCR reactions were performed with thermal cycling conditions: denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, primer annealing at respective annealing temperatures for the 30 s and extension at 72 °C for 30 s. Relative mRNA concentrations of the transcripts in the experimental groups were determined as fold change to the control by following the $2^{-\Delta\Delta CT}$ method [38, 39]. The melt curve analysis was performed to check for any non-specific amplification during qPCR analysis.

## 2.11 Molinspiration

Molinspiration tool is used to execute QSAR studies to identify possible activators of biological objects. This online tool helps to calculate significant molecular properties of ligands including logP, polar surface area, number of hydrogen bond donors, number of hydrogen bond acceptors, and prediction of the bioactivity score for the majority of chief drug targets [40]. Lipinski's rule of five was applied to Garcinol and HCA [41]. The mol files and smile formula of Garcinol and HCA were obtained from the CHEMSPIDER database [42].

## 2.12 Molecular docking

### 2.12.1 Protein preparation

The PDB structures of iNOS (PDB ID: 2NSI) and COX-2 (PDB ID: 6COX) were retrieved from RCSB Protein Data Bank (http://www.rcsb.org/pdb/). The protein molecules were optimized (Fig. 1). The water molecules, hetero atoms were removed from the crystal structures with the help of Chimera 1.15. Polar hydrogens and Kollman charges were added, and the autodock structures (pdbqt

file) were constructed with the help of the Autodock tool (ADT) [43].

### 2.12.2 Ligand preparation

The structures of Garcinol (PubChem CID-5281560) and HCA (PubChemCID-185620) were retrieved as an SDF file from PubChem database (https://pubchem.ncbi.nlm.nih.gov) [44]. The file was converted to PDB format with the help of Open Babel software (http://openbabel.org) [45]. To carry out molecular docking, the ligand was optimized, energy minimization was performed, and the pdbqt file was constructed with the help of the Autodock tool (ADT) [43].

### 2.12.3 Grid box set-up and docking

Molecular docking was carried out with garcinol and HCA against iNOS and COX-2 with the help of the AutoDock 4.2.6 program [43]. The binding site residues of the proteins were predicted using CASTp 3.0 [46]. A grid box enclosing the binding site residues of the target proteins was created to achieve the best conformational space. The grid box parameters are shown in Tables 2, 3. Lamarckian Genetic Algorithm was used to carry out the docking. The resultant docked poses were chosen based on their binding energies and intermolecular H-bonds. The H-bond and hydrophobic interactions between protein–ligand complexes were analyzed using the Lig-Plot + tool [47].

## 2.13 Statistical analysis

Experiments were conducted in triplicates, and all the parameters were presented as mean ± SEM. The statistical significance was calculated by one-way ANOVA, followed by Tukey's post hoc test using SPSS software. The results were considered statistically significant when $p < 0.05$.

## 3 Results

### 3.1 Effects of GME, Garcinol, and HCA on RAW264.7 cell viability

The present study reported significant ($p < 0.05$) inhibition of RAW264.7 cell viability post-treatment with GME, Garcinol, and HCA for 48 h. The $IC_{50}$ of GME, Garcinol and HCA for RAW264.7 cells as calculated

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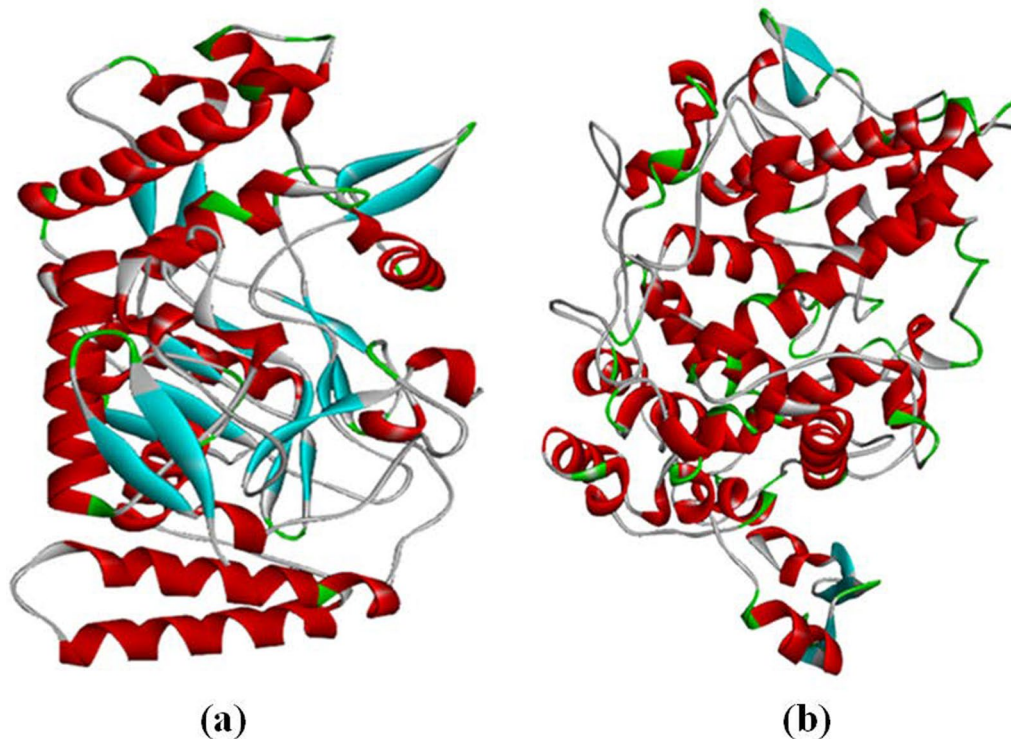


**Fig. 1** Optimized structures of (**a**) iNOS and (**b**) COX-2 (BIOVIA Discovery StudioVisualizer)

**Table 2** Properties of ligands analyzed using Molinspiration

| Ligands | CHEMSPIDER ID | LogP | H-bond acceptor | H-bond donor | Mol wt | No. of violation | TPSA |
|---|---|---|---|---|---|---|---|
| Hydroxycitric acid | 110,439 | − 2.90 | 8 | 5 | 208.12 | 0 | 152.35 |
| Garcinol | 10,199,485 | 8.26 | 6 | 3 | 602.81 | 2 | 111.90 |

from the regression equation was found to be 460 μg/ml, 24 μM, and 70 μg/ml, respectively. Based on the $IC_{50}$, two different doses for GME (230 μg/ml and 115 μg/ml); Garcinol (6 μM and 12 μM); and HCA (17.5 μg/ml and 35 μg/ml) were selected for treatment of the cells (Figs. 2, 3, 4).

### 3.2 Determination of LPS dose by NO assay
The cells on treatment with different concentrations of LPS after 24 h showed a significant increase in the production of total nitric oxide. However, the maximum amount of total nitric oxide produced 24 h after LPS treatment was found at the concentration of 1 μg/ml LPS by 6.99fold ($p < 0.001$) as compared to control. Therefore, the LPS dose selected for the present study was 1 μg/ml (Fig. 5).

### 3.3 Effects of GME, Garcinol, and HCA on morphology of LPS-stimulated RAW264.7 cells
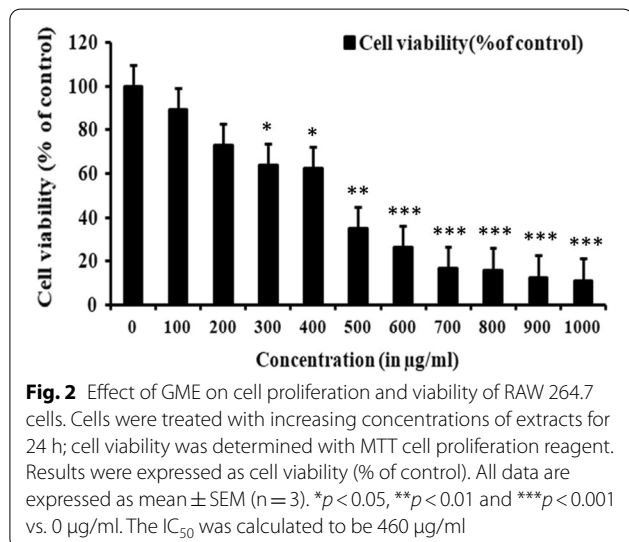The cells were observed under the optical microscope (400X) 24-h post-treatment. The cells without LPS treatment showed a rounded form whereas the LPS-stimulated cells showed an asymmetrical form as well as spindle shape with pseudopodia formation. The pre-treatment of LPS-stimulated cells with GME, Garcinol, and HCA showed reduced level of pseudopodia formation. (Fig. 6).

### 3.4 Effects of GME, Garcinol, and HCA on TNO production
The results of the nitric oxide assay revealed that there was a significant increase in the TNO level by 4.30-fold ($p < 0.001$) after 24 h of LPS exposure as compared to control. On the other hand, its levels was found to significantly decreased by -2.43-fold ($p < 0.05$) in 230 μg/ml GME; -1.92-fold ($p < 0.05$) and -3.19-fold ($p < 0.01$),

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**Table 3** Grid box parameters for docking and binding site residues

| Protein | PDB ID | Binding site residues | Center grid box (points) | Grid size (points) |
|---|---|---|---|---|
| iNOS | 2NSI | MET120,THR121,LEU125,TRP194,ALA197,PRO198,ARG199, CYS200,ILE201,GLY202,ILE204,GLN205,LEU209,SER242,ILE2 44,ARG258,TRP260,ASN261,ALA262,GLN263,ARG266,ALA2 82,ASN283,GLU285,PHE286,VAL305,TRP346,TYR347,PRO3 50,ALA351,VAL352,ASN354,MET355,PHE369,ASN370,GLY3 71,TRP372,TYR373,MET374,THR376,GLU377,ARG381,ASP3- 82,ASP385,GLN387,ARG388,MET434,ALA439,SER442,ILE46 2,TRP463,LEU464,VAL465,PRO466,PRO467,PHE488,TYR489, TYR490,TYR491,GLN492,GLU494,ALA495,TRP496,LYS497 | $12.327 \times 61.831 \times 15.179$ | $108 \times 92x96$ |
| COX-2 | 6COX | ASN34,CYS36,CYS37,ASN39,PRO40,CYS41,GLN42,ASN43 ,ARG44,GLY45,GLU46,CYS47,MET48,ASP58,CYS59,THR60 ,ARG61,THR62,GLY63,PHE64,GLU73,LEU75,THR76,LYS79, LEU80,LEU82,LYS83,PRO84,THR85,PRO86,ASN87,VAL89,HI S90,LEU93,MET113,LYS114,TYR115,VAL116,THR118,SER11 9,ARG120,SER121,TYR122,LEU123,ILE124,ASP125,SER126, PRO127,PRO128,THR129,TYR130,GLY135,TYR136,LYS137, THR149,ARG150,ALA151,LEU152,PRO153,PRO154,VAL155 ,ALA156,CYS159,GLN192,PHE205,PHE209,GLY227,VAL228, VAL344,ILE345,TYR348,VAL349,LEU352,SER353,TYR355,PH E357,LEU359,LEU366,GLN369,GLN370,PHE371,GLN372,GL N374,ASN375,ARG376,ILE377,ALA378,PHE381,LEU384,TYR 385,TRP387,GLN461,GLU465,LYS468,ARG469,PHE470,SER4 71,LEU472,LYS473,PRO474,ARG513,ALA516,ILE517,PHE518 ,GLU520,MET522,VAL523,GLU524,GLY526,ALA527,PRO528, PHE529,SER530,LEU531,LYS532,GLY533,LEU534 | $29.245 \times 14.866x35.875$ | $102 \times 118x124$ |



**Fig. 2** Effect of GME on cell proliferation and viability of RAW 264.7 cells. Cells were treated with increasing concentrations of extracts for 24 h; cell viability was determined with MTT cell proliferation reagent. Results were expressed as cell viability (% of control). All data are expressed as mean ± SEM (n = 3). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. 0 μg/ml. The $IC_{50}$ was calculated to be 460 μg/ml



**Fig. 3** Effect of Garcinol on cell proliferation and viability of RAW 264.7 cells. Cells were treated with increasing concentrations of Garcinol for 24 h; cell viability was determined with MTT cell proliferation reagent. Results were expressed as cell viability (% of control). All data are expressed as mean ± SEM (n = 3). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. 0 μM. The $IC_{50}$ was calculated to be 24 μM
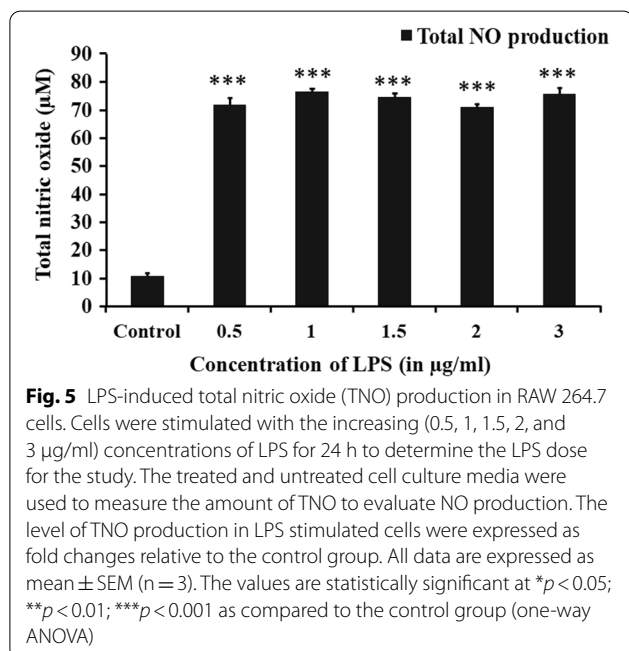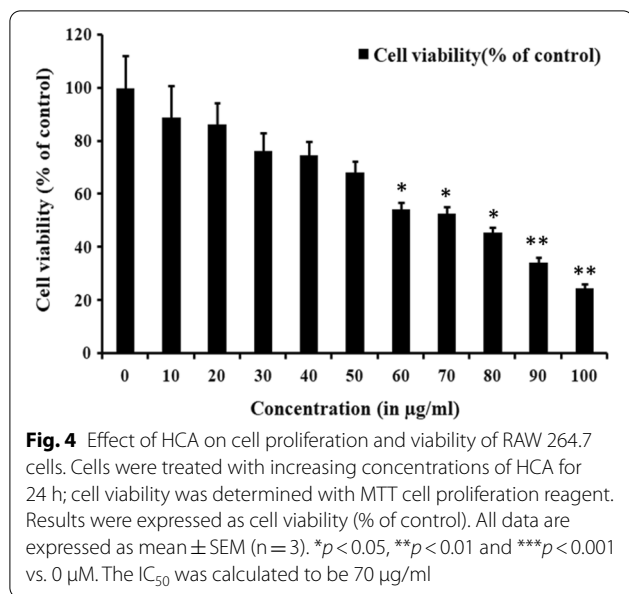
respectively, in the 6 μM and 12 μM Garcinol; -2.50-fold ($p < 0.05$) and -2.71-fold ($p < 0.05$), respectively, in 17.5 μg/ml and 35 μg/ml HCA pre-treated cells as compared to the LPS-treated cells (Fig. 7a).

### 3.5 Effects of GME, Garcinol, and HCA on PGE2 levels

The results of the PGE2 assay revealed that there was a significant increase in the PGE2 level by 13.33-fold ($p < 0.001$) 24-h post-LPS exposure as compared to control. On the other hand, its levels significantly decreased
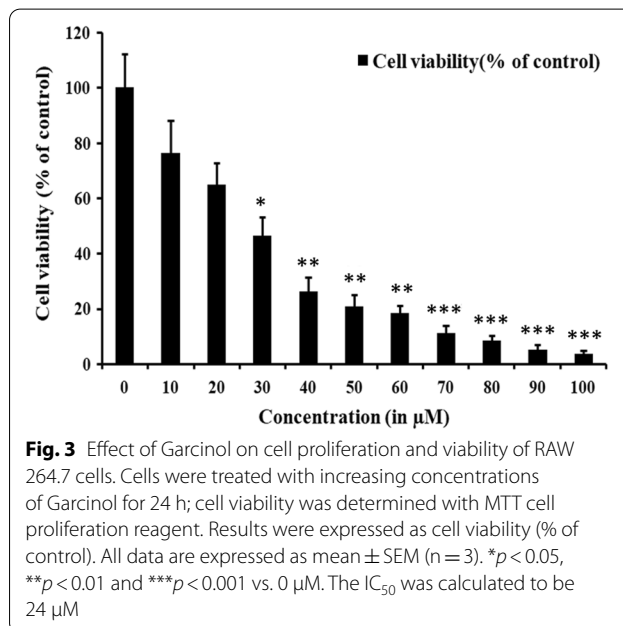
by -3.17-fold ($p < 0.01$) and -5.28-fold ($p < 0.01$), respectively, in the 115 μg/ml and 230 μg/ml GME; -5.97-fold ($p < 0.01$) and -7.31-fold ($p < 0.01$), respectively, in 6-μM and 12-μM Garcinol; -8.52-fold ($p < 0.01$) and -9-fold ($p < 0.01$), respectively, in 17.5 μg/ml and 35 μg/ml HCA pre-treated cells as compared to the LPS treated cells (Fig. 7b).

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**Fig. 4** Effect of HCA on cell proliferation and viability of RAW 264.7 cells. Cells were treated with increasing concentrations of HCA for 24 h; cell viability was determined with MTT cell proliferation reagent. Results were expressed as cell viability (% of control). All data are expressed as mean ± SEM (n = 3). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. 0 µM. The $IC_{50}$ was calculated to be 70 µg/ml



**Fig. 5** LPS-induced total nitric oxide (TNO) production in RAW 264.7 cells. Cells were stimulated with the increasing (0.5, 1, 1.5, 2, and 3 µg/ml) concentrations of LPS for 24 h to determine the LPS dose for the study. The treated and untreated cell culture media were used to measure the amount of TNO to evaluate NO production. The level of TNO production in LPS stimulated cells were expressed as fold changes relative to the control group. All data are expressed as mean ± SEM (n = 3). The values are statistically significant at *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ as compared to the control group (one-way ANOVA)

## 3.6 Effects of GME, Garcinol, and HCA on mRNA expression of iNOS and COX-2

The real-time qPCR studies revealed a significant increase in the transcript levels of inos, and cox-2 after 24 h of LPS treatment. Their transcript levels increased by 46.68-fold ($p < 0.001$), and 12.98-fold ($p < 0.01$) post-LPS stimulation, respectively, as compared to control. The mRNA expressions of inos were however observed to be decreased significantly in the GME (230 µg/

ml) pre-treated cells by -6.30-fold ($p < 0.01$) and cox-2 mRNA expression was found to be decreased significantly in both the concentrations of GME (115 µg/ml and 230 µg/ml) by -1.90-fold ($p < 0.05$), and -2.69-fold ($p < 0.05$), respectively, as compared to the LPS treated cells. The inos and cox-2 transcripts levels were found to be decreased in Garcinol pre-treated cells, respectively, by -1.54-fold ($p < 0.05$), and -3.47-fold ($p < 0.05$) for 6 µM; -2.89-fold ($p < 0.01$) and -3.02-fold ($p < 0.05$) for 12 µM concentrations of Garcinol as compared to the LPS treated cells. Similarly, inos transcript level was found to be decreased by -3.76-fold ($p < 0.05$) in 35 µg/ml of HCA pre-treated cells. Also, the cox-2 transcripts levels were found to be decreased, respectively, by -5.69-fold ($p < 0.05$) and -6.06-fold ($p < 0.05$) in 17.5 µg/ml and 35 µg/ml of HCA pre-treated cells as compared to the LPS treated cells (Fig. 8a and b).
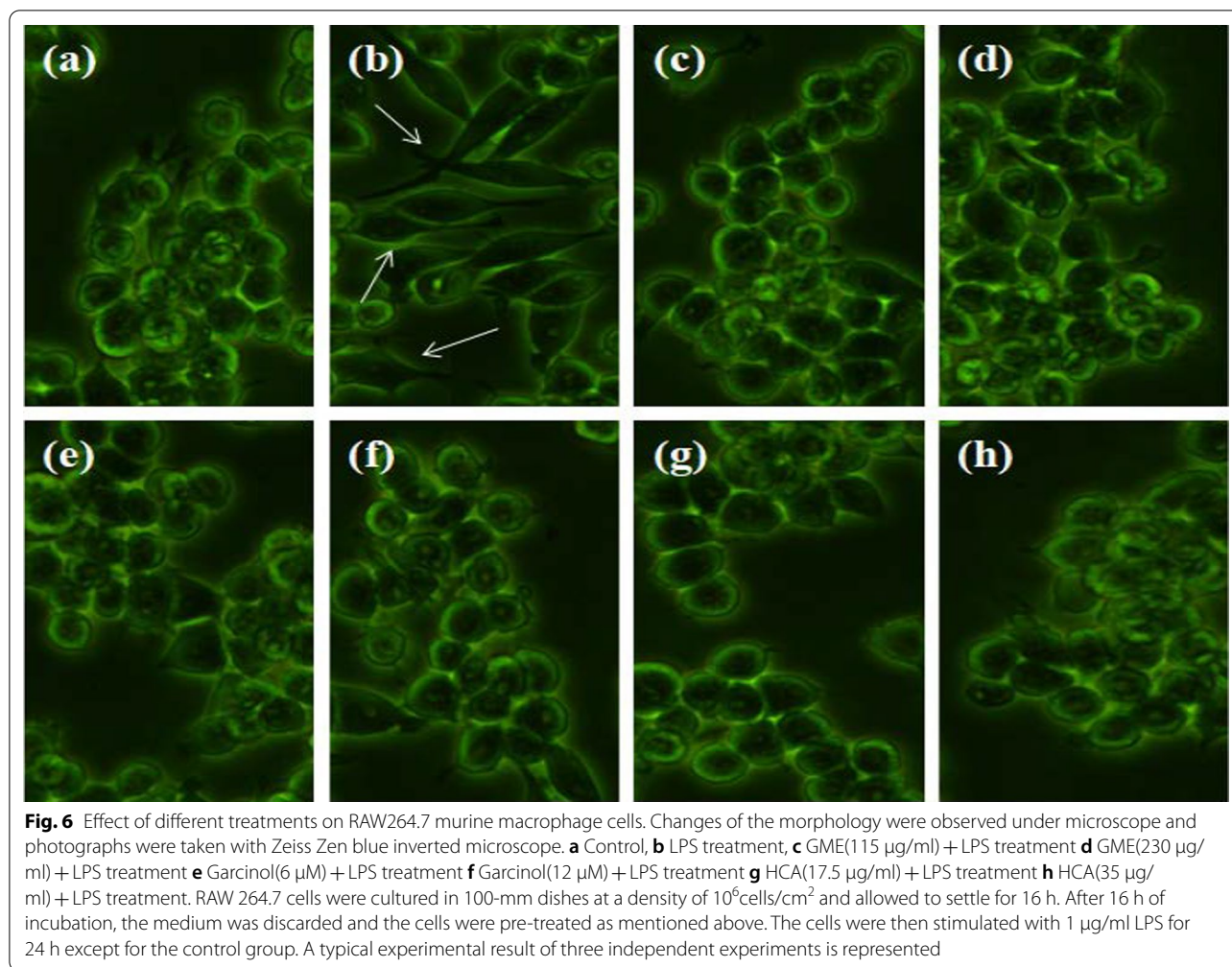
## 3.7 Molinspiration analysis of Garcinol and HCA molecular properties

The molecular properties of ligands Garcinol and HCA such as logP, number of hydrogen bond donors, number of hydrogen bond acceptors, the molecular weight was calculated using the Molinspiration tool (Table 2). HCA showed zero violations against Lipinski's rule of five. However, Garcinol with a molecular weight greater than 500, i.e., 602.81 g/mol and logP value of 8.26 shows two violations against the rule of five.

## 3.8 Molecular docking analysis

Molecular docking studies were performed to elucidate the interaction between the targets (iNOS and COX-2) and chief constituents (Garcinol and HCA) of *Garcinia* as a potent anti-inflammatory agent. The chain A of both iNOS and COX-2 were selected for molecular docking (Fig. 1a and b). The docking analysis indicates significant binding affinities of Garcinol and HCA with the protein targets. The present study revealed that Garcinol showed hydrogen bonding interactions with Arg199, Ile201, Leu464 and hydrophobic interactions with Met374, Tyr491, Phe369, Met355, Trp463, Val352, Tyr373, Pro350 residues of iNOS with binding energy (ΔG) of -9.46 kcal/mol (Fig. 9); hydrogen bonding interactions with Arg120 and hydrophobic interactions with His90, Val523, Tyr355, Arg513, Ala516, Met 522, Phe518, Leu384, Tyr385, Trp387, Phe381, Leu352, Val349, Tyr348, Leu531, Val116, Leu359 residues of COX-2 with a binding energy (ΔG) of -4.2 kcal/mol (Fig. 10). Similarly, our study also revealed that HCA showed hydrogen bonding interactions with Thr121, Lys123, Thr126 and hydrophobic interactions with Thr109, Ile119, Pro122 residues of iNOS with binding energy (ΔG) of -3.11 kcal/mol (Fig. 11); hydrogen bonding interaction with Lys83, Tyr115, Arg120, Glu524

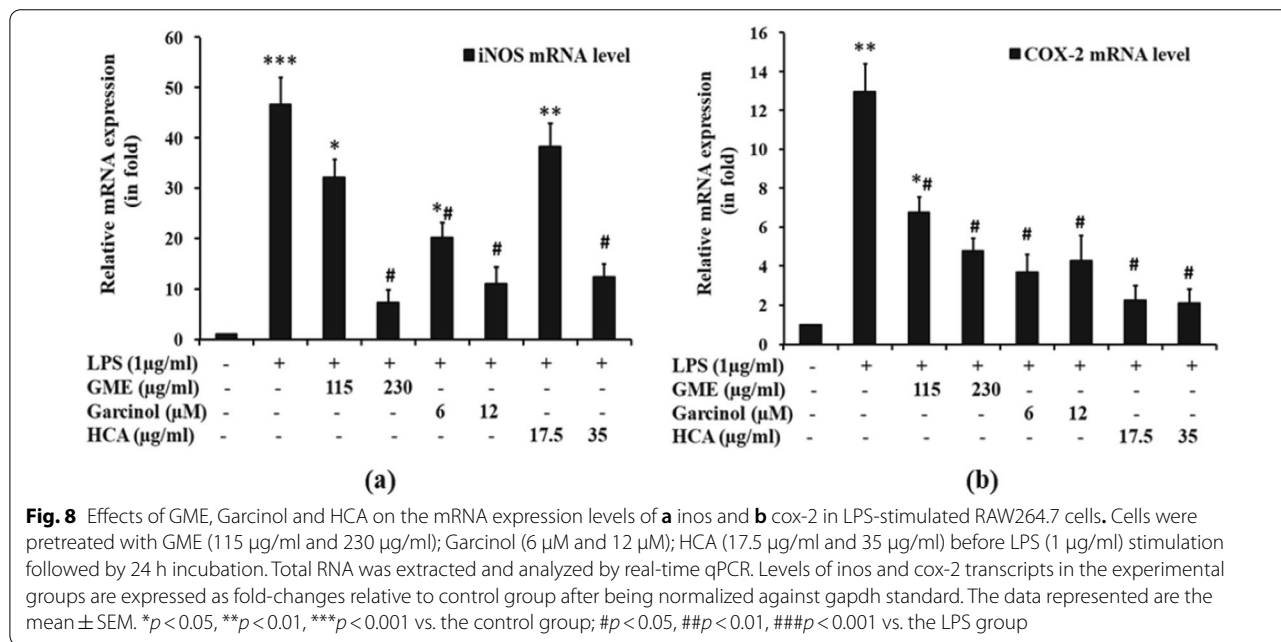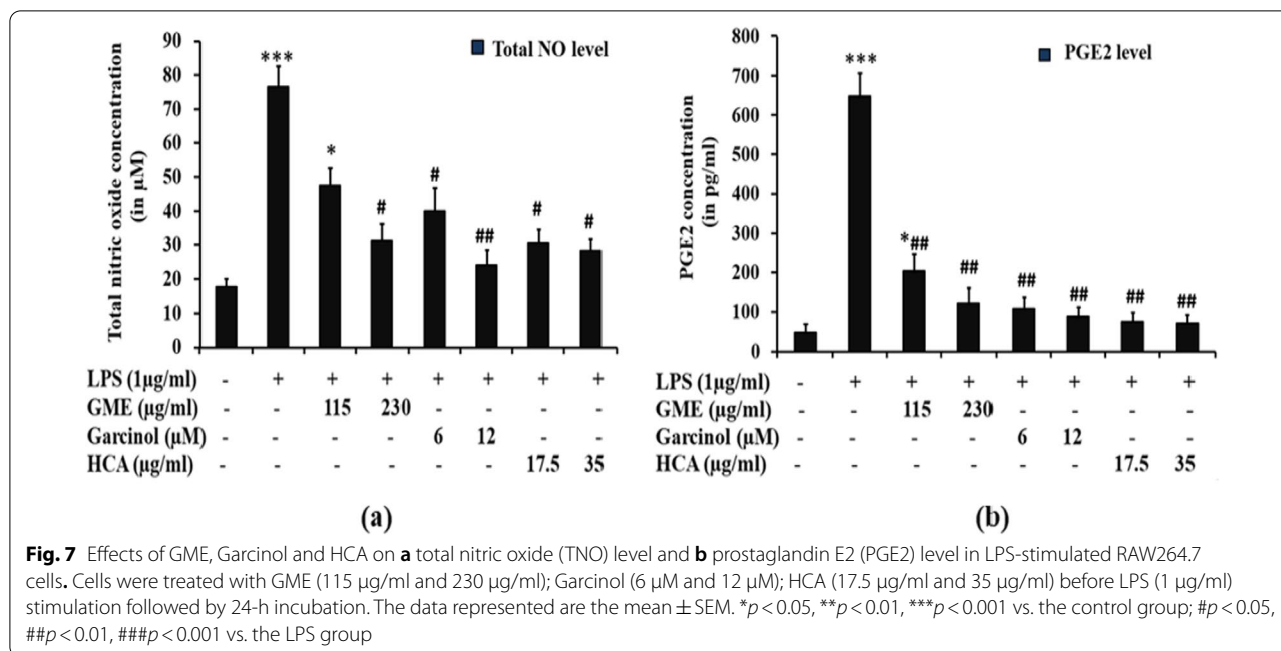**Fig. 6** Effect of different treatments on RAW264.7 murine macrophage cells. Changes of the morphology were observed under microscope and photographs were taken with Zeiss Zen blue inverted microscope. **a** Control, **b** LPS treatment, **c** GME(115 μg/ml) + LPS treatment **d** GME(230 μg/ml) + LPS treatment **e** Garcinol(6 μM) + LPS treatment **f** Garcinol(12 μM) + LPS treatment **g** HCA(17.5 μg/ml) + LPS treatment **h** HCA(35 μg/ml) + LPS treatment. RAW 264.7 cells were cultured in 100-mm dishes at a density of $10^6$cells/cm$^2$ and allowed to settle for 16 h. After 16 h of incubation, the medium was discarded and the cells were pre-treated as mentioned above. The cells were then stimulated with 1 μg/ml LPS for 24 h except for the control group. A typical experimental result of three independent experiments is represented

and hydrophobic interactions with Pro84, Pro86, Ser 119, Tyr122 residues of COX-2 with binding energy (ΔG) of -3.15 kcal/mol (Fig. 12).

## 4 Discussion

*Garcinia* is widely distributed in the tropical region and has been traditionally used in medicinal folklore with little understanding of the actual mechanism of its therapeutic action. It is one of the most important medicinal plants that have been used traditionally for its medicinal value. Earlier studies have demonstrated that *Garcinia* possesses anti-bacterial, anti-cancer, antioxidant activities as well as emerging anti-inflammatory potential [48–50]. The pharmacological effects of *Garcinia* may be primarily due to the presence of compounds such as hydroxycitric acid (HCA), Garcinol, and isogarcinol. [22–24]. From our previous studies, it has been established that *Garcinia* herbal extract consists of several important phytoconstituents like Garcinol and HCA. In our previous studies using the LPS-challenged rat model, it was
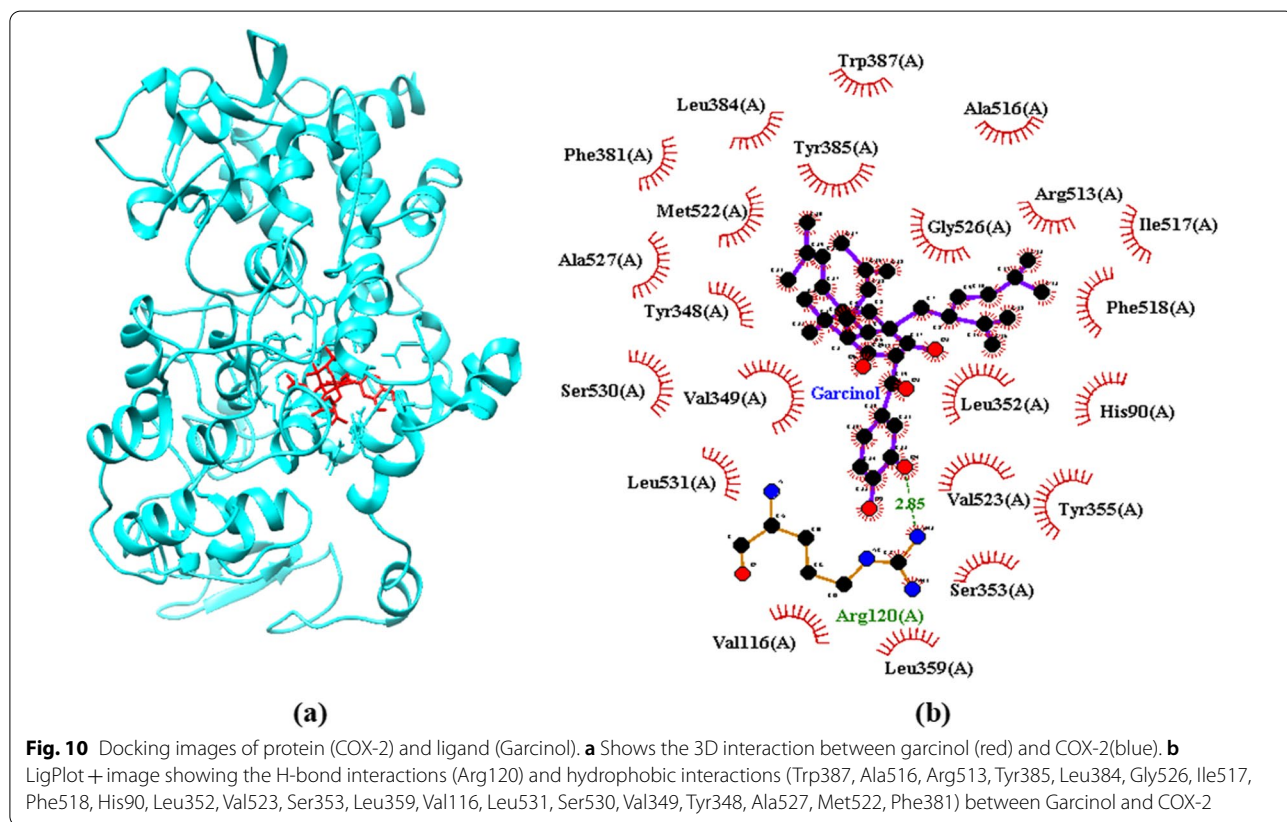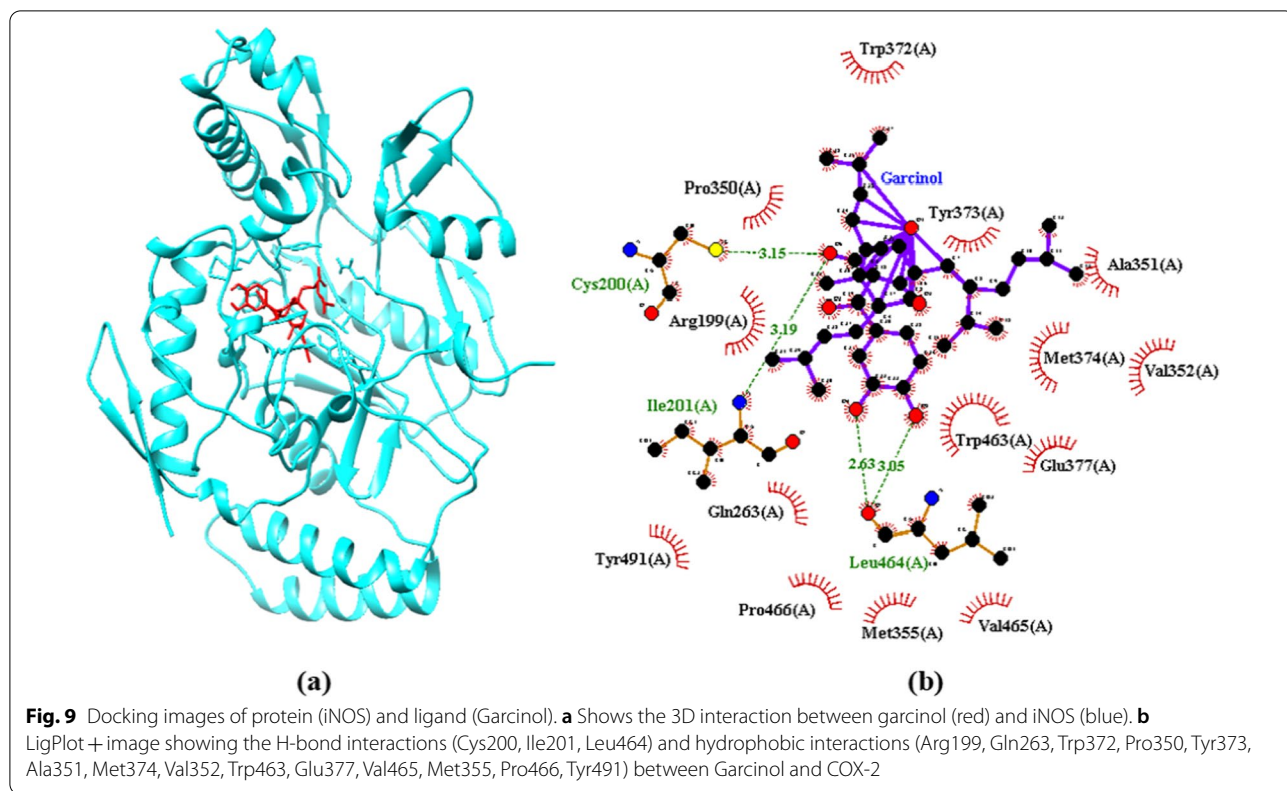
established that *Garcinia* herbal extract could potentially inhibit the inos and cox-2 mRNA expressions and decrease subsequent production of NO and PGE2 [33]. Therefore, this study was undertaken to establish the inhibitory action of *Garcinia* extract supplement and its important phytoconstituents Garcinol and HCA against iNOS and COX-2 targets in vitro. The present study investigated the inhibitory action of GME, Garcinol, and HCA against inos and cox-2 mRNA expressions. In addition, quantitative analysis was done to study the effects of GME, Garcinol, and HCA on the production of TNO and PGE2 in LPS-stimulated murine macrophage RAW264.7 cells. Also, molecular docking analysis was performed to study the interactions of Garcinol and HCA with iNOS and COX-2 proteins. In this study, the test for *Mycoplasma* contamination was performed before the experiments and was found to be negative. The cells on treatment with different concentrations of LPS after 24 h showed a significant increase in the production of total nitric oxide levels. The results of the nitric oxide assay
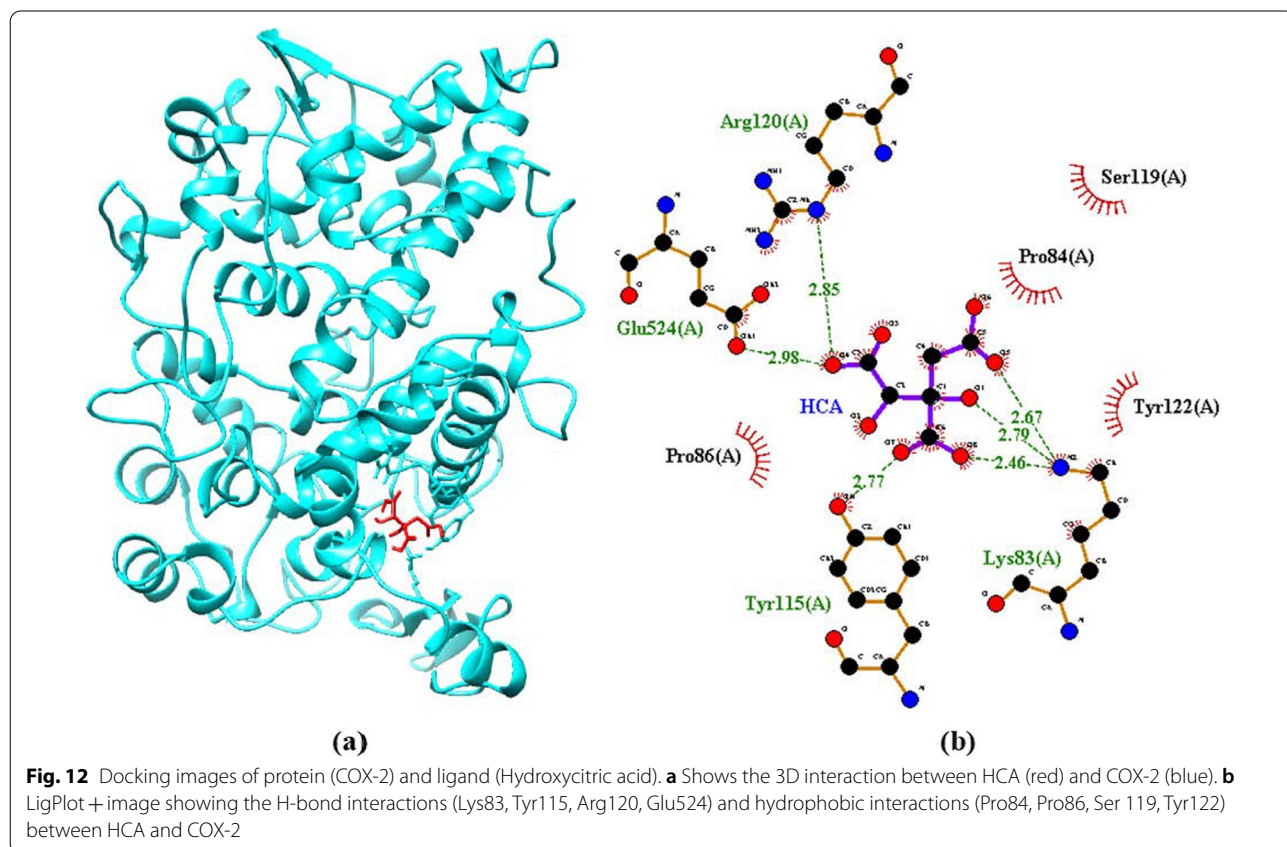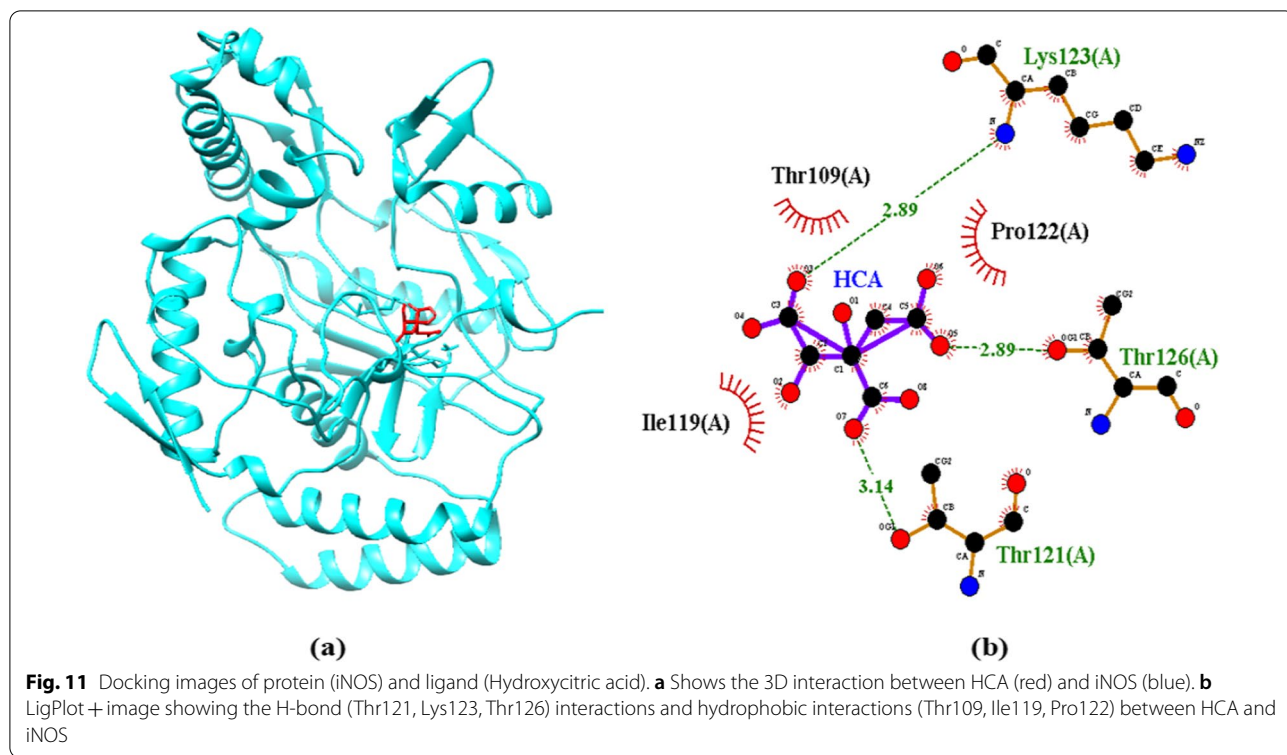
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**Fig. 7** Effects of GME, Garcinol and HCA on **a** total nitric oxide (TNO) level and **b** prostaglandin E2 (PGE2) level in LPS-stimulated RAW264.7 cells**.** Cells were treated with GME (115 μg/ml and 230 μg/ml); Garcinol (6 μM and 12 μM); HCA (17.5 μg/ml and 35 μg/ml) before LPS (1 μg/ml) stimulation followed by 24-h incubation. The data represented are the mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. the control group; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ vs. the LPS group



**Fig. 8** Effects of GME, Garcinol and HCA on the mRNA expression levels of **a** inos and **b** cox-2 in LPS-stimulated RAW264.7 cells**.** Cells were pretreated with GME (115 μg/ml and 230 μg/ml); Garcinol (6 μM and 12 μM); HCA (17.5 μg/ml and 35 μg/ml) before LPS (1 μg/ml) stimulation followed by 24 h incubation. Total RNA was extracted and analyzed by real-time qPCR. Levels of inos and cox-2 transcripts in the experimental groups are expressed as fold-changes relative to control group after being normalized against gapdh standard. The data represented are the mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. the control group; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ vs. the LPS group

and PGE2 estimation revealed that there was a significant increase in the TNO level after 24 h of LPS exposure as compared to control. On the other hand, its levels were found to significantly decrease in GME, Garcinol and HCA pre-treated cells as compared to the LPS treated cells (Fig. 7a and b).

The real-time qPCR studies revealed a significant increase in the transcript levels of inos, and cox-2 after 24 h of LPS treatment which was found to significantly decrease in GME, Garcinol and HCA pre-treated cells as compared to the LPS treated cells (Fig. 8a and b). It has been well-established that NO is a pro-inflammatory mediator [51]. The reduction in NO and PGE2 production in GME, Garcinol, and HCA-treated cells can thus be attributed to the decreased expression of inos and cox-2, respectively. iNOS is the enzyme principally

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**Fig. 9** Docking images of protein (iNOS) and ligand (Garcinol). **a** Shows the 3D interaction between garcinol (red) and iNOS (blue). **b** LigPlot + image showing the H-bond interactions (Cys200, Ile201, Leu464) and hydrophobic interactions (Arg199, Gln263, Trp372, Pro350, Tyr373, Ala351, Met374, Val352, Trp463, Glu377, Val465, Met355, Pro466, Tyr491) between Garcinol and COX-2



**Fig. 10** Docking images of protein (COX-2) and ligand (Garcinol). **a** Shows the 3D interaction between garcinol (red) and COX-2(blue). **b** LigPlot + image showing the H-bond interactions (Arg120) and hydrophobic interactions (Trp387, Ala516, Arg513, Tyr385, Leu384, Gly526, Ile517, Phe518, His90, Leu352, Val523, Ser353, Leu359, Val116, Leu531, Ser530, Val349, Tyr348, Ala527, Met522, Phe381) between Garcinol and COX-2

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**Fig. 11** Docking images of protein (iNOS) and ligand (Hydroxycitric acid). **a** Shows the 3D interaction between HCA (red) and iNOS (blue). **b** LigPlot + image showing the H-bond (Thr121, Lys123, Thr126) interactions and hydrophobic interactions (Thr109, Ile119, Pro122) between HCA and iNOS



**Fig. 12** Docking images of protein (COX-2) and ligand (Hydroxycitric acid). **a** Shows the 3D interaction between HCA (red) and COX-2 (blue). **b** LigPlot + image showing the H-bond interactions (Lys83, Tyr115, Arg120, Glu524) and hydrophobic interactions (Pro84, Pro86, Ser 119, Tyr122) between HCA and COX-2

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responsible for NO in inflammation. iNOS is not usually expressed in resting cells but is however induced by some cytokines as well as microbial agents [52, 53]. Studies have reported stimuli like LPS and certain cytokines cause the expression of iNOS along with the production of pro-inflammatory mediators, such as prostaglandin and prostacyclin, through the COX pathway [54, 55]. NO is known to elevate the synthesis of prostaglandin by activating the constitutive and inducible cyclooxygenases in many cells [56, 57]. Evidence suggests that in macrophages, the activity of iNOS and COX-2 induces the release of several pro-inflammatory mediators including NO and certain cytokines (Tumor necrosis factor-α and interleukins) [58, 59]. Hence, the inhibition of iNOS and COX-2 is an important step toward the prevention of inflammation. Inducible nitric oxide synthase catalyzes the production of a large amount of NO during the inflammatory condition. Therefore, iNOS inhibitors are essential for healing nitric oxide-mediated inflammatory responses [60]. Moreover, herbal inhibitors such as *Garcinia* might play an important role as safe modulators of NO in the pathogenesis of inflammation. Similarly, COX-2 catalyzes the production of proinflammatory PGE2 and is known to be highly expressed during inflammation [61, 62]. From the present observations, it is found that *Garcinia* can act as a potential inhibitor of LPS-induced NO and PGE2 production. This inhibition might be due to the blocking of major downstream signaling involved in the production of these inflammatory mediators. However, the actual mechanism of inhibition is still unclear. It has been reported that *Garcinia mangostana* extracts induce anti-inflammatory action by decreasing the LPS-induced cytokine and PGE2 levels in immortalized human gingival fibroblasts cells [11]. Similarly, Cho and Cho studied the anti-inflammatory activities of ethanol extracts of *Garcinia subelliptica* in macrophages. They established that non-cytotoxic concentrations of the extracts could decrease the NO and PGE2 generation by altering the iNOS and COX-2 expression, respectively, in LPS-induced RAW 264.7 cells. This observation is in line with our study. Further, they established that the decreased secretion of inflammatory mediators by *Garcinia subelliptica* was associated with a decrease in the activation of c-Jun N-terminal kinase (JNK) [62]. Evidence suggests that LPS significantly induces the secretion of proinflammatory mediators in macrophages by triggering the mitogen-activated protein kinase (MAPK) signaling. Therefore, blocking the downstream signaling including suppressing of p38, ERK, and JNK phosphorylation suggests a vital target for a therapeutic approach against inflammation [63–65].

Macrophages when stimulated by bacterial endotoxin (LPS) lead to induction of inflammatory response. Such responses involve the release of several pro-inflammatory mediators like NO and PGE2 whose production is induced by the expression of iNOS and COX-2, respectively [66, 67]. Several signaling pathways such as mitogen-activated protein kinase (MAPK) have been reported to be involved in response to LPS stimulation. Reports suggest that the binding of LPS to its receptor TLR-4 in macrophages leads to the signal transduction cascades [68–70]. The receptor-ligand complex stimulates the transcription factor nuclear factor kappaB (NF-κB) via the activation of JNK and p38 MAPK signaling. p38MAPK is the chief signaling molecule that modulates LPS-induced iNOS and COX-2 expression in macrophages [71, 72]. Stimulation of the p38MAPK is known to carry out NF-κB translocation via phosphorylation of IkBα [73]. Garcinol has been found to suppress the NF-κB signaling in LPS-activated macrophages. Karin et al. established that Karcinol could inhibit phosphorylation of IkBα on serine 32 in RAW264.7 cells [74]. NF-κB is bound to IκBα in the cytoplasm and its activation leads to its translocation from cytoplasm to the nucleus thus leading to the expression of inflammatory genes such as iNOS, COX2, and IL-6 [75]. Garcinol has been known to inhibit LPS-induced COX-2 expression through decreased expression of NF-κB and suppression of the p38 MAPK signaling pathway [50]. Earlier reports have suggested that Garcinol inhibits LPS-induced iNOS expression [49]. Therefore, based on previous reports our results indicate that Garcinol might inhibit the LPS-induced NF-κB translocation through suppression of p38 MAPK and thereby decreasing the expression of COX-2 and iNOS. Similarly, studies in animal models suggest that HCA suppresses inflammatory markers in certain tissues and serum [76]. HCA has been reported to modulate experimental autoimmune encephalomyelitis (EAE), somewhat, by inhibiting the serum NO production. HCA could decrease the serum NO levels possibly under the controlled regulation of NF-κB and MAPKs pathways in EAE mice [77]. Also, reports suggest that HCA could regulate the cytokine signaling in many of cell types, such as macrophages, via the inhibition of NF-κB and mostly by inhibiting STAT-1 nuclear transfer and DNA binding [78].

Molecular docking studies were performed to elucidate the interaction between the targets (iNOS and COX-2) and Garcinol as well as HCA as a potent inhibitor. The molecular properties of ligands (Garcinol and HCA) calculated using the Molinspiration tool showed that HCA showed zero violations against Lipinski's rule of five. However, Garcinol showed two violations against the rule of five (Table 2). Therefore, it suggests that the bioavailability of HCA is more since it follows Lipinski's rule of five, and Garcinol is therefore considered to be poorly

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absorbed. Although the 'rule-of-five' is used to detect the bioavailability of the oral drug, however, no more than 51% of all FDA-approved small-molecule drugs are both used orally and obey the thumb rule which does not even include the increasing number of biological compounds of which more than a few have achieved success [79].

Certain bioactive compounds having anti-inflammatory effects isolated from various medicinal plants have been studied through molecular docking against iNOS and COX-2 [80]. In the present study, molecular docking analysis of Garcinol and HCA against iNOS and COX-2 proteins showed good binding affinities (Figs. 9, 10, 11, 12). Molecular docking studies were performed to elucidate the interaction between the targets (iNOS and COX-2) and chief constituents (Garcinol and HCA) of *Garcinia* as a potent anti-inflammatory agent. The docking analysis indicates significant binding affinities of Garcinol and HCA with the protein targets. The H-bond formation together with the hydrophobic interactions indicates that Garcinol, as well as HCA other than the anti-inflammatory drugs, could prove to be a potent inhibitor of iNOS and COX-2. Studies have shown that anti-inflammatory drugs (NSAIDs) such as dexamethasone and indomethacin inhibit iNOS and COX-2 [5, 81]. NSAIDs like sodium diclofenac and ibuprofen have shown interaction with iNOS with binding energy ($\Delta$G) of about -6.7 kcal/mol and -7.50 kcal/mol, respectively [82, 83]. Also, it has been reported that diclofenac binds to Ser530 and Tyr385 residues of the COX-2 active site [84]. Several studies have reported many natural inhibitory ligands for iNOS and COX-2 by molecular docking analysis [82, 84–88]. It is well-established that non-steroidal anti-inflammatory drugs (NSAIDS) operate by suppressing the release of prostaglandins by inhibiting COX-2. Synthetic drugs such as Ibuprofen and Naproxen have been reported to prevent the release of prostaglandins. Reports suggest that molecular docking of inhibitory drugs such as Ibuprofen as well as Naproxen against COX-2 showed involvement of Arg120 and Tyr355 amino acid residues [89]. Studies have also revealed Xanthone derivatives inhibit the COX enzyme that shows contact with Arg120, Ser 530, Met522, Tyr 355, Tyr385, Ser353 of the enzyme [86]. Similarly, our findings demonstrate that HCA interacts with COX-2 forming H-bonds with Arg120. Studies have reported that molecular docking of certain flavonoids including quercetin against iNOS involved the interactions with the active site residues Ile119, Thr109, Ser118, Trp461, Met480 that suggested causing inhibition of iNOS [90, 91]. This is in agreement with the present study which showed favorable interaction of HCA with iNOS effectively involving Ile119 as well as Thr109 amino acid residues. Such potential molecular affinity of HCA provides a vast possibility for safe drug design.

Curcuminoids have been used as potential agents to block iNOS and COX-2 [82, 92]. Studies have shown that curcumin binds with iNOS with $\Delta$G of-6.8 kcal/mol [82]. Other phytocompounds like quercetin have been found to efficiently interact with iNOS active residues compared to tetrahydrobiopterin, an iNOS inhibitor [93]. Similarly, curcumin analogues are known to interact with Ser530 residue of COX by H-bond [84]. Also, Xanthone derivatives were observed to be potent inhibitors of COX. Studies have shown the interaction of such derivatives with Arg120, Ser530, Met 522, Tyr355, Tyr 385, Ser353 residues of COX [87]. This report is in agreement with the present study. These findings showed the inhibition of the target proteins by ligand binding, which is in line with our study. The present study, therefore, provides information suggesting the possible anti-inflammatory role of *Garcinia*.

## 5 Conclusions

The present study demonstrated that *Garcinia* extract exhibited potent anti-inflammatory activity in LPS-induced RAW 264.7 cells. GME and the active phyto-constituents present, i.e., Garcinol and HCA efficiently reduced the relative mRNA expressions of inos and cox-2. A significant inos and cox-2 inhibition were observed and subsequent low levels of TNO and PGE2 in the GME, Garcinol and HCA pre-incubated cells were followed by LPS incubation. Also, the molecular docking of Garcinol and HCA against iNOS and COX-2 targets revealed potent protein–ligand binding affinities. These phytoconstituents showed significant interactions with the targets with low binding energies as compared to several NSAIDs. Such binding affinities of these compounds could be linked to their inhibitory action against these targets. All these observations can be correlated to the conclusion of the anti-inflammatory effect of *Garcinia*. These inhibitory activities of *Garcinia* extract can be the basis to promote the development of anti-inflammatory drugs.

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## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
Authors declare no conflicts of interest.

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