Molecular Docking Prediction and in Vitro Studies Elucidate Anti-inflammatory Effect of Garcinia Extract Against Inducible Nitric Oxide Synthase and Cyclooxygenase-2 Targets

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Abstract

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, Garcinia herbal extract (GHE) and one of its important phytocompound (garcinol) were evaluated for their inhibitory action against important inflammatory markers inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS)-induced RAW 264.7 cells. iNOS and COX-2 plays an 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 Garcinia (230µg/ml and 115µg/ml); garcinol (12µM and 6µM) followed by stimulation with 1µg/ml of LPS for 24h. The results of the study demonstrated that GHE and garcinol plays an important role in suppressing LPS- induced relative mRNA expression of iNOS, COX-2 and subsequent reduction in the levels of nitric oxide and prostaglandin E 2 . Molecular docking analysis of garcinol and hydroxycitric acid, the major active components of GHE with iNOS and COX-2 proteins showed potent interaction with low binding energies. This study suggests that GHE (containing high percentage of HCA) and garcinol may possess anti-inflammatory activity thus providing a possibility for drug designing as iNOS and COX-2 inhibitors.

Introduction

The process of inflammation is mediated by several important molecules, out of which the inducible nitric oxide synthase (iNOS or NOS2), 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 of this enzyme. However, a number of extracellular stimuli can trigger different signaling pathways causing the expression of iNOS. Lipopolysaccharide (LPS) is one of the most potent stimulus 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 researches have been going on to find out natural anti-inflammatory agents. From earlier times herbal medicines have been 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 have 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. lanceaefolia*, *G. xanthochymus*, and *G. cowa* [8, 9]. *Garcinia* is an important medicinal plant that possesses many important phytoconstituents. It has been traditionally used in the 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 extracts (GHE) are available in the form of oral supplements well-known for weight loss and are 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 garcinol could be a positive anti-inflammatory agent. 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, 26]. The dried fruit of the *Garcinia* consists of 30 to 50% HCA. HCA has been generally used for its anti-inflammatory activities [27]. It has been known to reduce oxidative stress in some experimental models of inflammation [28, 29]. 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 studies with the LPS-challenged rat model, it was observed that GHE significantly inhibited the iNOS and COX-2 and also led to a subsequent decrease in serum NO and PGE2 levels. The LC-MS analysis of GHE has shown the presence of many important phytocompounds [30]. The major active constituents found to be present in GHE were hydroxycitric acid (HCA) and garcinol. Therefore, in this study, we aim to investigate the inhibitory potential of the GHE containing HCA as major constituent as well as garcinol 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).

Results And Discussion

Garcinia is widely distributed in tropical region and has been traditionally used in medicinal folklore as 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 [31–33]. The pharmacological effects of *Garcinia* may be primarily due to presence of compounds such as hydroxycitric acid (HCA), garcinol, isogarcinol etc. [22–25]. From our previous studies, it has been established that GHE consists of several important phytocompounds and one of the potent active phytoconstituents is HCA. In our previous studies using the LPS-challenged rat model, it was established that GHE could potentially inhibit the inos and cox-2 expressions and decrease subsequent production

of NO and PGE2 [30]. Therefore, this study was undertaken to establish the inhibitory action of GHE and one of its important phytocompounds garcinol against iNOS and COX-2 targets in vitro. The present study investigated the inhibitory action of GHE and garcinol against inos and cox-2 mRNA expressions. In addition, quantitative analysis was done to study the effects of GHE and garcinol on the production of NO and PGE2 in lipopolysaccharide (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 prior to the experiments and was found to be negative. Our results demonstrated the effects of GHE and garcinol on RAW264.7 cell viability. The present study reported significant ($p < 0.05$) inhibition of RAW264.7 cell viability post treatment with GHE and garcinol for 12hr. The IC_{50} of GHE and garcinol for RAW264.7 cells as calculated from the regression equation was found to be 460µg/ml and 24µM respectively (Figs. 4 and 5). Based on the IC_{50} two different doses for GHE (230µg/ml and 115µg/ml) and garcinol (6µM and 12µM) were selected for treatment of the cells. Similarly, the LPS dose was determined by nitric oxide assay. The cells on treatment with different concentrations of LPS after 24h showed significant increase in the production of nitric oxide. However, the maximum amount of nitric oxide produced 24h after LPS treatment was found at the concentration of 1µg/ml LPS by 6.99fold ($p < 0.001$) as compared to control. Therefore, the LPS dose selected for the present study was 1µg/ml (Fig. 6). The results of the nitric oxide assay and PGE2 estimation revealed that there was a significant increase in the NO level after 24h of LPS exposure as compared to control. On the other hand its levels were found to significantly decrease in GHE and garcinol pre-treated cells as compared to the LPS treated cells. The results of the nitric oxide assay revealed that there was a significant increase in the total NO level by 4.30 fold ($p < 0.001$) after 24h of LPS exposure as compared to control. On the other hand its levels was found to significantly decreased by -2.43fold ($p < 0.05$) in 230µg/ml GHE; -1.92fold ($p < 0.05$) and − 3.19 fold ($p < 0.01$) respectively in the 6µM and 12µM garcinol pre-treated cells as compared to the LPS treated cells (Fig. 7). It has been well established that NO is a pro-inflammatory mediator [34]. The results of the PGE2 assay revealed that there was a significant increase in the PGE2 level by 13.33 fold ($p < 0.001$) 24h 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 115µg/ml and 230µg/ml GHE; -5.97 fold ($p < 0.01$) and − 7.31 fold ($p < 0.01$) respectively in the 6µM and 12µM garcinol pre-treated cells as compared to the LPS treated cells (Fig. 8).

Similarly, the results of the qPCR analysis revealed a significant increase in the transcript levels of inos and cox-2 after 24h of LPS treatment. Their transcript levels increased post LPS stimulation and found to decrease significantly in the GHE and garcinol pre-treated cells. The real-time qPCR studies revealed a significant increase in the transcript levels of inos, and cox-2 after 24h 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 was however observed to be decreased significantly in the GHE (230µg/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 GHE (115µg/ml and 230µg/ml) by -1.90 fold ($p < 0.05$), and − 2.69 fold ($p < 0.05$) respectively as compared to the LPS treated cells (Fig. 8). Similarly, inos and cox-2 transcripts levels were found to be decreased in garcinol pre-treated cells respectively by -1.54 ($p < 0.05$), and − 3.47($p < 0.05$) for 6µM; -2.89 ($p < 0.01$), and − 3.02($p < 0.05$) for 12µM concentrations of garcinol as compared to the LPS treated cells (Fig. 9). The reduction of NO and PGE2 production in GHE and garcinol treated cells can thus be attributed to the decreased expression of inos and cox-2 respectively. iNOS is the enzyme principally 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 [35, 36]. 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 COX pathway [37, 38]. NO is known to elevate the synthesis of prostaglandin by activating the constitutive and inducible cyclooxygenases in many cells [38, 39]. 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) [40, 41]. Hence, the inhibition of iNOS and COX-2 is an important step toward prevention of inflammation. The study by Liao et al. has shown that garcinol could suppress iNOS as well as COX-2 expression in LPS-stimulated macrophages and also inhibit the NFκB activation [33] which is in line with our study. 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 [42, 43]. 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 [44]. Moreover, herbal inhibitors such as GHE 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 [45, 46]. 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 antiinflammatory activities of ethanol extracts of *Garcinia subelliptica* in macrophages. They established that noncytotoxic 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 cJun Nterminal kinase (JNK) [47]. 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 [48–50].

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) such as LogP, number of hydrogen bond donors, number of hydrogen bond acceptors, molecular weight was calculated using Molinspiration tool (Table 2). HCA showed zero violations against the Lipinski's rule of five. However, garcinol with molecular weight greater than 500 i.e., 602.81g/mol and logP value of 8.26 shows two violations against the rule of five. Therefore, it suggests that the bioavailability of HCA is more since it follows the Lipinski's rule of five and garcinol is therefore considered to be poorly absorbed. Certain bioactive compounds having anti-inflammatory effects isolated from various medicinal plants have been studied through molecular docking against iNOS and COX-2 [51]. In the present study, molecular docking analysis of garcinol and HCA against iNOS and COX-2 proteins showed good binding affinities. 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 potent anti-inflammatory agent. The docking analysis clearly indicates significant binding affinities of garcinol and HCA with the protein targets. The present study revealed that garcinol

showed hydrogen bonding interactions with Cys200, Ile201, Leu464 and hydrophobic interactions with Arg199, Gln263, Trp372, Pro350, Tyr373, Ala351, Met374, Val352, Trp463, Glu377, Val465, Met355, Pro466, Tyr491 residues of iNOS with a binding energy (ΔG) of -9.46kcal/mol (Fig. 10); hydrogen bonding interactions with Arg120 and hydrophobic interactions with Trp387, Ala516, Arg513, Tyr385, Leu384, Gly526, Ile517, Phe518, His90, Leu352, Val523, Ser353, Leu359, Val116, Leu531, Ser530, Val349, Tyr348, Ala527, Met522, Phe381 residues of COX-2 with a binding energy (ΔG) of -4.2kcal/mol (Fig. 11). 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 -3.11kcal/mol (Fig. 12); hydrogen bonding interaction with Lys83, Tyr115, Arg120, Glu524 and hydrophobic interactions with Pro84, Pro86, Ser 119, Tyr122 residues of COX-2 with binding energy (ΔG) of -3.15kcal/mol (Fig. 13). 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 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, 52]. NSAIDs like sodium diclofenac and ibuprofen have shown interaction with iNOS with binding energy (ΔG) of about − 6.7kcal/mol and − 7.50kcal/mol respectively [53, 54]. Also, it has been reported that diclofenac binds to Ser530 and Tyr385 residues of COX-2 active site [55]. Our study has shown lower binding energy (ΔG= -9.46kcal/mol) between garcinol and iNOS rendering it a potent inhibitor. Several studies have reported many natural inhibitory ligands for iNOS and COX-2 by molecular docking analysis [51, 54, 56–59]. 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 [60]. 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 [57]. 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 [61]. This is in agreement with the present study which showed favourable 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 designing. Curcuminoids have been used as potential agents to block iNOS and COX-2 [53, 62]. Studies have shown that curcumin binds with iNOS with ΔG of-6.8 kcal/mol [53]. Other phytocompounds like quercetin has been found to efficiently interact with iNOS active residues compared to tetrahydrobiopterin, an iNOS inhibitor [63]. Similarly, curcumin analogues are known to interact with Ser530 residue of COX by H-bond [56]. Also, Xanthone derivatives were observed to be potent inhibitors of COX. Studies have shown interaction of such derivatives with Arg120, Ser530, Met 522, Tyr355, Tyr 385, Ser353 residues of COX [57].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* and its important compound garcinol.

Conclusion

The present study demonstrated that Garcinia extract exhibited potent anti-inflammatory activity in LPS-induced RAW 264.7 cells. GHE and garcinol 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 NO and PGE2 in the GHE as well garcinol pre-incubated cells followed by LPS incubation. Also, the molecular docking of garcinol and HCA which are the major active components of Garcinia 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 a number of NSAIDs. Such binding affinities of these compounds could be linked to its 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 and garcinol can be the basis to promote the development of anti-inflammatory drugs.

Materials And Method

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, 100µg/ml Streptomycin. The cell line was maintained at 37°C and 5% CO_2 in CO_2 incubator. The cells were tested for any kind of Mycoplasma contamination prior to the experiments following the PCR-based method [64, 65].

Chemicals

LPS (E. coli serotype O111: B4, Sigma Aldrich, USA) stock solution of 1mg/ml was prepared in 1X PBS and stored in aliquots at − 20°C until the experiment. Similarly, the methanolic extract of commercially available *Garcinia* extract supplement (Himalaya, Lot No.: 11702264) was prepared for in vitro treatment. Similarly, garcinol (Sigma Aldrich, USA) stock solution was prepared by dissolving in DMSO to make 20mM stock solution [66].

Determination of LPS dose

The LPS dose was determined using the NO assay. The production 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. To determine the LPS dose to stimulate RAW 264.7 cells for the study, RAW 264.7 cells were seeded in a 6-well culture plate at a density of 2×10^5 cells/well and allowed to settle for 16h. After 16h 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). 24h post-treatment, 100 µl of cell culture supernatant was used for NO estimation. A triplicate set was taken for the measurement of each sample.

Cell viability assay and IC_{50} determination

To ascertain the IC_{50} dose for GHE and garcinol, cell viability was quantified using the MTT reagent (HiMedia). The effect of GHE and garcinol on cell viability was investigated using the MTT assay. RAW264.7cells were seeded in 96-well plates at a density of 1×10^4 cells/well and allowed to settle for 12hr at 37°C. The culture medium was then discarded and fresh media containing different concentrations of GHE (0, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 µg/ml) and garcinol (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM) was added and then incubated for 48h at 37°C. Post incubation, 10µl of MTT was added to each well and further incubated for 3–4 hr 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 570nm and 630nm with a microplate reader. The IC_{50} value of GHE and garcinol was evaluated from the MTT assay results.

Determination of total nitric oxide level

RAW 264.7 cells were cultured in 100mm dishes at a density of 10^6cells/cm^2 and allowed to settle for 16h. After 16h of incubation the medium was discarded and the cells were pre-treated with medium containing 115µg/ml and 230µg/ml of GHE; 6µM and 12µM of garcinol for 12h 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 24h except for control group. 24h post LPS treatment, 100 µl of cell culture supernatant was used for NO estimation. Triplicate set was taken for measurement of each sample. The production of total nitric oxide in the culture medium of treated cells was measured using the Nitric oxide estimation kit (HiMedia) following the manufacturer's protocol.

Prostaglandin E_2 assay

RAW 264.7 cells were cultured in 100mm dishes at a density of 10^6cells/cm^2 and allowed to settle for 16h. After 16h of incubation, the medium was discarded and the cells were pre-treated with a medium containing 115µg/ml and 230µg/ml of GHE; 6µM and 12µM of garcinol for 12h 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 24h except for the control group. 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×g. The medium was discarded and cells were washed 3 times with pre-cooled PBS. For every 1×10^6 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 10min at 1500×g at 2–8℃. The resultant supernatant was used to carry out the assay using the PGE_2 ELISA kit (Elabscience®, USA) following the manufacturer's instructions.

RNA Isolation and cDNA Synthesis

RAW 264.7 cells were cultured in 100mm dishes at a density of 10^6cells/cm^2 and allowed to settle for 16h. After 16 h of incubation, the medium was discarded and the cells were pre-treated with a medium containing 115µg/ml and 230µg/ml of GHE; 6µM and 12µM of garcinol for 12h except for the control and LPS treated groups and then the medium was discarded. The cells were then stimulated with 1µg/ml LPS for 24h except for the control group. 24h post LPS treatment the cells were washed with ice-cold PBS. 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 (0.5µg per sample) was reverse transcribed to cDNA using a first-strand cDNA synthesis kit (High Capacity, ThermoFisher) following the manufacturer's protocol. For qPCR studies, the cDNA samples were diluted 50 times with sterile miliQ water.

Designing of primers

For quantitative real-time 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 [67]. 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 have been shown in Table 1.

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), 2.8µl of each forward and reverse primers (10µM) and 2µl of cDNA template was used; and qPCR reactions were performed with thermal cycling conditions: denaturation at 95°C for10 min, followed by 45 cycles of denaturation at 95°C for 15s, primer annealing at respective annealing temperatures for 30s and extension at 72°C for 30s. Relative mRNA concentrations of the transcripts in the experimental groups were determined as fold change to the control by following $2^{-\Delta\Delta CT}$ method [68, 69]. The melt curve analysis was performed to check for any non-specific amplification during qPCR analysis.

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, prediction of the bioactivity score for majority of chief drug targets [70]. Lipinski's rule of five was applied to garcinol and HCA [71]. The mol files and smile formula of garcinol and HCA were obtained from CHEMSPIDER database [72] (Figs. 1 and 2).

Molecular Docking

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/) (Fig. 3). The protein molecules were optimized. 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 Autodock tool (ADT) [73].

Ligand preparation:

The structures of garcinol (PubChem CID-5281560) and hydroxycitric acid (HCA) (PubChemCID-185620) was retrieved as an SDF file from PubChem database (https://pubchem.ncbi.nlm.nih.gov) [74]. The file was converted to PDB format with the help of Open Babel software (http://openbabel.org) [75]. 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) [73].

Grid box set up and docking

Molecular docking was carried out with garcinol and HCA on iNOS and COX-2 with the help of AutoDock 4.2.6 program [73]. The binding site residues of the proteins were predicted using CASTp 3.0 [74]. A grid box enclosing the binding site residues of the target proteins was created to achieve the best conformational space. The Grid box parameters have been shown in Table 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 analysed using the LigPlot + tool [75].

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 $p < 0.05$.

Declarations

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Tables

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

Table 2

Properties of ligands analysed using Molinspiration

Ligands	CHEMSPIDER ID	LogP	H-bond acceptor	H-bond donor	Mol wt.	No. of violation	TPSA
Hydroxycitric acid	110439	-2.90	8	5	208.12	0	152.35
Garcinol	10199485	8.26	6	3	602.81	2	111.90

Table 3

Grid box parameters for docking and binding site residues

Protein	PDB ID	Binding site residues
iNOS	2NSI	MET120,THR121,LEU125,TRP194,ALA197,PRO198,ARG199,CYS200,ILE201,GLY202,ILE204,GLN205,LEU209,SER242,ILE244,ARG258,TRP
COX-2	6COX	ASN34,CYS36,CYS37,ASN39,PRO40,CYS41,GLN42,ASN43,ARG44,GLY45,GLU46,CYS47,MET48,ASP58,CYS59,THR60,ARG61,THR62,GLY6

Figures

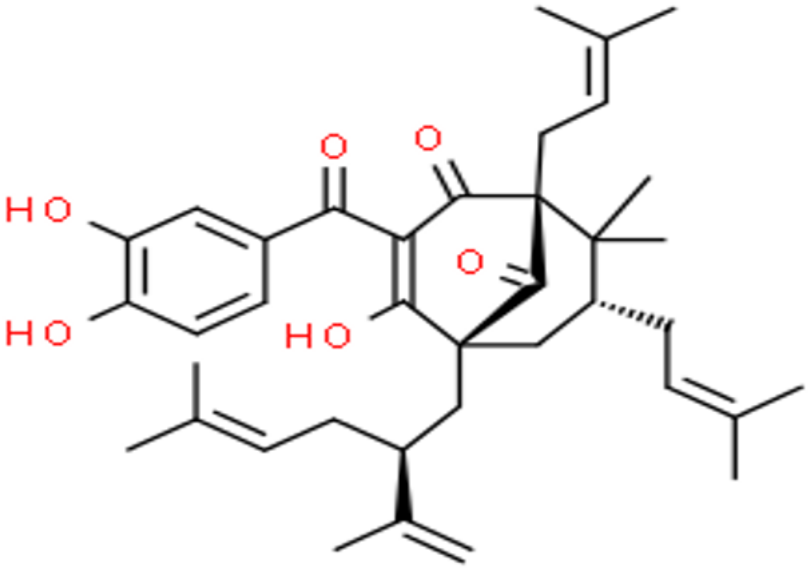

Figure 1

Chemical structure of Garcinol (ChemSpider ID 10199485)

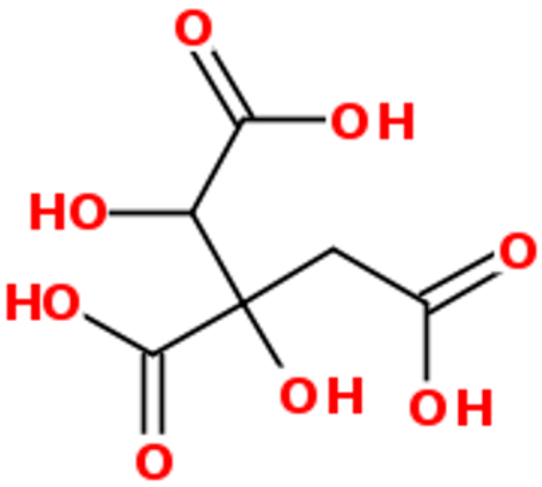

Figure 2

Chemical Structure of Hydroxycitric acid (ChemSpider ID 110439)

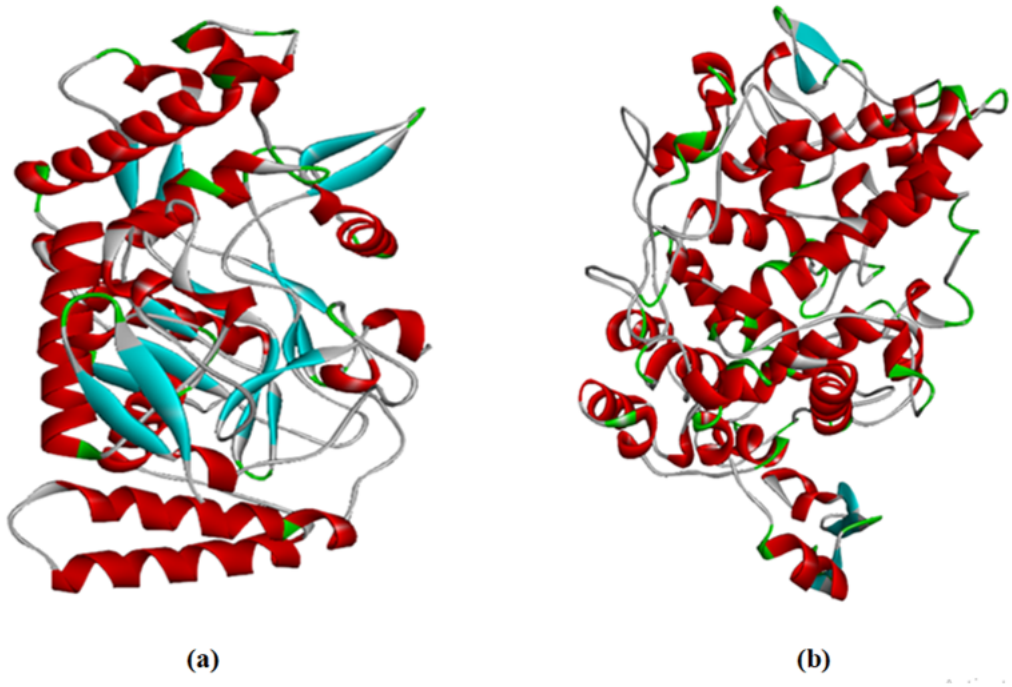

Figure 3

Optimized structures of (a) iNOS and (b) COX-2 (BIOVIA Discovery StudioVisualizer)

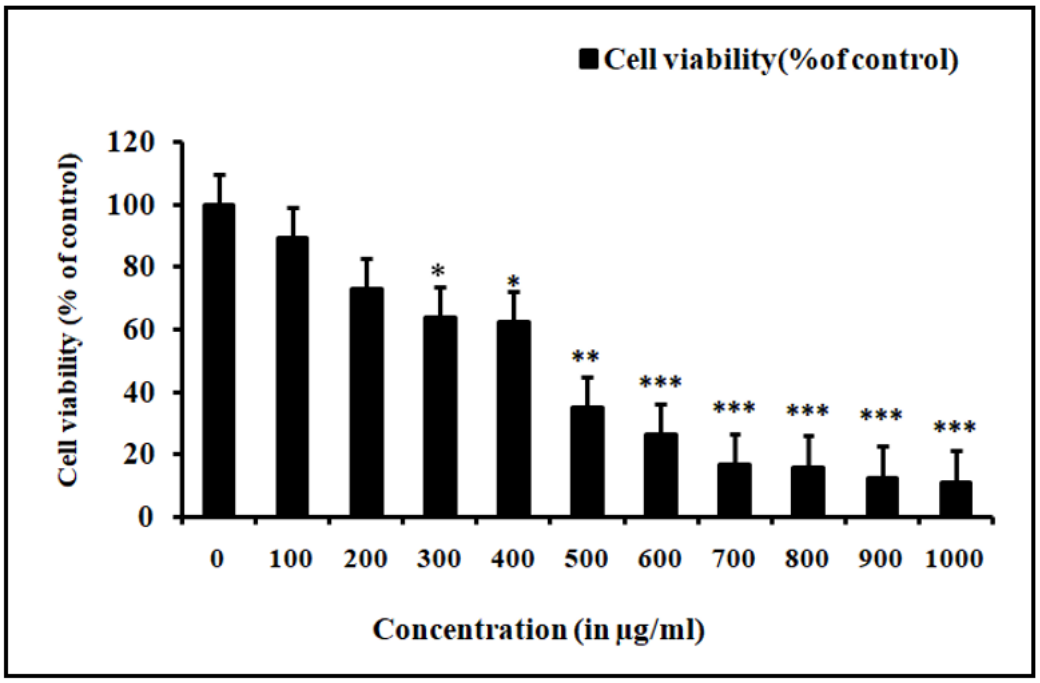

Figure 4

Effect of GHE 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 ± SD (n=3). *p < 0.05, **p <0.01 and ***p < 0.001 vs. 0 µg/ml. The IC50 was calculated to be 460µg/ml

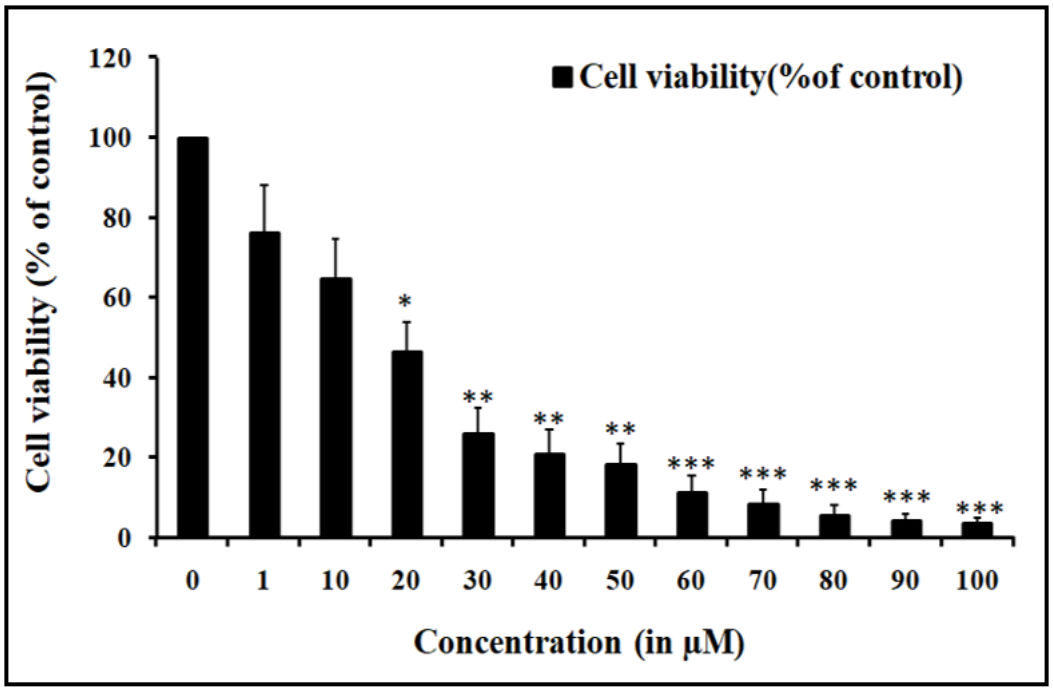

Figure 5

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 ± SD (n=3). *p < 0.05, **p <0.01 and ***p < 0.001 vs. 0 µM. The IC50 was calculated to be 24uM

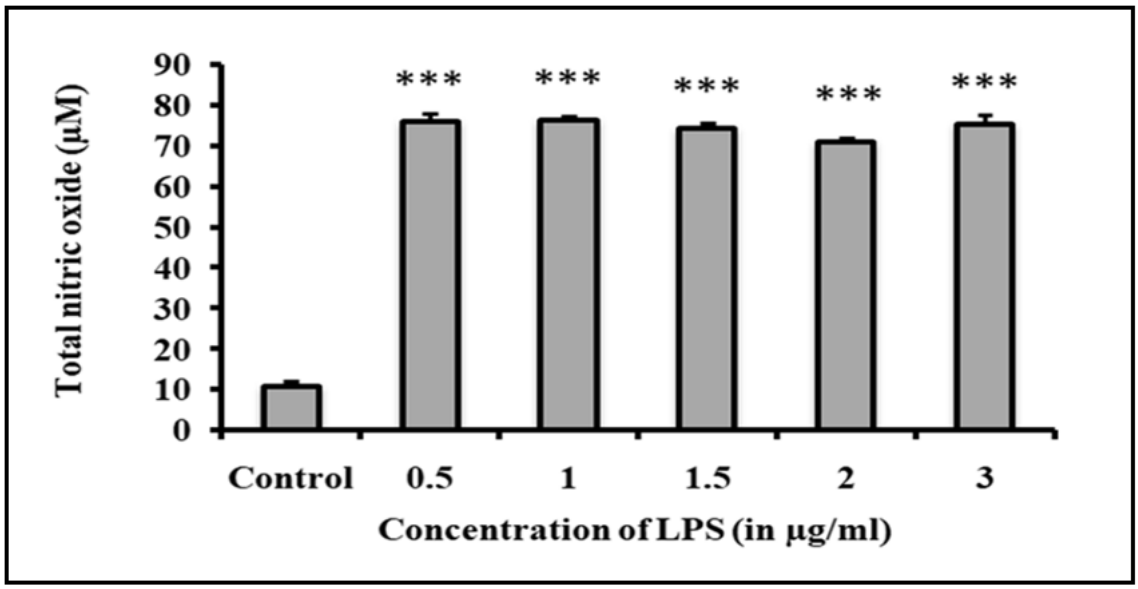

Figure 6

LPS-induced NO production in RAW 264.7 cells. Cells were stimulated with the indicated 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 nitrite to evaluate NO production. Level of NO production in LPS stimulated cells are expressed as fold changes relative to the control group. The values are statistically significant at *p<0.05; **p<0.01; ***p<0.001 as compared to the control group (one-way ANOVA).

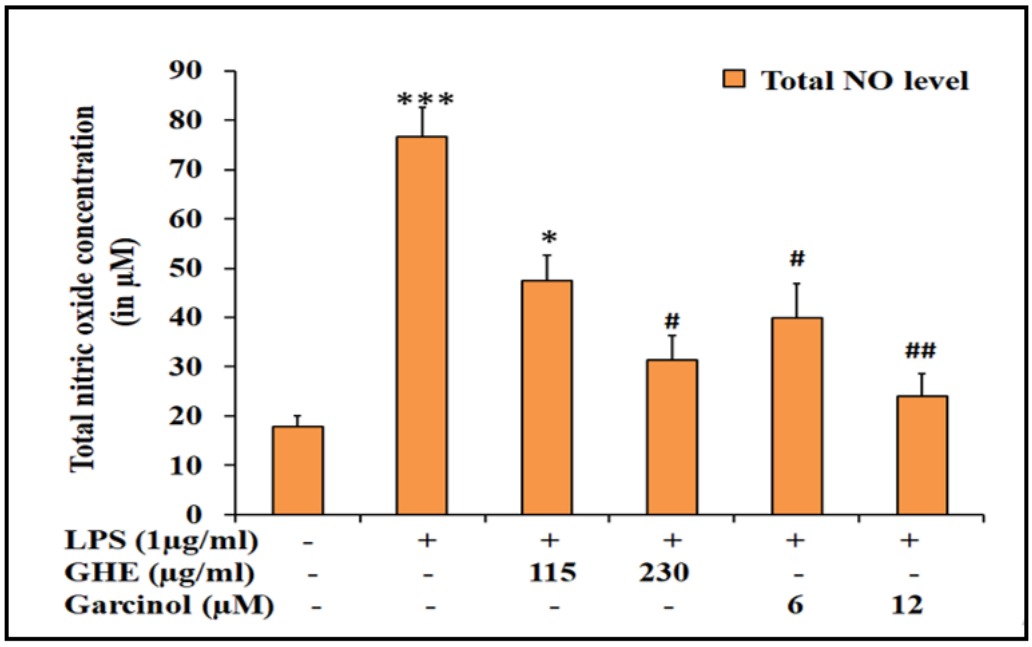

Figure 7

Effects of GHE and Garcinol on the nitric oxide (NO) level of in LPS-stimulated RAW264.7 cells. Cells were treated with GHE (115µg/ml and 230µg/ml) and Garcinol (6µM and 12µM) before LPS (1 µg/mL) stimulation followed by 24h 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

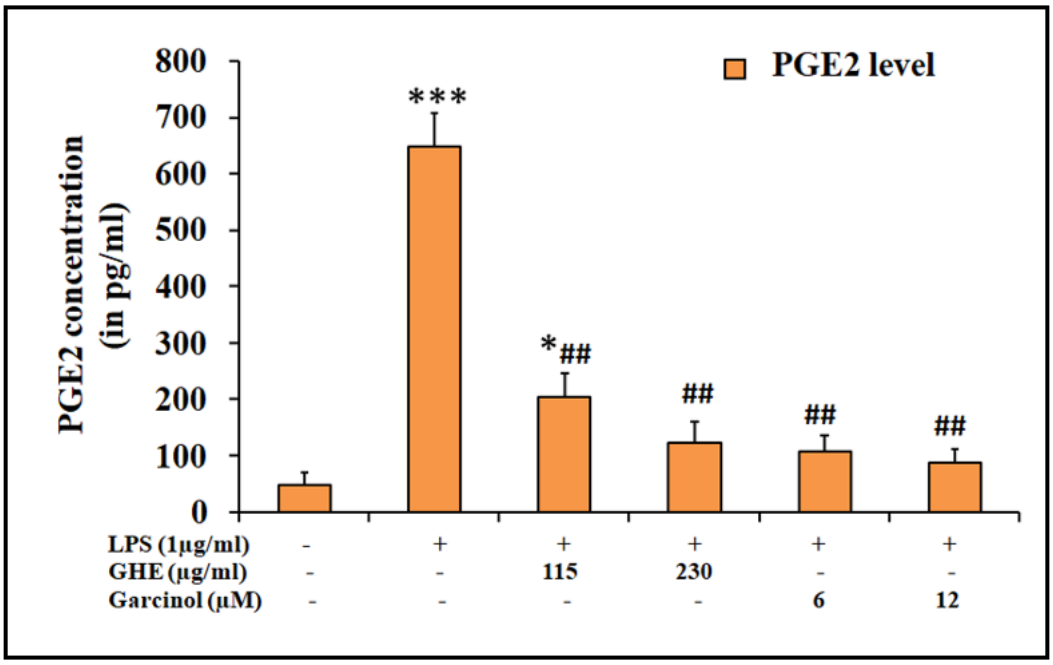

Figure 8

Effects of GHE and Garcinol on the prostaglandin E2 (PGE2) level of in LPS-stimulated RAW264.7 cells. Cells were treated with GHE (115µg/ml and 230µg/ml) and Garcinol (6µM and 12µM) before LPS (1 µg/mL) stimulation followed by 24h 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

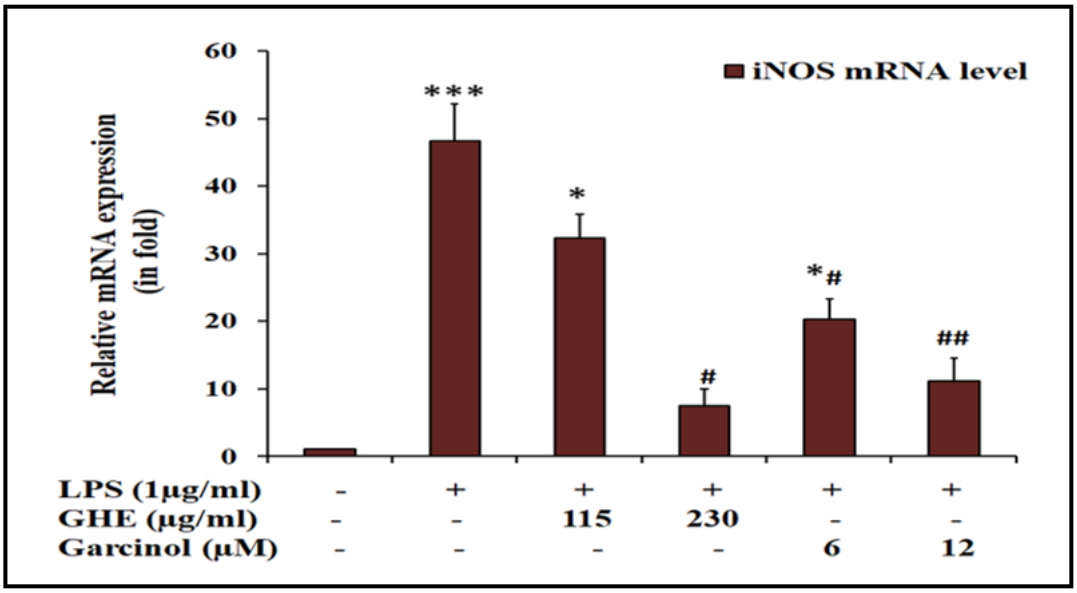

Figure 9

Effects of GHE on the mRNA expression levels of inos in LPS-stimulated RAW264.7 cells. Cells were pretreated with GHE (115μg/ml and 230μg/ml) and Garcinol (6μM and 12μM) before LPS (1 μg/ml) stimulation followed by 24h incubation. RNA was extracted and analyzed by real-time qPCR. Levels of inos 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.

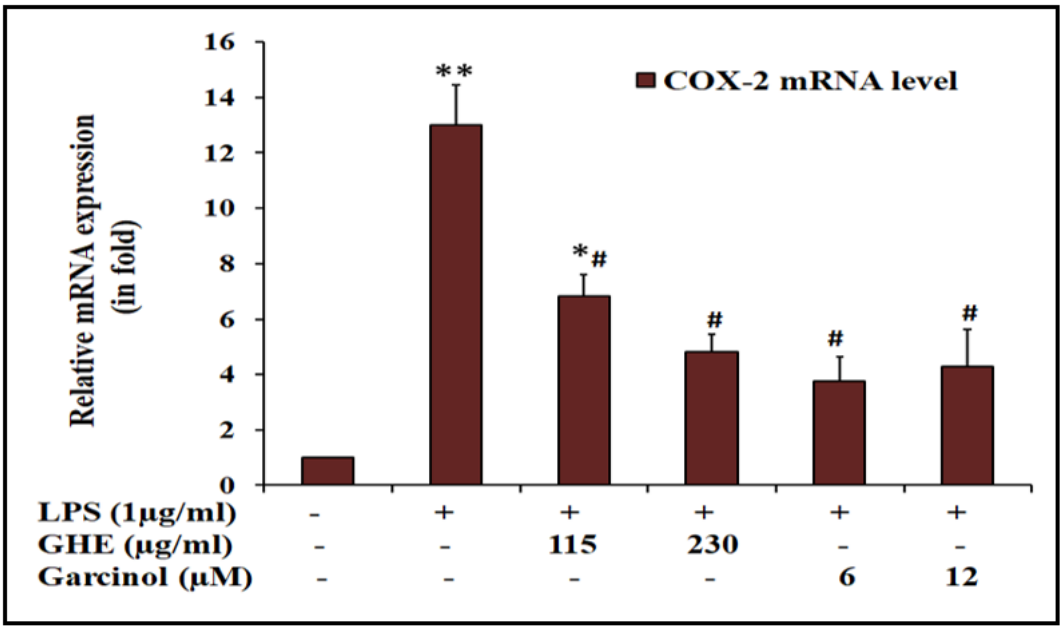

Figure 10

Effects of GHE on the mRNA expression levels of cox-2 in LPS-stimulated RAW264.7 cells. Cells were pretreated with GHE (115μg/ml and 230μg/ml) and Garcinol (6μM and 12μM) before LPS (1 μg/ml) stimulation followed by 24h incubation. RNA was extracted and analyzed by real-time qPCR. Levels of 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

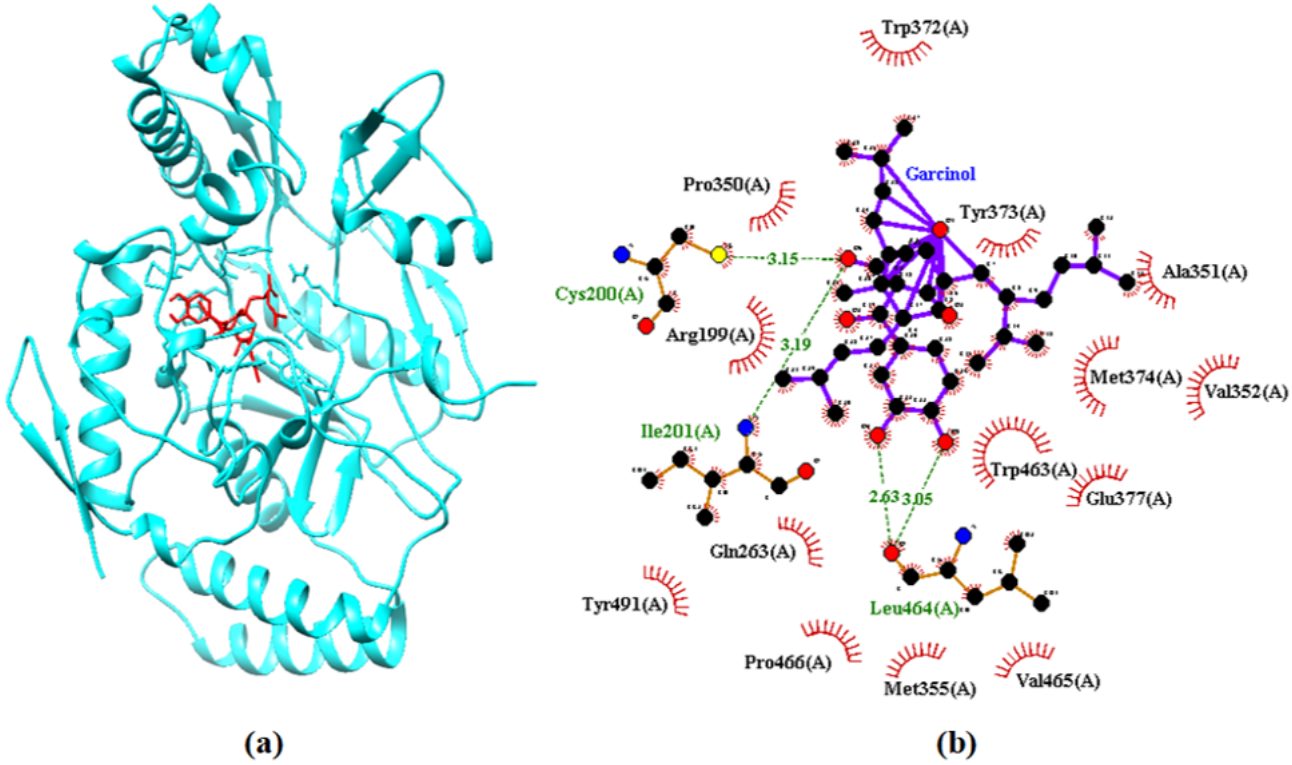

Figure 11

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.

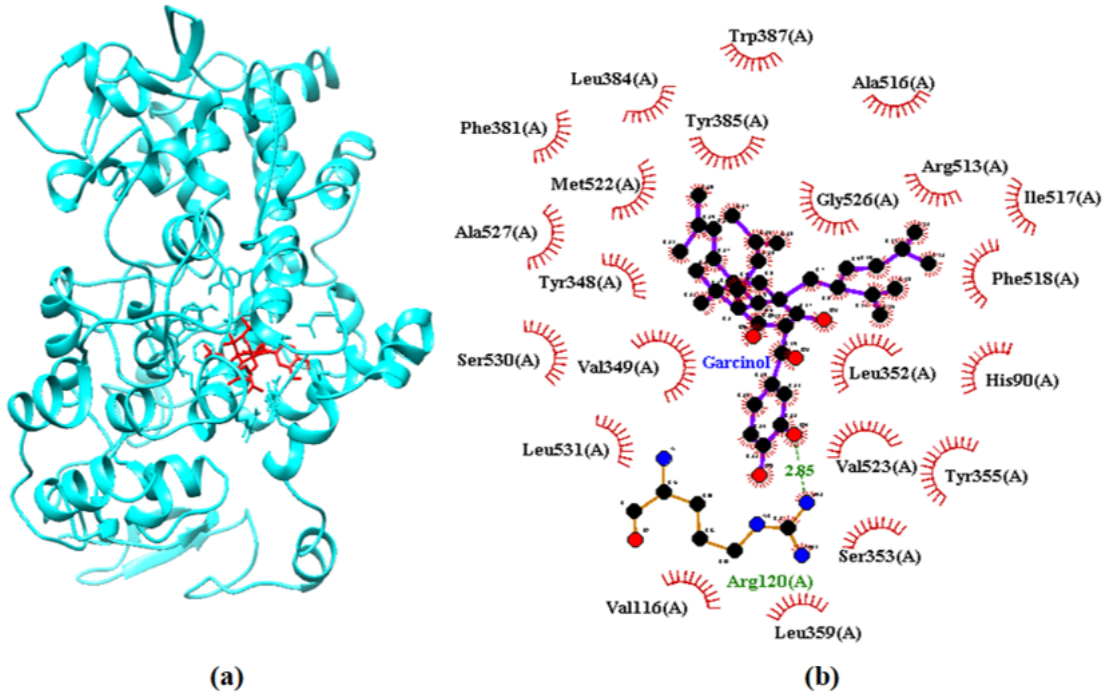

Figure 12

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.

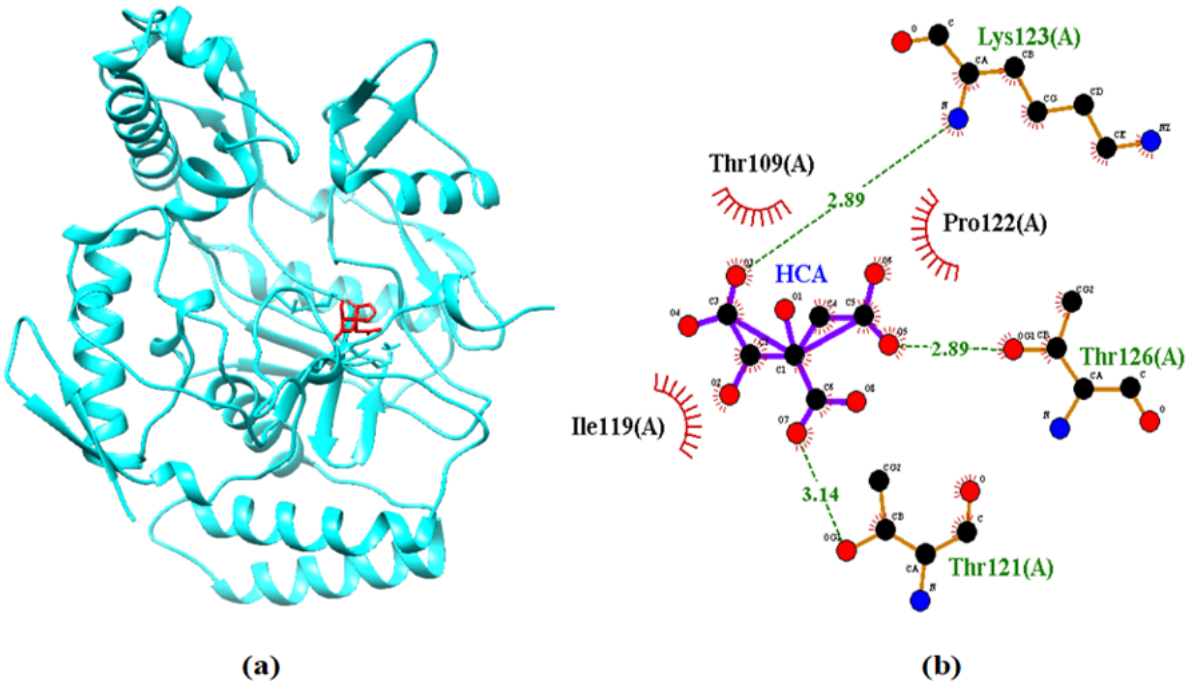

Figure 13

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.

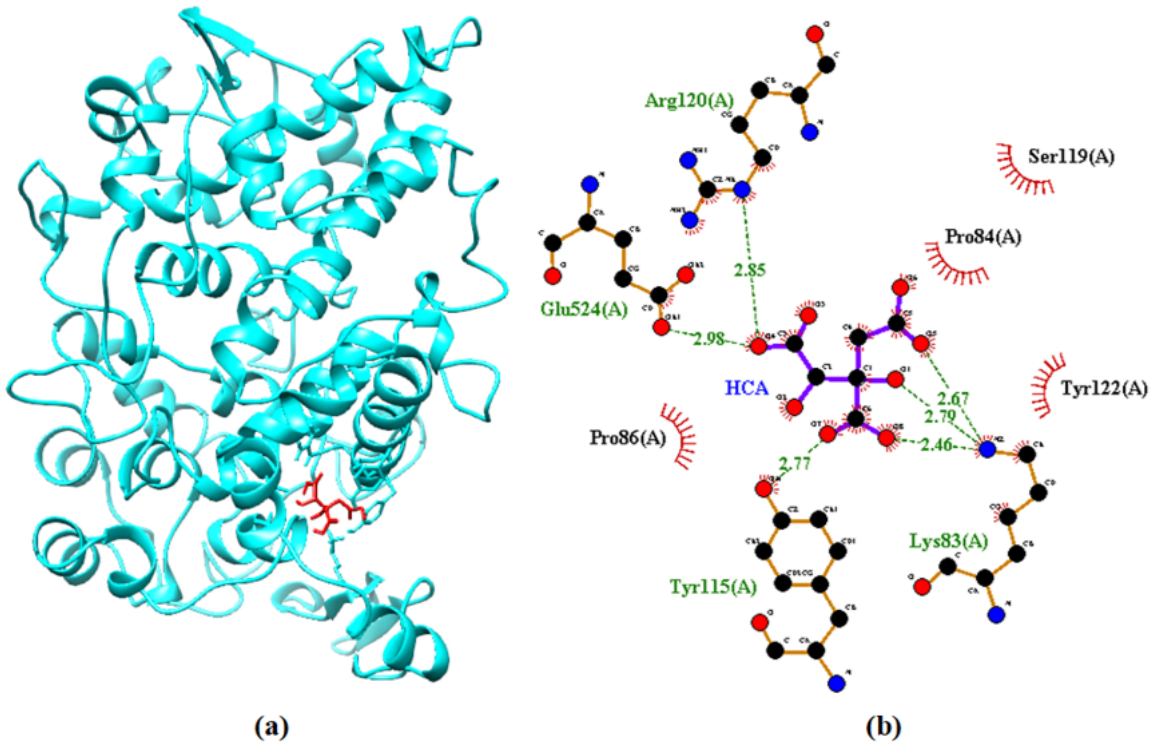

Figure 14

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.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementary.docx