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ORIGINAL ARTICLE

In silico Molecular Docking and Comparative *in-vitro* Analysis of Ethyl 3, 4, 5-Trihydroxybenzoate and its Derivative Isolated from *Hippophae rhamnoides* Leaves as Free Radical Scavenger and Anti-Inflammatory Compound

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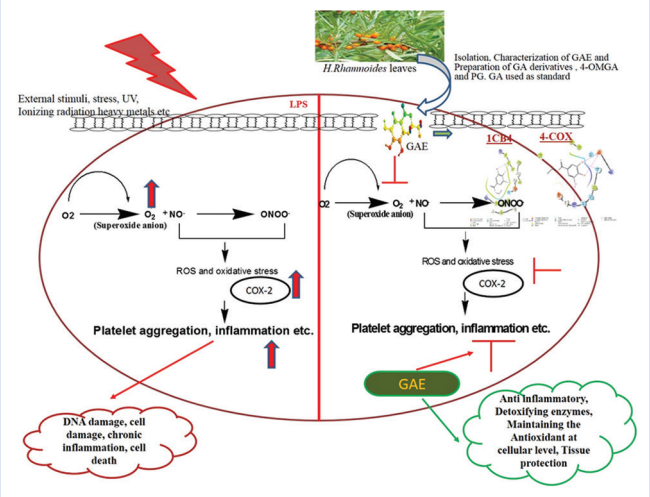
ABSTRACT

Background: Excessive production of reactive oxygen species (ROS) associated with oxidative stress induce tissue injury that might trigger the inflammatory process. Superoxide dismutase (SOD) and cyclooxygenase 2 (COX-2) play a significant role in the inflammation prompt after the overproduction of ROS. Polyphenols compound play an important role in alleviating problem associated with oxidative stress. *Hippophae rhamnoides* leaves extract contain major bioactive polyphenol compound Ethyl 3,4,5-trihydroxybenzoate (gallic acid ethyl ester [GAE]), Gallic acid (GA) and need to be investigate for its anti-oxidant and anti-inflammatory properties. **Objective:** The objective of this study is to determine the antioxidant and anti-inflammatory potential of GAE and GA derivatives isolated from *H. rhamnoides* leaves, *in vitro* and *in silico* approach target on COX-2 and SOD receptor. **Materials and Methods:** The isolated compounds GAE, GA, and derivatives 4-O methyl gallic acid (4-OMGA), pyrogallol (PG) were docked using Schrodinger's (LLC, Cambridge, USA) tools. Further *in vitro* antioxidant activity was determined using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide radical anion scavenging activity. The anti-inflammatory activity was evaluated using COX-2 inhibitory assay in lipopolysaccharide-stimulated RAW 264.7 cell line. **Results:** *In silico* result showed notable binding affinity of GAE with the SOD and COX-2 receptors followed by GA > PG > 4-OMGA. Experimentally, GAE confirmed promising antioxidant potential (DPPH; half-maximal inhibitory concentration 20.3 ± 2.65), SOD anion at 50 µM as well anti-inflammatory activity by inhibiting the COX-2 activity in RAW 264.7 cell line. **Conclusion:** The result demonstrated the potential biological activities of GAE, GA, and derivatives. *In silico* finding may act as precious tools to further unlock these potential therapeutic agents against oxidative stress.

Key words: 4-O methyl gallic acid, cyclooxygenase 2, gallic acid, gallic acid ethyl ester, pyrogallol, superoxide dismutase

SUMMARY

- *Hippophae rhamnoides* extract have a promising antioxidant, anti-inflammatory activity. This study contributes to the knowledge of the biological activity of Gallic acid ethyl ester (GAE) and gallic acid derivatives. The result indicated that GAE isolated from the *Hippophae rhamnoides* leaves showed promising antioxidant and anti-inflammatory activity. Future detail investigation was required for these compounds to understand the mechanistic superoxide dismutase and cyclooxygenase-2 pathway accountable for diminish oxidative stress induce inflammation.



Abbreviations used: ΔG: Gibbs energy; 4-OMGA: 4-O methyl gallic acid; COX-2: Cyclooxygenase 2; GA: Gallic acid; GAE: Gallic acid ethyl ester; PG: Pyrogallol; SOD: Superoxide dismutase.

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INTRODUCTION

An imbalance between oxidant/antioxidant levels is partially involved in the pathology of different disorders. Under the excessive cause of oxidative stress, superoxide dismutase (SOD) act as an endogenous cellular defense system to degrade superoxide (O_2^-) into oxygen and hydrogen peroxide.[1] The excess level of free radical generation leads to inflammation and prompt a threat factor for cancer progression and other diseases. Several enzymes and a proinflammatory molecule

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involved trigger the inflammation, one of these cellular enzymes, cyclooxygenase-2 (COX-2) play a key role in inflammation. Metabolites of arachidonic acid were involved in several biological processes in inflammation, platelet aggregation, and several immunological functions. These arachidonic acid produce prostaglandin by the action of COX. It has two isoform COX-1 and COX-2, COX-1 is a constitutive form of the enzyme expressed in the body and involves in different function such as maintain the normal gastric mucosa and renal blood flow. Whereas COX-2 in inducible form is expressed in inflammation and other physiological condition. It also involved in the production of prostaglandin which mediates pain and supports the inflammation process.[2,3] Several bioactive compounds present in the fruits and vegetables exhibited antioxidant and anti-inflammatory properties and have ability to fight against oxidative stress induced by several ways such as radiation exposure, UV exposure, and accidental damage. Oxidative stress produces due to overproduction of free radicals such as hydroxyl ion ($OH^{\cdot}$), superoxide anion (O_2^{-}), hydrogen ion (H+) and it interferes with different process in the physiology system and disturbed endogenous and exogenous cellular enzymatic activity which attacks nucleic acid, lipid membrane, and protein at nucleus to produce secondary radicals cause intracellular toxicity.[4] Antioxidants have the ability to counter the hazardous effect of oxidative stress by neutralizing the free radicals process and thus preventing from many life-threatening disease. These free radicals are unstable with an unpaired electron and reactive with another molecule such as reactive oxygen species and reactive nitrogen species. These free radicals are responsible to induce many problems such as inflammatory disease, carcinogen, tumor genesis, Parkinson's disease, and Alzheimer's disease.[5] Thus, to counter the serious medical problem, the elaboration of the antioxidant compound is significant in the field of modern drug design and development. Free radicals interrupt the antioxidant enzymatic system including different enzymes such as SOD, glutathione reductase, glutathione peroxidase and catalase as well as mediate proinflammatory molecule such as COX-2. Screening of various antioxidant properties of medicinal plants has been investigated over the last decade in the hope of finding a novel compounds as an effective therapy for modern disease related to overproduction of free radical generation which leads to several diseases.

Certain polyphenols, flavonoids and their derivatives are known to have their own important biological properties such as antimicrobial, antioxidant, antiproliferative, and anti-inflammatory activity.[6-9]

Ethyl 3,4,5-trihydroxybenzoate is commonly known as Gallic acid, ethyl ester (GAE), $C_9H_{10}O_5$ consist of molecular weight 198.17 g/mol is colorless or slightly yellow crystalline compound. It is one of the major bioactive constituents of *Hippophae rhamnoides* aqueous extract SBL-1.[10] SBL-1 is reported as a radioprotective aqueous extract in preclinical studies;[11] the presence of the bioactive constituent in this extract showed radioprotective properties and proposed to be a potent aqueous extract for radiation-induced damage associated with free radical generation. However, a comparative study of these molecules and derivatives on the expression of SOD and COX-2 by using *in silico* tools has not been explored so far. Therefore, in this study, we investigated *in silico* and *in vitro* based comparative study of *H. rhamnoides* extract bioactive constituent GAE and its derivative (Gallic acid [GA], 4-O-methyl gallic acid [4-OMGA] and pyrogallol [PG]) in order to regulate the COX-2 and SOD expression.

The objective of this study *in silico* ligand-protein docking was to explore their predominant binding model of four different ligands with the three-dimensional (3D) structure of SOD and COX-2 receptors. The intermolecular flexible docking simulation was performed to explore the binding site of the four compounds GA, 4-OMGA, GAE, and PG with target on two different receptors SOD and COX-2. Energy values were calculated from the information obtained while docking the confirmations of different receptor complexes. The strategically use of *in silico* molecular docking and *in vitro* studies of GAE, GA, 4-OMGA, and PG could help in understanding and identifying the potential of lead compound; which inhibit the process of free radical generation.

MATERIALS AND METHODS

Chemical and reagents

Analytical grade organic solvent was purchased from Merck (Mumbai, India). PG, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), dimethyl sulfoxide molecular grade (99.9%), lipopolysaccharide (LPS) serotype were purchased from Sigma-Aldrich (Germany), acetonitrile (high-performance liquid chromatography [HPLC] gradient grade), orthophosphoric acid, hydrochloric acid were purchased from Merck (Mumbai, India). Prostaglandin-Endoperoxidase Synthase 2 primary monoclonal antibody was purchased from Cloud-Clone Corporation, USA.

Extraction and isolation

The aerial part of *H. rhamnoides* leaves were washed thoroughly with distilled water and shade dried for 48 h and then crushed to coarse powder and extracted in a solvent apparatus with 95% alcohol. The extract was concentrated under reduce pressure by using a vacuum rotary evaporator. The dried extract was dissolved in water and filtered using Whatman filter paper no 1. The filtrate was treated with an aqueous solution of sodium bicarbonate and filtered. The residue discarded and the filtrate was neutralized by adding concentrated hydrochloric acid. After keeping for some time, the precipitate so obtained was filtered and washed with cold water to remove the traces of hydrochloric acid and impurities. The solid residue was dried and recrystallized from boiling aqueous ethanol and then on cooling crystal were collected and dried in desiccator, and their melting point was recorded. The purity of the compound was ascertained by thin-layer chromatography and HPLC.

Preparation of gallic acid derivatives

GA was dissolved in dried absolute ethyl alcohol followed by passing dried fumes of hydrochloric acid through a glass tube, for about 2–3 h, and then, the contents were poured in ice-cold water. The precipitates obtained was filtered and washed with cold water, dried, and recrystallized from aqueous boiling ethanol 1:1% on cooling crystal of ester appeared and collected.

The 4-OMGA derivative was prepared from GA using dimethyl sulfate and in the presence of tetrabutylammonium bromide in a semi-continuous process.[12] PG was procured from Sigma, Mumbai, India, for the comparative analysis.

Molecular docking studies

Molecular docking was applied on reported chemical entities in section by using Schrodinger, (LLC, Cambridge, USA). The SOD and COX-2 receptors were retrieved from the protein data bank (PDB) (http://www.rscrb.org/pdb). Molecular docking of ligands receptor deals with ligand interaction, H-bonding, and hydrophobic effects with receptors.

Target selection+

Identification and selection of appropriate drug target is an important part in drug designing. 3D structure of SOD and COX-2 were obtained from PDB with id's 1CB4 and 4-COX respectively. The complexes bound to the receptor molecule, such as nonessential water molecule, including heteroatoms were removed from the target receptor molecule.

Tool used

ChemDraw was used for designing the ligands. The receptors (PDB ID: 1CB4 and 4-COX) were accessed from the database of Schrodinger tools. These receptors refined, optimized, and energy minimized using protein preparation wizard. The binding sites were identified using Sitemap (version 3.4) for the receptors reported without co-crystallized ligands.[13,14]

Ligand preparation

Four compounds GA, GAE, 4-OMGA, and PG were used for docking studies [Figures 1-4]. Structure of compounds obtained from the PubMed and PubChem literature showed antioxidant and inhibitory action toward inflammation.[15] The ligand was sketched in ChemDraw and the mol/sdf files were imported to LigPrep followed by ligand preparation and energy minimization by using LigPrep v2.9 module under the force field OPLS3. The possible ionization states of all the ligands were generated using the ionization module in the pH range.

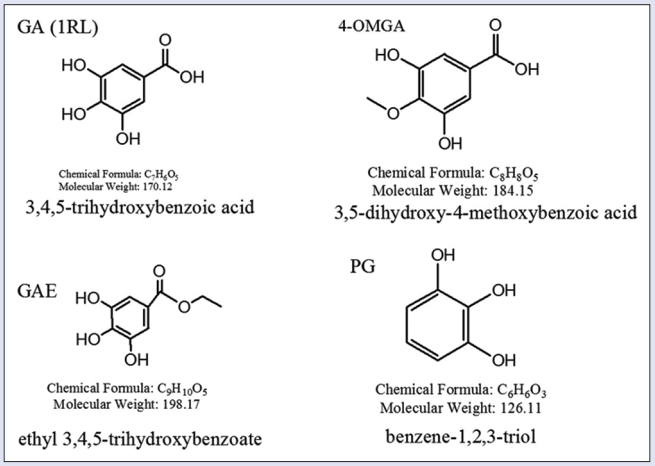

Figure 1: Ligands detailed structure with its molecular formula and its weight, Gallic acid (1RL), 4-OMGA, GAE, PG. 4-OMGA: 4-O methyl gallic acid; GAE: Gallic acid ethyl ester; PG: Pyrogallol

In vitro free radical scavenging activity induced

2, 2-diphenyl-1-picrylhydrazyl assay

The antioxidant capacity was determine by measuring DPPH radical scavenging by use the method of Brand-Williams. Test samples including GA, GAE, 4-OMGA, and PG, 5 mg were dissolved in 100 mL diluent stock. The different concentration stock solution were prepared 10, 20, 40, 60, 80, and 100 µg/mL solution from stock solution. In different concentration solution, 1 mL DPPH was added and the solution was kept for 30 min in dark place. Absorbance was observed at 515 nm.[16] GA used as standard and calculated on based formula. Each experiment was performed in triplicate.

$$\text{Scavenging effect in percent } (\%) = \frac{1-\text{absorbance (sample)}}{\text{Absorbance (control)}} \times 100$$

Superoxide anion radical scavenging

Superoxide radical scavenging activity was done based on the described method.[17] The radicals were generated by oxidation in phenazine methosulfate-nicotinamide adenine dinucleotide reduced (PMS-NADH) and measured through reduction of nitro blue tetrazolium (NBT). Superoxide radicals were generated in sodium phosphate buffer 100 mM, pH 7.4 containing 1 mL of NBT, and NADH (468 mM) solution. The reaction was initiated by adding 1 mL PMS (60 mM) to the mixture. The reaction mixture

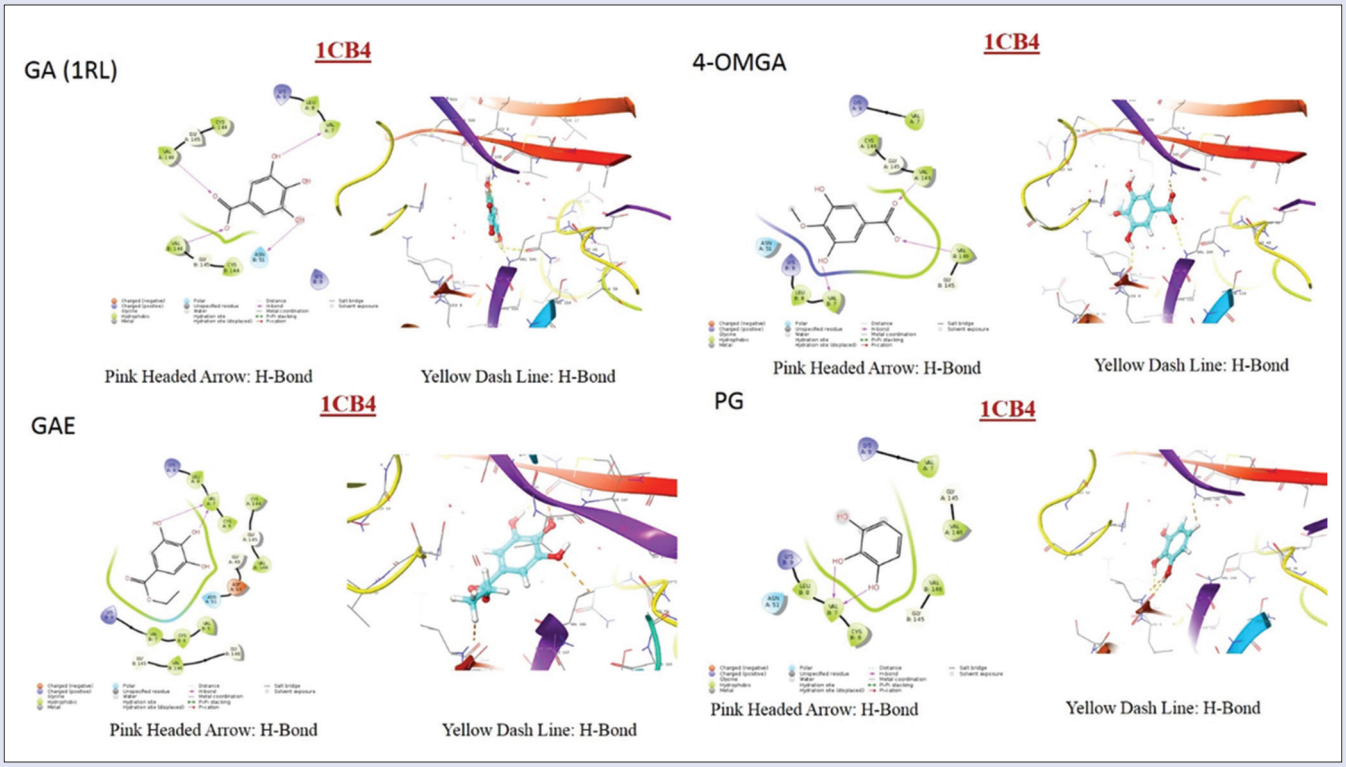

Figure 2: *In silico* molecular docking with superoxide dismutase receptor protein data bank: 1CB4 with different ligands, Gallic acid (1RL), 4-OMGA, GAE, PG. 4-OMGA: 4-O methyl gallic acid; GAE: Gallic acid ethyl ester; PG: Pyrogallol

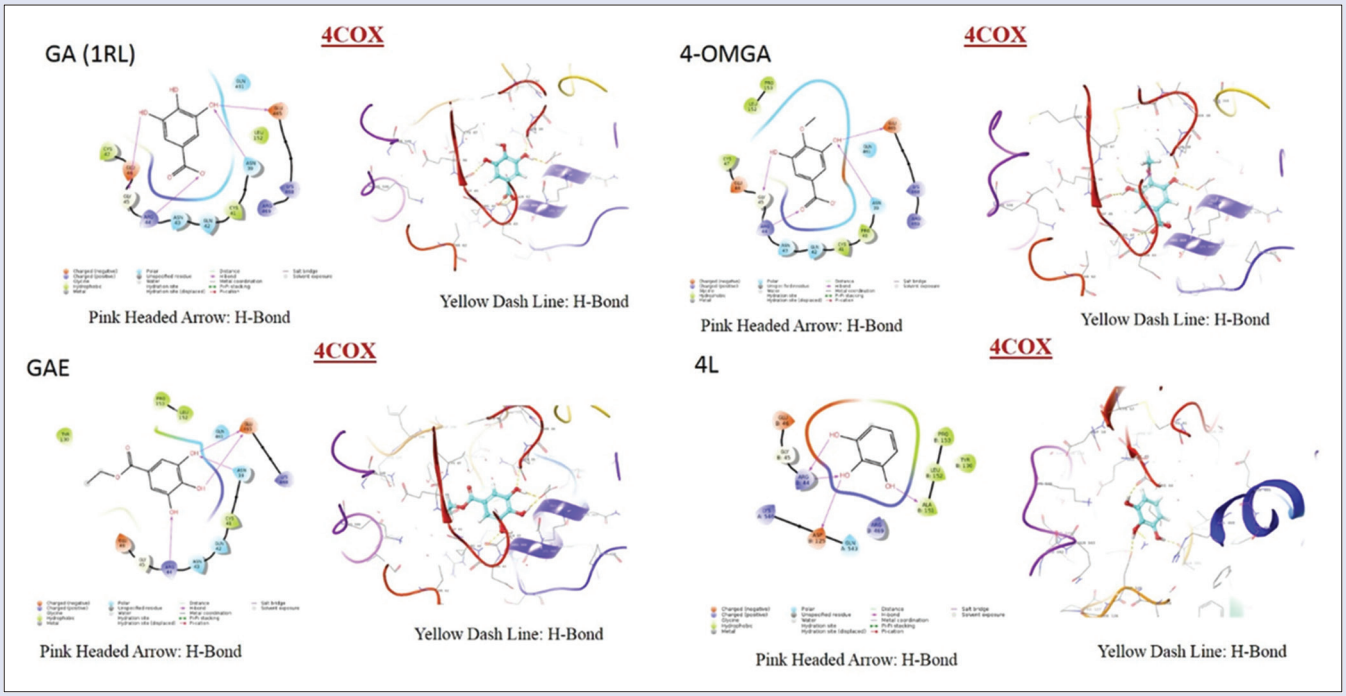

Figure 3: *In silico* molecular docking with cyclooxygenase 2 receptor protein data bank: 4-cyclooxygenase with different ligands, Gallic acid (1RL), 4-OMGA, GAE, PG. 4-OMGA: 4-O methyl gallic acid; GAE: Gallic acid ethyl ester; PG: Pyrogallol

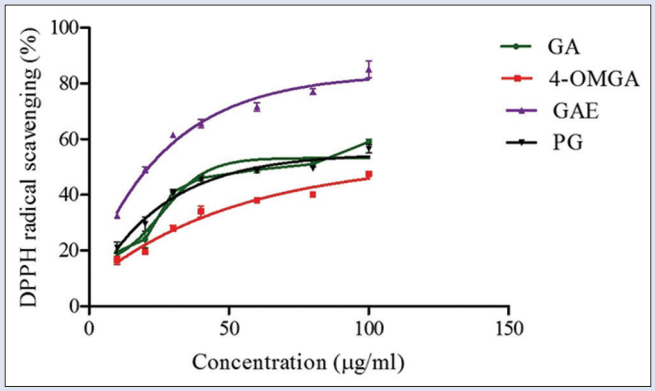

Figure 4: Effect of GA, 4-OMGA, GAE and PG on DPPH radicals. GA use as positive control. GA: Gallic acid; 4-OMGA: 4-O methyl gallic acid; GAE: Gallic acid ethyl ester; PG: Pyrogallol; DPPH: 2, 2-diphenyl-1-picrylhydrazyl

was incubated at 25°C for 5 min and the absorbance was measured against the blank. GA was used as positive control. Decrease rate in the extent of NBT reduction, measured by the absorbance of the reaction mixture, correlates with the superoxide radicals scavenging activity of GAE and its derivative. The slope of the correlation and absorbance with the time was calculated. Reaction mixture without sample was considered as the control and the SOD scavenging ability was calculated as

$$\frac{(1-\text{Slope of the sample})}{\text{Slope of the control}} \times 100\%$$

Anti-inflammatory activity

The COX-2 inhibitory assay was carried out as described by Walker and Gierse[18] to determine the anti-inflammatory potential of isolated compounds and their derivatives on RAW 264.7 cells lines. It grown to 75% confluence followed by cell line activation with 1 µL LPS (1 µg/mL). The standard anti-inflammatory drug indomethacin (Sigma Aldrich, Germany) was used as reference anti-inflammatory drug. Corresponding the different concentration of isolated compounds and derivatives, including GA, GAE, 4-OMGA, and PG were added at different concentration 10, 20, 30, 40. 50, 60, and 100 µg/mL in cells and incubated for 24 h. Post incubation *in vitro* COX-2 activity was performed to determine the anti-inflammatory activity by using the cell lysate of RAW 264.7 cell line. Cell lysate incubated in Tris-HCl buffer (pH 8.0), glutathione (5 mM/L), and hemoglobin (5 mM/L) for 1 min at 25°C. The investigation were carried out by the incorporation of arachidonic acid (200 mM/L) and concluded after 20 min incubation at 37°C by the addition of 1% thiobarbiturate. COX-2 activity was resolved by interpreting absorbance at 632 nm.[19]

RESULT AND DISCUSSION

The intermolecular flexible docking simulation was performed to explore the binding site of different ligands with different receptors. Energy values were calculated from the information obtained while docking the confirmation of different receptor complexes. To determine the best compound affinity and develop a lead molecule, different compounds were compared to reference ligands and screening were based on Gibbs free energy values, affinity, confirmation of the structure, and hydrogen bonding interaction between compounds and target proteins.

Superoxide dismutase (protein data bank: 1CB4)

The interpretation of ligands with different receptors showed polar and hydrophobic interaction, some ligands are forming H-bonds and some form salt bridges. The detail of the ligand receptor is tabulated in Table 1. The GA considered as reference ligand was found to bind with the residues Val7, Leu8, Val144, and Val146 of chain-A through

Table 1: Ligands detail with polar and hydrophobic interaction with receptor and amino acid residue

Ligand	Protein			
	1CB4		4COX	
	Polar interactions	Hydrophobicinteractions	Polar interactions	Hydrophobic interactions
GA	Chain A: Lys9	Chain A: Leu8, Val7, Cys144, A: Val146	Chain A: Asn 39, Gln42, Asn43, Arg44	Chain A: Cys41, Cys47
(1RL)	Chain B: Lys9, Asn51	Chain B: Cys144, Val146	Chain B: Gln461, Lys468, Arg469	Chain B: Leu152
4-OMGA	Chain A: Lys9	Chain A: Val7, Cys144, Val146	Chain A: Asn39, Gln42, Asn43, Arg44	Chain A: Pro 40, Cys41, Cys47
	Chain B: Lys9, Asn51	Chain B: Leu8, Val7, Val146	Chain B: Gln 461, Lys 468, Arg469	Chain B: Leu 152, Pro153
GAE	Chain A: Lys9, Asn51	Chain A: Cys6, Val7, Leu8, Cys144, Val146	Chain A: Asn39, Gln42, Asn43, Arg44	Chain A: Cys41
	Chain B: Lys9	Chain B: Val5, Cys6, Val7, Val146	Chain B: Gln461, Lys468	Chain B: Tyr130, Pro153, Leu152
PG	Chain A: Lys9	Chain A: Val7, Val146	Chain A: Lys546, Gln543	Chain B: Pro153, Leu152,
	Chain B: Lys9, Asn51	Chain B: Leu8, Val7, cys6, Val146	Chain B: Arg44, Arg469	Ala151, Tyr130

Ligands with SOD and COX-2 receptor. Amino acid residue represent in table formed salt bridges or Hydrogen bond between ligand receptor. SOD: Superoxide dismutase; COX-2: Cyclooxygenase-2; GA: Gallic acid; 4-OMGA: 4-O methyl gallic acid; GAE: Gallic acid ethyl ester; PG: Pyrogallo

hydrophobic interaction. The molecule represented asymmetrical orientation to both the chains of the dimeric protein. It observed that the molecule interacts at the symmetrical orientation to both the chain of dimeric protein and molecule interacts at the symmetrical interface of the protein. The interfacial positioning of the ligand offered maximum stability to the receptor-ligand complex and thus suggested the suitable site of interaction with optimum activity.

4-OMGA was found to interact through three H-bonds. The carboxylate anion formed an H-bond with the chain-B residue Val146, while carbonyl oxygen interacted with a methyl group resulted change in the overall conformation of the ligand with respect to the orthosteric site of the protein. In spite of undergoing an orientation change in the structure, the ligand accommodated itself at the protein interfaces, indicating its activity almost similar to the 1RL. It can be said that change in the conformation or functionally of 4-OMGA did not change the overall ligand interactions with the protein. Therefore, the molecule more or less remains the same inside the active site, reflecting similar behavior toward the protein (two-dimensional [2D] and 3D ligand interaction diagram of the ligand with ICB4 protein).

GAE was found to strongly interact via two H-bonds and bind with residue with Cys6, Val7, Leu8, Cys144, Val146 of chain-A through hydrophobic interaction. On the other hand, the chain-B residue Val5, Val7, Cys6, Val146 and Lys9 were interacted via the hydrophobic, polar and positively charged interactions. Two of the three hydroxyl groups formed an H-bond with chain-A residue Val7. The chain-A and chain-B residue formed the hydrophobic cavity around the ligand, whereas the chain-A residue Lys9, Asn51, and Chain-B residue Lys9 were interacting through polar and positively charged interaction. In spite of undergoing an orientation change in structure, the ligand accommodated itself at the protein interface, indicating it's higher compared to the 1RL. The observation was further assured from the G-score values obtained for 1RL (−5.6) and GAE (−6.8) which were mostly higher. Therefore, the molecule more remains the same inside the active, reflecting similar behavior toward the protein (2D and 3D ligand interaction diagram of the ligand with ICB4 protein).

PG was found to strongly interact via two H-bonds and bind with Val7 of chain-A through hydrophobic interaction, while Lys9 of the same protein chain-A interacted via charged polar interaction. Two of three hydroxyl group formed an H-bond with chain-B residue Val7 via the hydrophobic interaction. In spite of undergoing an orientation change in the structure, the ligand accommodated itself at the protein interface, indicating its activity almost similar to the 1RL. Therefore, the molecule remains same inside the active site, reflecting similar behavior toward the protein (2D and 3D ligand interaction diagrams of the ligands with 1CB4 proteins).

Cyclooxygenase 2 (protein data bank: 4-cyclooxygenase)

GA (1RL) was found to be bind with the residue Glu465 of chain-B through hydrophobic interactions with negative charged. While Lys468, Arg469 of the same protein chain-And Asn39 were interacted via the hydrophobic, polar and positively charged interactions. Two of the three hydroxyl group formed an H-bond with chain-A residue Gly45, whereas the second hydroxyl group was interacting with the chain-B Glu46 residue through H-bond. The carboxylate carbonyl oxygen was observed to interact with the hydrophobic chain-A residue Arg144 with positive charge. The molecule represented asymmetrical orientation to both the chains of the dimeric protein. It was observed that the molecule interacts at the symmetrical interfaces of the dimeric protein. The interfacial positioning of the ligand offered maximum stability to the receptor-ligand complex and thus suggesting a suitable site of interaction with optimum activity.

4-OMGA was found to interact via three H-bonds. The carboxylate anion was forming an H-bond with the chain-A residue Arg44. The substitution of a phenyl hydrogen with a methyl group resulted change in overall conformation of the ligand with respect to the orthosteric site of the protein. We observed that one of the hydroxyl protons was interacted with chain-B residue Glu465 via an H-bond interaction with a negative charge. Moreover; the large size of methyl group drifted the ligand in an orientation where, the polar chain-A residue Asn43, Arg44 and Gly45 became closer to methoxy group. Second hydroxyl proton was observed to interact with chain residue Asn39 through a polar and positive charged interaction. The chain-A residues Leu152, Pro153 and chain-B residues Pro40 and Cys41 formed the polar cavity with a negative charge around the ligand, while the chain-A residue Lys468, Arg469and Gln461and Chain-B residues Gln42, Asn43 and Arg44 were interacting through polar and positively charged interactions. In spite of undergoing an orientational change in the structure, the ligand accommodated itself at the protein interface, indicating its activity almost similar to the 1RL. It can be said that the change in the conformation or functionality did not change the overall ligand interactions with the protein. The observations were further assured from the G-Score values obtained for 1RL (−5.6) and 4-OMGA (−5.2), which were almost similar, suggesting a very small change in the ligand potency. Therefore, the molecule more or less remains.

GAE was found to strongly interact via four H-bonds and binds with residue with Asn39 and Arg44 of chain-B through hydrophobic interaction with polar and positive charge. On the other hand, the two hydroxyl group interacted with chain-A residue Glu465 negatively charged via the hydrophobic, polar and positively charged interactions. Two of three hydroxyl formed an H-bond with chain-A residue Glu465

and other two hydroxyl group was forming an H-bond with chain-B residue Asn39 and Arg44. The chain-A and chain-B residue formed the hydrophobic cavity with polar and positively charged interaction. In spite of undergoing an orientation change in the structure, the ligand accommodated itself at the protein interface, indicating it's higher activity in comparison to 1RL. The observation was further assured from the G-score value obtained for 1RL and GAE which were mostly higher. Therefore, the molecule more remains same inside the active, reflecting similar behavior toward the protein (2D and 3D ligand interaction diagram of the ligand with 4-COX protein).

PG was found to strongly interact via four H-bonds. PG was found to bind with Ala151, Arg44 of chain-B through positively charged interaction, while Leu152, Pro153 and Tyr130 of the same protein chain-B interacted via hydrophobic interaction. All of three hydroxyl group formed an H-bond with chain-B residue Ala151 and Arg44 via the hydrophobic and positive charge interaction whereas only one of the hydroxyl groups was forming H-bond with chain-B residue Asp125 with a negative charge. In spite of undergoing an orientational change in the structure, the mixed cavity with polar interaction carried out both positive and negative charged form around the ligand. Therefore, the molecule remains same inside the active site, reflecting similar behavior towards the protein (2D and 3D ligand interreaction diagrams of the ligands with 4-COX proteins). Detailed G-score value of four ligands affinity with SOD and COX-2 receptor were observed in the following order GAE > GA > PG > 4-OMGA and tabulated in Table 2.

Table 2: G-score index of ligands with superoxide dismutase and cyclooxygenase-2 receptor

Ligands	Receptor G-score	
	1CB4	4COX
GA (1RL)	−5.6	−5.7
4-OMGA	−5.2	−4.8
GAE	−6.8	−6.3
GA	−5.0	−5.4

GA: Gallic acid; 4-OMGA: 4-O methyl gallic acid; GAE: Gallic acid ethyl ester; PG: Pyrogallol; COX: Cyclooxygenase

In vitro free radical scavenging activity of Gallic acid ethyl ester and its derivative

In vitro, antioxidant assay of GAE and GA derivative was determined by DPPH assay. The graph was plotted against percentage inhibition versus concentration. Increase in percentage inhibition depends upon the concentration increase and free radical scavenging and quenching activity intensifies. The half maximal inhibitory concentration (IC_{50}) value of GAE and its derivative 4-OMGA and PG were 20.3 ± 2.65, 48.64 ± 2.13, 38.97 ± 4.17 μg/mL. IC_{50} value of standard GA was found to be 28.76 ± 3.26 μg/ml. This again confirmed the antioxidant potential of the different molecule in comparison to reference compound by DPPH assay [Figure 4].

In vitro superoxide anion radical scavenging activity

The superoxide anion induces damage directly or indirectly by producing H_2O_2,-OH or singlet oxygen under different pathology. It has been also observed that superoxide directly initiate malondialdehyde formation by-product of lipid peroxidation.[20-22] Superoxide anion radical scavenging activity of GAE increased markedly with increase in concentration [Figure 5]. GAE had the highest inhibition effect at 50 μM on superoxide anion formation when compared to GA, 4-OMGA and PG. However, result suggested that GAE has a promising effect on inhibition of superoxide anion formation and the radical scavenging activity of all derivatives decreased in the following order GAE > GA > PG > 4-OMGA.

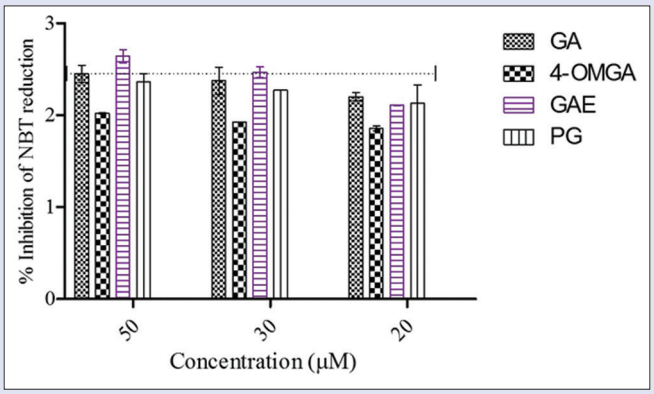

Figure 5: Effect of GA, 4-OMGA, GAE, and PG in *in vitro* superoxide dismutase activity. Superoxide dismutase was measured in the % inhibition of nitro blue tetrazolium reduction with pretreated with different concentration of compounds. GA: Gallic acid; 4-OMGA: 4-O methyl gallic acid; GAE: Gallic acid ethyl ester; PG: Pyrogallol

In vitro anti-inflammatory activity

Anti-inflammatory effect of gallic acid ethyl ester and its derivative on cultured RAW 264.7 cells line by cyclooxygenase 2 inhibitory assay

In vitro anti-inflammatory activity of GAE and its derivative was investigated by using LPS stimulated RAW 264.7 macrophages cell line. prostaglandin E2 (PGE_2) production in cell supernatant of RAW264.7 cell line was determined to evaluate the inhibitory effect of GAE and GA derivatives on COX-2 activity. LPS stimulation induce the production of PGE_2 whereas very low amount of PGE_2 were observed in unstimulated cells. Different concentration of molecules like 10, 20, 30, 40, 50, 60 and 100 μg/mL were used for *in vitro* anti-inflammatory study and inhibition of COX-2 level is measured [Figure 6]. The inhibitory effect of the samples were statistically significant when compared with LPS stimulated cells ($P < 0.01$). The result suggested that GAE at 50 μM induce an inhibitory effect on COX-2 activity strongest suggested that the inhibition obtained with indomethacin.

CONCLUSION

The results of the above study suggested that the bioactive compounds present in the *H. rhamnoides* have a noticeable effect on the scavenging of free radicals. The major bioactive constituent GAE when compared with different metabolites the radical scavenging activity were increased or reduced depends upon the property of compounds and its concentration. The result suggested that comparative analysis of GAE isolated from *H. rhamnoides* leaves showed the best binding affinity *in-silico* with SOD and COX-2 receptor at highest G-score (−6.8 and −6.3) with hydrophobic and polar interaction of amino acid residue and its strongly interacted with four H-bonds two of three hydroxyl was formed H-bonds with chain-A residue of COX-2 receptor in comparison to 1RL. Whereas in SOD receptor GAE was found to strongly interact via 2H-bonds and interacted with hydrophobic reactions. Two of three hydroxyl groups was forming an H-bonds with chain-A residue. Whereas *in vitro* studies suggested that GAE exhibited and confirm the potential of radical quenching in

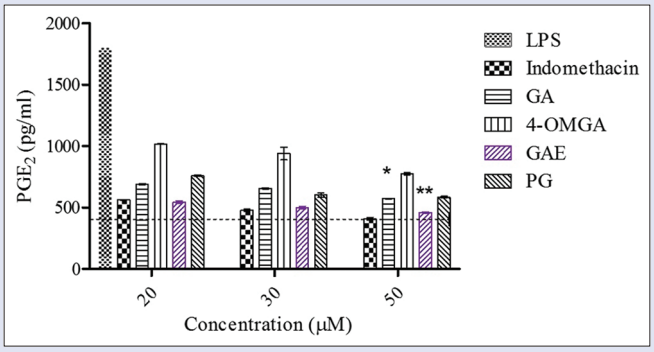

Figure 6: Effect of GA, 4-OMGA, GAE, and PG on LPS stimulated PGE_2 production in RAW 264.7 cells. PGE_2 was measured in the culture medium of the cells with pretreated with different concentration of compounds and then stimulated with LPS, Indometahcine use as positive control. GA compared with LPS *$P > 0.05$, GAE compared with LPS **$P > 0.01$. GA: Gallic acid; 4-OMGA: 4-O methyl gallic acid; GAE: Gallic acid ethyl ester; PG: Pyrogallol; LPS: Lipopolysaccharide; PGE_2: Prostaglandin E2

DPPH assay showed radical inhibiting at 20.3 ± 2.65 μg/mL of GAE when compared to reference compound GA 28.76 ± 3.26 μg/mL. SOD activity and anti-inflammatory of GAE showed the potent activity and inhibiting the superoxide anion radicals as well it also ameliorate the COX-2 activity in RAW 264.7 macrophage cell lines stimulated by LPS. The radical scavenging activity of GAE, GA, 4-OMGA and PG were observed and their activity were decreased in the following order GAE > GA > PG > 4-OMGA and showed that GAE exhibited a potent compound inhibit the generation of free radicals associated with superoxide anions and inhibiting the COX-2 activity which will further help in inhibiting the progression of several disease at initial levels associated with toxic free radicals induce inflammation.

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Conflicts of interest

There are no conflicts of interest.

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