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Preliminary phytochemical, insilico, invitro and invivo anti-inflammatory activity of ethanolic extract of Hippopahe rhamnoides.Linn

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ABSTRACT

Inflammation is a complex biological response that plays a crucial role in the body's defense mechanism. However, chronic inflammation is implicated in various diseases. The present study investigated the anti-inflammatory potential of the ethanolic fruit extract of *Hippophae rhamnoides* L. (Synonym:-sea buckthorn) through an integrated approach involving in-silico molecular docking, in-vitro bioassays and in-vivo evaluation. Preliminary phytochemical screening confirmed the presence of flavonoids, phenolic acids, tannins, saponins, carbohydrates, proteins, amino acids, phytosterols, triterpenoids, fixed oils, and carotenoids. UV-visible spectral analysis identified key bioactive compounds, including quercetin, kaempferol, isorhamnetin, gallic acid, caffeic acid, ellagic acid, β-sitosterol, and lycopene. In-silico docking analysis revealed that the flavonoids and phenolic acids exhibited strong binding affinity towards COX-2, indicating selective inhibition, which may offer a safer alternative to conventional NSAIDs. In-vitro anti-inflammatory assays, including the egg albumin denaturation, HRBC membrane stabilization and COX-2 inhibition assays, demonstrated significant inhibition, comparable to standard drugs like Diclofenac and celecoxib. In-vivo studies using the carrageenan-induced paw edema model in albino rats confirmed a dose-dependent anti-inflammatory effect. The percentage inhibition of edema after 4 hours was 57.57% for Diclofenac, 48.48% for the 250 mg/kg extract, and 54.54% for the 500 mg/kg extract.

The above findings suggested that *Hippophae rhamnoides* L. possesses significant anti-inflammatory properties, primarily through COX-2 inhibition, making it a promising medication for natural anti-inflammatory drug development.

Keywords: *Hippophae rhamnoides L., anti-inflammatory, molecular docking, egg albumin denaturation, HRBC membrane stabilization, COX-2 inhibition, UV-visible spectrum analysis, carrageenan-induced paw edema.*

1. INTRODUCTION

Medicinal plants have been essential in healthcare for centuries, providing bioactive compounds with anti-inflammatory, antimicrobial, antioxidant, and anticancer properties. With growing concerns over antibiotic resistance, drug side effects and high pharmaceutical costs, they are being re-explored for their therapeutic potential. The WHO estimates that 80% of people in developing countries rely upon medicinal plants for primary healthcare (WHO, 2021) [1].

Inflammation is a complex physiological response of vascular tissues to harmful stimuli such as pathogens, injury or irritants, playing a crucial role in eliminating the cause of injury, clearing damaged cells and promoting tissue repair. It involves immune cells, blood vessels and molecular mediators [2]. Acute inflammation is a short-term defense mechanism characterized by redness, heat, swelling, pain and loss of function, whereas chronic inflammation, often due to unresolved acute responses, contributes to diseases like arthritis, cardiovascular disorders and neurodegenerative conditions [3]. Common causes include infections, trauma, autoimmune diseases, and obesity-induced metabolic dysfunction [4]. The inflammatory

process involves white blood cells releasing chemicals that increase blood flow, causing redness, warmth and swelling. Cyclooxygenase (COX) enzymes play a key role, with COX-1 maintaining physiological functions and COX-2 amplifying inflammation, making them major targets for anti-inflammatory drugs like NSAIDs [5].

The main drug classes used for the management of inflammation are NSAIDs, corticosteroids, DMARDs, and biologic agents. NSAIDs, including non-selective inhibitors like aspirin, ibuprofen, and naproxen, block COX-1 and COX-2 to relieve pain, fever, and inflammation but may cause GI ulcers, renal toxicity, and cardiovascular risks [6]. Selective COX-2 inhibitors like celecoxib and Etoricoxib reduce inflammation with fewer GI effects, while salicylates like aspirin irreversibly inhibit COX-1 for cardiovascular protection. Corticosteroids, such as hydrocortisone (short-acting), prednisone (intermediate-acting), and dexamethasone (long-acting), suppress NF-κB and phospholipase A_2, treating conditions like asthma, rheumatoid arthritis and cerebral edema but causing metabolic disturbances, immunosuppression and osteoporosis [7]. DMARDs, including methotrexate and sulfasalazine, inhibit immune function for rheumatoid arthritis but may lead to hepatotoxicity and bone marrow suppression [8]. Biologic DMARDs like Infliximab target cytokines or B-cells for autoimmune diseases. Biologic agents, such as TNF-α inhibitors (Infliximab, Adalimumab), IL-6 inhibitors (Tocilizumab) and JAK inhibitors (Tofacitinib), modulate immune pathways for inflammatory diseases but pose risk of infections and malignancies [9].

The development of new anti-inflammatory drugs with minimal side effects is essential for enhancing patient safety and treatment adherence. Growing interest in natural and alternative remedies has emerged for managing various diseases, though scientific validation remains limited.

Figure 1*: Hippophae rhamnoides L* berry

Hippophae rhamnoides L. (Sea Buckthorn) from Elaeagnaceae family has been selected for this study, with its berries being the focus due to their rich composition of bioactive compounds. The ethanolic extract contains flavonoids (Quercetin, kaempferol, isorhamnetin), phenolic acids (Gallic acid, caffeic acid, ellagic acid), triterpenoids (Oleanolic acid, ursolic acid), carotenoids (β-carotene, lycopene) and fatty acids & sterols (Palmitoleic acid, β-sitosterol), which contribute to its anti-inflammatory property [10-11]. Sea Buckthorn offers diverse therapeutic benefits, including skin health and wound healing due to its moisturizing, regenerative and anti-aging effects; cardiovascular support by reducing cholesterol, improving circulation and preventing atherosclerosis; and digestive health benefits by alleviating gastritis, peptic ulcer and liver disorders. Its high vitamin C content boosts immunity, while its strong anti-inflammatory and antioxidant properties make it effective in managing arthritis and oxidative stress-related diseases [12]. Additionally, it aids in weight management by enhancing metabolism, supports eye health through its beta-carotene content and exhibits potential anti-cancer and anti-diabetic effects by regulating blood sugar and inhibiting cancer cell growth. Furthermore, its anti-inflammatory and immune-boosting properties make it beneficial for respiratory conditions like bronchitis and asthma, highlighting its significance as a natural therapeutic agent [13].

Despite its known medicinal potential, the precise anti-inflammatory activity and mechanism of *Hippophae rhamnoides .L.*, berry extract have not been fully explored through a combined **in-silico**, ***in vitro*** & ***in vivo*** approach so far. So the present study aim to bridge this gap by evaluating the ethanolic extract of *Hippophae rhamnoides .L.*, berries using **in-silico molecular docking studies** to predict active compounds responsible for anti-inflammatory activity and in-vitro & in-vivo **anti-inflammatory assays** to validate its efficacy for the first time.

2. MATERIALS AND METHODS

Materials:

Chemicals : Wagner's reagent (Iodine-Potassium Iodide solution), Mayer's reagent (Potassium mercuric iodide solution), Hager's reagent (Saturated picric acid solution), Dragendorff's reagent (Potassium bismuth iodide solution), Dilute sulfuric acid (H_2SO_4), Organic solvent (e.g., chloroform), Dilute ammonia (NH_3), Pyridine, Sodium nitro-prusside, Sodium picrate, Picric acid, Glacial acetic acid, Ferric chloride ($FeCl_3$), Bromine water, Lead acetate, Molisch's reagent (α-Naphthol in alcohol), Concentrated hydrochloric acid (HCl), Fehling's A solution (Copper sulfate), Fehling's B solution (Potassium sodium tartrate in sodium hydroxide), Benedict's reagent (Copper sulfate, sodium carbonate, sodium citrate), Barfoed's reagent (Copper acetate in acetic acid), Ethanol (95%), Magnesium turnings, Zinc, Sodium hydroxide (NaOH), Biuret reagent (Copper sulfate, sodium hydroxide, potassium sodium tartrate), Concentrated nitric acid (HNO_3), Millon's

reagent (Mercuric nitrate in nitric acid), Acetic anhydride, 0.5N alcoholic potassium hydroxide (KOH), Phenolphthalein, 90% Alcohol, Antimony trichloride ($SbCl_3$), Blood sample (for the hemolytic test), phosphate-buffered saline (PBS) (pH 6.4), Diclofenac sodium, egg albumin, Alsever's solution (2 % dextrose, 0.8 % sodium citrate, 0.05% citric acid and 0.42 % sodium chloride in water), isosaline, fresh whole human blood, EIA kit (Cayman Chemical Company, MI), Celecoxib, carrageenan.

Instruments: Simple distillation apparatus, Shimadzu UV-Visible spectrophotometer, centrifuge apparatus, Plethysmometer.

Docking software: Pubchem, Molegro molecular viewer, Protein Data Bank, Auto dock tools (Python 2.7.1, MGL tools 1.5.4) and Autodock vina tools (**AutoDock Vina v1.2.x (2021–present),** UCSF Chimera

Methods:

Collection and preparation of plant materials:

The berries of *Hippophae rhamnoides .L.*,(Sea buckthorn) were procured from Winzera Pvt. Ltd.,unit No. C-801, Tower C, Unit No. 801, Plot No. 8, Block-B, Sec-62, KLJ Noida One, Noida-201301, India. The plant material (voucher specimen) was authenticated by DR.R. MURUGAN, Ph.D., Botanist in Centre for Research and postgraduate studies in Botany, Ayya Nadar Janaki Ammal College, Sivakasi. The berries were thoroughly washed with water and air dried in shadow. The shade-dried berries were then coarsely powdered and used for further studies.

Preparation of ethanolic extract of *Hippophae rhamnoides*.L. berry :

The ethanolic extract of *Hippophae rhamnoides* L. berries was prepared using the cold maceration method. Coarsely powdered berries (50 g) were placed in a clean, dry glass container and ethanol was added in a 1:10 (w/v) ratio to ensure complete immersion. The container was sealed and left to macerate at room temperature (25 ± 2°C) for 72 hours with occasional shaking. After maceration, the extract was filtered using Whatman No. 1 filter paper, and the filtrate underwent solvent removal through distillation. The filtrate was transferred to a distillation flask and heated at 40–50°C using a heating mantle, allowing ethanol to evaporate gradually and be collected separately for potential reuse. The process continued until a thick, semi-solid extract was obtained, which was then stored in a clean glass container at 4°C for further studies. The percentage yield of the extract was also calculated (w/w) [14].

Preliminary phytochemical screening [15-16]:

I. Alkaloids

1. **Wagner's Test**: The test extract was mixed with Wagner's reagent (Iodide-KI solution), resulting in a reddish-brown precipitate, indicating the presence of alkaloids.
2. **Mayer's Test**: The test extract was treated with Mayer's reagent (potassium mercuric iodide solution), forming a cream-colored precipitate, confirming the presence of alkaloids.
3. **Hager's Test**: The test extract was mixed with Hager's reagent (saturated picric acid solution), producing a yellow precipitate, indicating alkaloids.
4. **Dragendorff's Test**: The test extract was treated with Dragendorff's reagent (potassium bismuth iodide solution), resulting in an orange-brown precipitate, confirming the presence of alkaloids.

II. Glycosides

5. **Borntrager's Test**: The test sample was mixed with dilute sulfuric acid, boiled, filtered and the filtrate was shaken with an organic solvent. The lower layer was separated and treated with dilute ammonia, which turned pink or red, indicating the presence of anthraquinone glycosides.
6. **Legal's Test**: The aqueous or alcoholic extract was treated with pyridine and sodium nitro-prusside, producing a pink to red color, confirming the presence of cardiac glycosides.
7. **Baljet's Test**: The test solution was treated with sodium picrate or picric acid, turning yellow to orange, confirming the presence of cardiac glycosides.
8. **Keller-Killiani Test**: The test extract in chloroform was treated with glacial acetic acid containing $FeCl_3$ and concentrated H_2SO_4. A reddish-brown color appeared at the junction of two layers, with a bluish-green upper layer, indicating cardiac glycosides.

III. Saponins

9. **Foam Test**: The test extract was vigorously shaken with water, forming persistent foam, indicating the presence of saponins.
10. **Hemolytic Test**: The test extract was mixed with a drop of blood on a glass slide, where a hemolytic zone appeared,

confirming the presence of saponins.

IV. Tannins & Phenolic Acids

11. **Ferric Chloride Test**: The test extract was treated with ferric chloride solution, resulting in a blue or green color, indicating the presence of tannins and phenols.
12. **Bromine Water Test**: The ethanolic extract was mixed with bromine water, leading to decolorisation, confirming tannins and phenols.
13. **Lead Acetate Test**: The ethanolic extract was treated with lead acetate solution, forming a white precipitate, indicating tannins and phenols.

V. Carbohydrates

14. **Molisch's Test**: The test extract was mixed with Molisch's reagent (alpha-naphthol in alcohol) and concentrated sulfuric acid. A purple to violet ring formed at the junction of two layers, indicating carbohydrates.
15. **Fehling's Test**: The test extract was treated with dilute HCl, heated, and then mixed with Fehling's A & B reagents and boiled. A brick-red precipitate appeared, confirming reducing sugars.
16. **Benedict's Test**: The test extract was mixed with Benedict's reagent and boiled, leading to the appearance of green, yellow, or red color, indicating reducing sugars.
17. **Barfoed's Test**: The test extract was treated with Barfoed's reagent, boiled and cooled. A red precipitate appeared, confirming the presence of monosaccharides.

VI. Flavonoids

18. **Sulfuric Acid Test**: The test extract was treated with 66% or 80% sulfuric acid, turning orange to red, confirming flavonols and flavones.
19. **Shinoda Test**: The test extract was treated with 95% ethanol, concentrated HCl, and magnesium turnings, leading to orange, pink, red, or purple coloration, indicating flavonoids.
20. **Test with Zinc and HCl**: The test solution was treated with zinc and HCl and heated, producing a pink to red color, confirming flavonoids.
21. **Test with Sodium Hydroxide**: The test residue was mixed with NaOH, turning yellow, and the color disappeared upon acid addition, indicating flavonoids.
22. **Test with Lead Acetate**: The test residue was treated with lead acetate, forming a yellow precipitate, confirming flavonoids.

VII. Proteins & Amino Acids

23. **Biuret Test**: The test solution was mixed with Biuret reagent, turning violet or pink, indicating proteins.
24. **Xanthoproteic Test**: The test solution was treated with concentrated HNO_3, boiled, cooled, and then treated with NaOH. A yellow precipitate appeared, turning orange after alkali addition, confirming proteins.
25. **Millon's Test**: The alcoholic extract was treated with Millon's reagent, forming a white precipitate that turned red upon heating, indicating amino acids.

VIII. Phytosterols & Triterpenoids

26. **Salkowski Test**: The test extract was mixed with chloroform and concentrated sulfuric acid and shaken well, resulting in a red-colored chloroform layer, confirming steroids and triterpenoids.
27. **Libermann-Burchard's Test**: The test extract was mixed with chloroform and acetic anhydride, boiled, cooled and concentrated sulfuric acid was added. A brown ring at the junction with an upper green layer was observed, indicating steroids and triterpenoids.

IX. Phenolic Compounds

28. **Libermann's Test**: The test extract was treated with acetic anhydride, heated, cooled and concentrated sulfuric acid was added, producing a blue color, indicating phenolic compounds.

X. Fixed Oils & Fats

29. **Spot Test**: The extract was pressed between two filter papers, leaving a permanent stain, confirming the presence of fixed oils.
30. **Alkali Test**: The extract was treated with 0.5N alcoholic KOH and phenolphthalein, then heated on a water bath.

Soap formation or partial neutralization of alkali was observed, confirming the presence of fixed oils.

XI. Volatile Oils

31. **Filter Paper Test**: The distillate obtained from the test extract was placed on filter paper, which did not leave a permanent stain, confirming volatile oils.
32. **Solubility in Alcohol**: The distillate obtained from the test extract was mixed with 90% alcohol, showing solubility, indicating the presence of volatile oils.

XII. Carotenoids

33. **Carr-Price Test**: The test extract was dissolved in chloroform, and a few drops of $SbCl_3$ solution were added, producing a blue color, confirming carotenoids.
34. **Sulfuric Acid Test**: The test extract was treated with concentrated H_2SO_4, forming layered colors: a blue-green top layer, orange middle layer, and yellow bottom layer, indicating the presence of carotenoids.

UV-Visible spectral analysis of phytocompounds [17]:

The test extract was examined under Visible and UV light for phytochemical analysis. For UV-Visible spectrophotometer analysis, the test extract was centrifuged at 3000 rpm for 10 min and filtered through Whatmann No. 1 filter paper. The filtrate was diluted to 1:10 ratio with the same solvent. The resultant extract was scanned at wave length ranging from 200 to 800 nm using Shimadzu UV-Visible spectrophotometer and the characteristic peaks were detected. The peak values of the UV-Visible spectrum were recorded.

Insilico molecular docking study for anti-inflammatory activity [18-19]:

Docking method: In the docking study of lead compounds, the following steps were taken:

Step 1: Ligand preparation

The chemical structure of the ligand (Bioactive compounds with potent anti-inflammatory activity can be determined from the literature survey) was obtained from open chemistry database Pubchem. The chemical composition of the compounds were downloaded in the Standard Data Format (SDF) which is converted to Protein Data Bank (PDB) format by using Molegro molecular viewer.

Step 2: Protein preparation

The target protein for inflammation such as **Cyclooxygenase 1 (PDB ID: 6Y3C) and Cyclooxygenase 2 (PDB ID: 1CX2)** were downloaded from Protein Data Bank. The protein is downloaded in their crystal structure and after protein processing, it was converted to the input format.

Step 3: Protein processing

Protein structure processing / refinement of protein includes removal of unwanted chains, hydrogen, water molecules, heteroatoms and bound ligands (if any).

Step 4: Docking analysis

Docking analysis for chosen target ligand against COX 1 & 2 was done by Auto dock and Auto dock vina software. For this installed Auto dock tools (Python 2.7.1, MGL tools 1.5.4) and Autodock vina tools (**AutoDock Vina v1.2.x (2021–present))**.

Step 5: Molecular visualization

UCSF Chimera is the software used for the protein preparation and molecular visualization. It produces high quality 3D images of protein.

In-vitro anti-inflammatory activity assay:

Egg albumin denaturation assay [20-22]:

a. **Egg Albumin separation process for protein denaturation assay:**

Egg white was first separated from the yolk. 25 mL of egg white was measured and diluted with distilled water to obtain a 100 mL solution. It was mixed and stirred vigorously until notable quantities of whitish substance get decreased. Then, the prepared egg white solution was centrifuged at 4000 rpm for 20 minutes. The precipitated globulin was removed and the resulting egg albumin solution was used for the albumin denature assay.

b. **Preparation of positive control / standard (Diclofenac Sodium)**:

The standard positive control, having 1000 µg/mL (w/v) strength, was prepared by dissolving 0.25 g of Diclofenac sodium in 50 mL of distilled water. From this stock solution, 4 mL was further diluted with 20 mL of distilled water to achieve a concentration of 1000 µg/ml. This dilution process was extended to create a series of test solution concentrations at 10 µg/ml,

20µg/ml, 30 µg/ml, 40 µg/ml and 50 µg/ml.

c. **Preparation of the negative control / blank:**

Negative control was prepared by combining 2.8 ml Phosphate Buffer Saline (PBS) (6.4 pH), 0.2ml of egg albumin solution and 2 ml of distilled water.

d. **Preparation of the serial dilutions:**

Stock solution with 1000 µg/mL (w/v) of strength was prepared by dissolving 0.25 g of plant extract (ethanolic extract of *Hippophae rhamnoides* .L., berry) in 50 mL of distilled water. From these stock solution, 4 mL was further diluted with 20 mL of distilled water to achieve a concentration of 1000 µg/mL. This dilution process was extended to create a series of test solution concentration at 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL and 50 µg/mL.

e. **Egg albumin denaturation assay procedure:**

The reaction mixtures were prepared using centrifuged egg albumin fraction (0.2mL), PBS of 6.4 pH (2.8mL) and 2mL of each different concentration of ethanolic extract of *Hippophae rhamnoides*.L., berry, and Diclofenac sodium separately. Then, the mixtures were incubated in a water bath at 37°C ± 2°C for 20 minutes. The temperature increased to 70°C, and the reaction mixtures were maintained for 5 minutes. Then the mixtures were cooled to room temperature, and absorption was measured by using UV spectrometer at 288 nm. The results were made in triplicate. Relevant intensities for the maximum wavelength were obtained to calculate the percentage inhibition.

The percentage inhibition of protein denaturation was calculated relative to the control by using the following formula

Percentage inhibition = (1-[Vt/Vc]) × 100

Where,

Vt = Absorbance of the test sample

Vc = Absorbance of control

HRBC Membrane stabilization assay [23-25]:

a. Preparation of human red blood cells (HRBC) suspension:

Fresh whole human blood was collected and mixed with equal volume of sterilized Alsever solution (2 % dextrose, 0.8 % sodium citrate, 0.05% citric acid and 0.42 % sodium chloride in water). The blood was centrifuged at 3000 rpm for 10 min and packed cells were washed three times with isosaline (0.85%, pH 7.2). The volume of the blood was measured and reconstituted as 10% v/v suspension with isosaline.

b. Hyposaline induced hemolysis:

The principle involved here is stabilization of human red blood cell membrane by hypotonicity induced membrane lysis. The assay mixture composes 1ml phosphate buffer [pH 7.4, 0.15 M], 2 ml hypo saline [0.36 %], 0.5 ml HRBC suspension [10 % v/v] with various concentrations of test extract and standard drug Diclofenac sodium of various concentrations (10, 20, 30, 40& 50µg/ml) and control (distilled water in place of hyposaline to produce 100 % hemolysis) were incubated at 37ºC for 30 min and then centrifuged. The hemolysis of RBC in the suspension was estimated by using UV spectrophotometer at 560 nm.

The percentage of hemolysis of HRBC membrane can be calculated as follows:

% Hemolysis = (Absorbance of Test Sample / Absorbance of Control) x 100

The percentage of HRBC membrane stabilization can be calculated as follows:

% Protection = 100 - [(Absorbance by Test sample / Absorbance by Control) x 100]

In-vitro cyclooxygenase-2 enzyme inhibition assay [26-27]:

In vitro COX-2 inhibition potency was estimated by Enzyme Immunoassay (EIA) method. EIA kit was used to examine the ethanolic fruit extract of *Hippophae rhamnoides* .L., anti-inflammatory activity towards COX-2 inhibition. The test extract was dissolved in 1 ml of DMSO (99%) in different amounts (50, 100 and 200 µg/ml), to estimate the inhibition activity according to the manufacturer's protocol. Celecoxib in different concentrations(50, 100 and 200 µg/ml) was used as a positive control ;10 µl of plant test extract and celecoxib were added to the reagents from EIA kit, 960 µl reaction buffer solution, 10 µl COX-2 enzyme and 10 µl heme. Then the resultant solution was incubated for 10 min at 36.5ºC, after that 10 µl of Arachidonic Acid (AA) was added immediately, afterwards 50 µl of 1 M HCl was added to finish the COX-2 reaction. Stannous chloride (100µl) was added to convert prostaglandin H2 (PG-H2) to prostaglandin F2α (PG-F2α) via reduction reaction, the COX-2 enzyme catalyzed reaction of arachidonic acid to produce PG-H2. When the solutions color became yellow and the concentration of the PG-F2α was estimated spectrophotometrically using UV-Visible spectrophotometer at

418 nm. The percentage inhibition of the enzyme was calculated by comparison of extract measurement with control assessment.

$$\textbf{Percentage of inhibition} = \frac{(\textbf{absorbance of control} - \textbf{absorbance of test})\ \ \textbf{X 100}}{\textbf{absorbance of control}}$$

Statistical analysis

The results are represented as Mean ± Standard Error (SE), the samples were done with three replicates (n=3). Statistical analysis was carried out by Analysis of Variance for one way (ANOVA), $p<0.05$ was considered statistically significant.

In-vivo anti-inflammatory activity assay: (Carrageenan-Induced paw edema model) [28-30]

Ethanolic fruit extract of *Hippophae rhamnoides*.L., was evaluated for in-vivo anti-inflammatory activity by using Carrageenan-Induced paw edema model. Male albino rats (180–200 g) were used taking into account international principles and local regulations concerning the care and use of laboratory animals. [86] The animals had free access to a standard commercial pelleted diet and water *ad libitum* and were kept in rooms maintained at 22 ± 1ºC with a 12 hrs light/dark cycle. The in-vivo anti-inflammatory activity was performed at the department of pharmacology, Arulmigu kalasalingam college of pharmacy, Krishnankoil. The institutional animal ethical committee has approved the protocol of the study *viz.* AKCP/IAEC/09/24-25.

Carrageenan-induced edema in rats:

For screening in vivo anti-inflammatory activity of ethanolic fruit extract of *Hippophae rhamnoides*.L.,, 4 Groups of six animals each were used.

Group I: Treatment with Vehicle/Control (Distilled water); 10 ml/Kg

Group II: Treatment with Standard drug, Diclofenac Sodium (10 mg/Kg)

Group III: Treatment with ethanolic fruit extract of *Hippophae rhamnoides*.L.; 250 mg/Kg

Group IV: Treatment with ethanolic fruit extract of *Hippophae rhamnoides*.L., 500 mg/Kg

Paw edema was induced by sub-plantar injection of 0.1 ml 1% sterile carrageenan in saline into the right hind paw. Diclofenac Sodium (10 mg/kg) and the ethanolic extract of *Hippophae rhamnoides*.L., at doses of 250 and 500 mg/kg were administered orally 60 minutes before carrageenan injection. Control group received the vehicle only (10 ml/kg). The inflammation was quantified by measuring the volume displaced by the paw, using a digital plethysmometer at time 1, 2 and 4 hrs after carrageenan injection. The difference between the left and the right paw volumes (indicating the degree of inflammation) was determined and the percent inhibition of edema was calculated in comparison to the control group of animals.

Inhibition (%) = (Control – Treated / Control) x 100

3. RESULTS AND DISCUSSION

Percentage Yield (%W/W):

Table 1: The percentage yield of the ethanolic fruit extract of *Hippophae rhamnoides*.L.,

S.NO	Solvent	Colour	Consistency	Average value of extract (%W/W)
1.	ETHANOL	REDDISH BROWN	SEMI SOLID	16.74%W/W

Coarse powder sample taken for extraction = 50g

Amount of ethanolic extract obtained = 8.37g

$$\textbf{Percentage Yield} = \left(\frac{\text{Weight of extract obtained}\quad \text{X 100}}{\text{Initial weight of plant material}}\right)$$

= (8.37 / 50) X 100

Therefore,

Percentage Yield = 16.74 % W/W

Preliminary phytochemical screening :

Qualitative phytochemical analysis results revealed that the ethanolic extract of *Hippophae rhamnoides*.L., berry contain **flavonoids, phenolic acids, tannins, saponins, carbohydrates, proteins & amino acid, phytosterols & triterpenoids, fixed oils and carotenoids**. Summary of preliminary phytochemical screening of ethanolic extract of *Hippophae rhamnoides* .L.,berry was shown in table no. 2

Table 2: Preliminary phytochemical screening of ethanolic fruit extract of *Hippophae rhamnoides*.L.,

S.NO	Identification test	Inference
	A. Test for alkaloids	
1.	Wagner's test	–
2.	Mayer's test	–
3.	Hager's test	–
4.	Dragendorff's test	–
	B. Test for glycosides	
5.	Borntrager's test	–
6.	Legal's test	–
7.	Baljet's test	–
8.	Keller-Killiani test	–
	C. Test for saponin	
9.	Foam test	+
10.	Hemolytic test	+
	D. Test for tannins & phenolic acids	
11.	Ferric chloride test	+
12.	Bromine water test	+
13.	Lead acetate test	+
	E. Test for carbohydrates	
14.	Molisch's test	+
15.	Fehling's test	+
16.	Benedict's test	+
17.	Barfoed's test	+
	F. Test for flavonoids	
18.	Sulphuric acid test	+
19.	Shinoda test	+

20.	Test with zinc & HCl	+
21.	Test with sodium hydroxide	+
22.	Test with lead acetate	+
	G. Test for proteins & amino acids	
23.	Biuret test	+
24.	Xanthoproteic test	+
25.	Millon's test	+
	H. Test for phytosterols & triterpenoids	
26.	Salkowski test	+
27.	Libermann-Buchard's test	+
	I. Test for phenolic compounds	
28.	Libermann's test	+
	J. Test for fixed oils & fats	
27.	Spot test	+
28.	Soap formation test	+
	K. Test for volatile oils	
29.	Spot test	_
30.	Alcohol test	_
	L. Test for carotenoids	
31.	Carr-price test	+
32.	Sulfuric acid test	+

(" + " indicates presence; " - " indicates absence)

UV Visible spectral analysis of phytocompound:

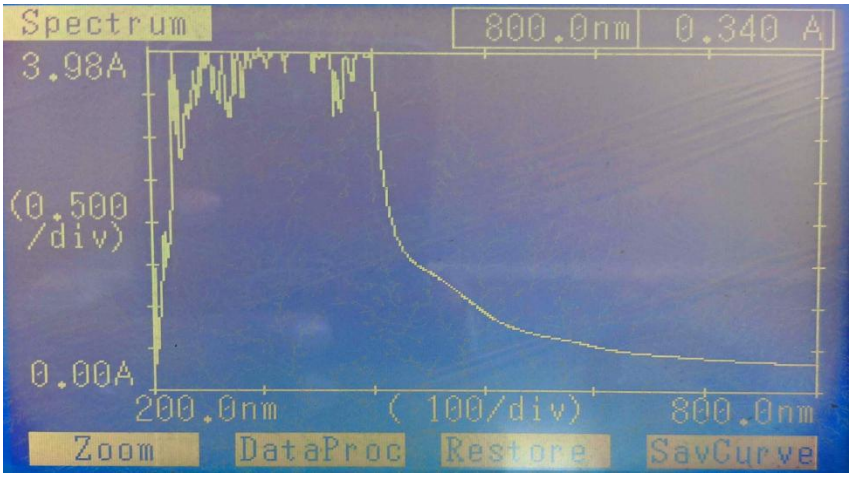

Figure 2: UV-Visible spectrum of ethanolic extract of *Hippophae rhamnoides*.L., berry

The UV-Visible fingerprint profile of the ethanolic extract of *Hippophae rhamnoides*.L., berry was observed at the wavelength of 200-800 nm due to sharpness of the peaks and proper baseline. The profile showed the compounds separated at the wavelength of 230, 245, 300,380, 400 and 450 nm with the absorption 3.98, 4.000,4.000,3.957. 4.000 and 1.786 respectively. These results showed that the ethanolic extract of *Hippophae rhamnoides*.L., berry contains flavonoids particularly quercetin, kaempferol & isorhamnetin (band I- 240-285 nm; band II- 300-400 nm)[31], phenolic acids particularly gallic acid, caffeic acid & ellagic acid (220-280 nm & 330-380 nm) [32] and carotenoids particularly β-sitosterol & lycopene (400-500 nm) [33].

Insilico molecular docking study for anti-inflammatory activity:

The **In-silico docking study** of the ethanolic fruit extract of *Hippophae rhamnoides.L.*, revealed that its **phytoconstituents (flavonoids-**quercetin, kaempferol and isorhamnetin**; phenolic acids –** gallic acid, caffeic acid and ellagic acid**) exhibit moderate binding affinity with COX-1 and excellent binding capacity with COX-2**, suggesting the strong anti-inflammatory potential with possible selectivity towards COX-2 inhibition. The moderate interaction with COX-1 indicates that these bioactive compounds may regulate prostaglandin synthesis without significantly affecting physiological functions like gastric mucosal protection, making them potentially safer than conventional NSAID like diclofenac, ibuprofen, indomethacin, etc. Their **strong binding to COX-2 than COX-1** was evidenced by hydrogen bonding and binding energy values are significantly lower (indicating stronger binding affinity). Comparisons with standard NSAIDs like **Diclofenac, and Celecoxib** showed that ethanolic fruit extract of *Hippophae rhamnoides.L.*, had competitive docking scores, indicating their potential as **natural alternatives to synthetic COX-2 inhibitors** with reduced risk of gastric complications.Fig 2 to 19 showed the docking image of phytocompounds with COX 1& COX 2 receptor respectively and table 3 & 4 showed the hydrogen bond interaction and binding affinity of various phytocompounds with COX 1& COX 2 receptors.

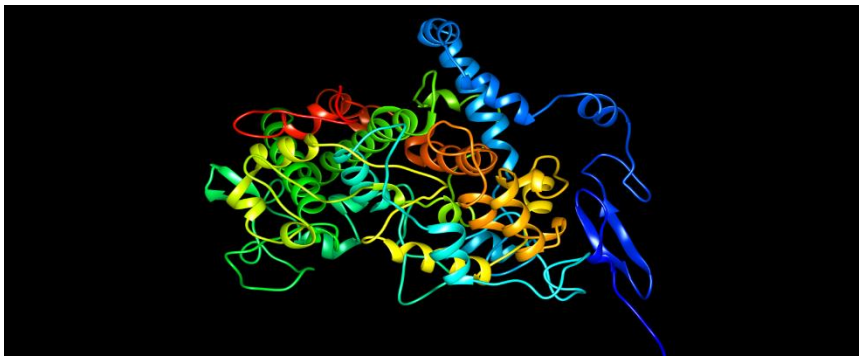

Figure 3: Cyclooxygenase (COX)-1 enzyme

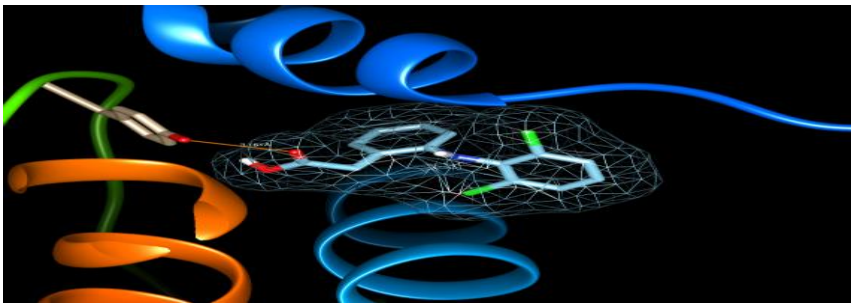

Figure 4: Docking image of diclofenac with COX-1

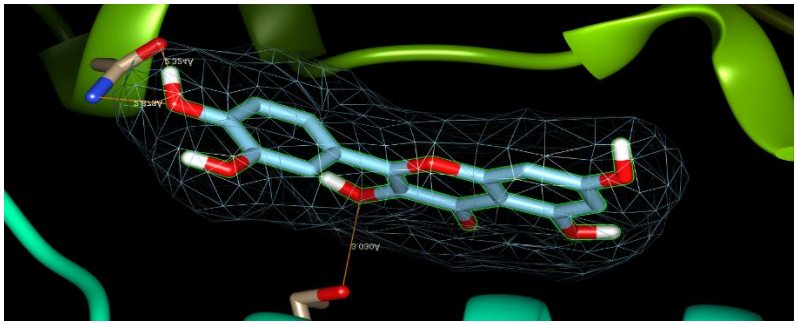

Figure 5: Docking image of quercetin with COX-1

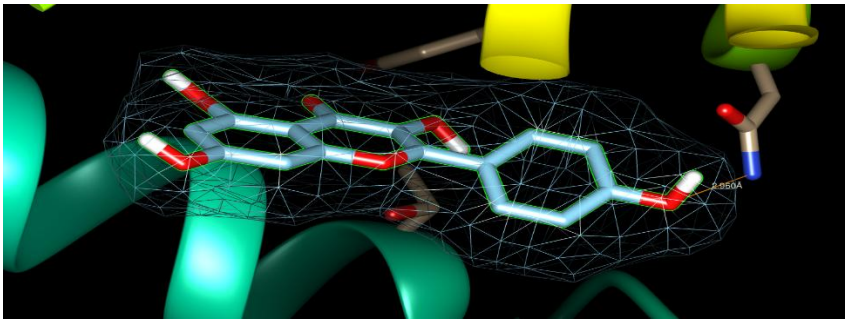

Figure 6: Docking image of kaempferol with COX-1

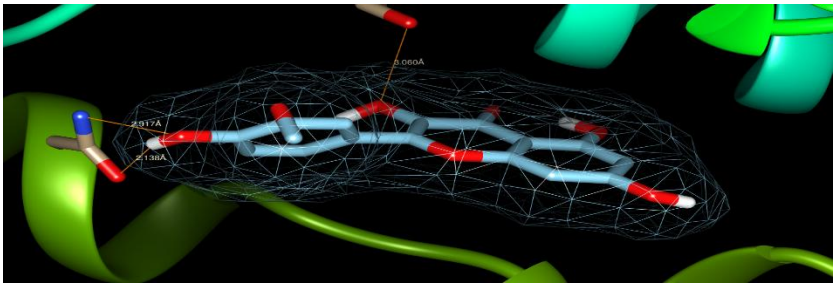

Figure 7: Docking image of isorhamnetin with COX-1

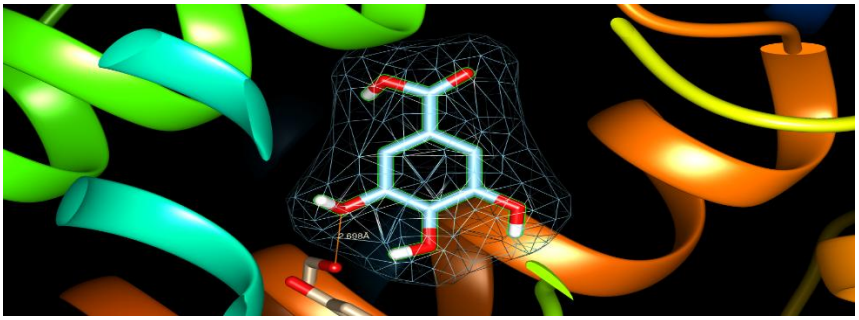

Figure 8: Docking image of gallic acid with COX-1

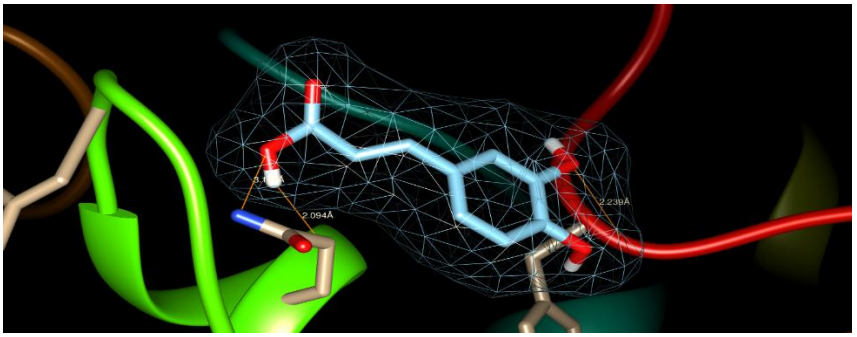

Figure 9: Docking image of caffeic acid with COX-1

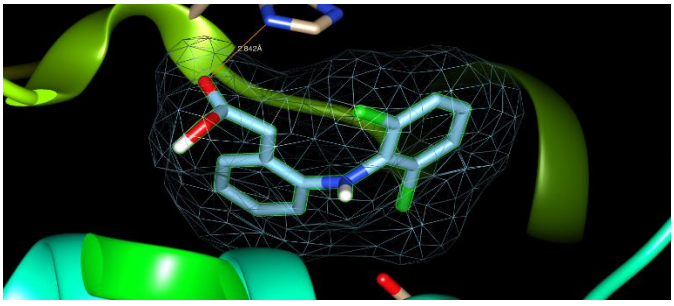

Figure 10: Docking image of ellagic acid with COX-1

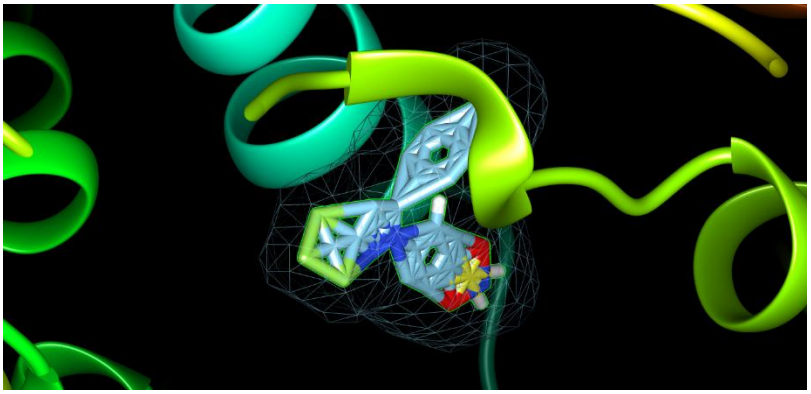

Figure 11: Docking image of celecoxib with COX-1

CLASS	PHYTOCOMPOUNDS	NUMBER OF HYDROGEN BOND INTERACTIONS	BINDING AFFINITY (Kcal/mol)
Standard	Celecoxib	1	-2.1
	Diclofenac	1	-8.2
Flavonoids	Quercetin	3	-4.2
	Kaempferol	2	-4.4
	Isorhamnetin	3	-4.2
Phenolic acids	Gallic acid	1	-4.2
	Caffeic acid	4	-5.2
	Ellagic acid	1	-3.5
Carotenoids	β-carotene	-	-
	Lycopene	-	-
	Ursolic acid	-	-

Triterpenoids	Oleonolic acid	-	-
Fatty acids and sterols	Palmitoleic acid	2	-0.2
	β-sitosterol	1	-2.0

Table 3: Hydrogen bond interaction and binding affinity of phytocompounds with COX-1

“ - ” indicates no docking

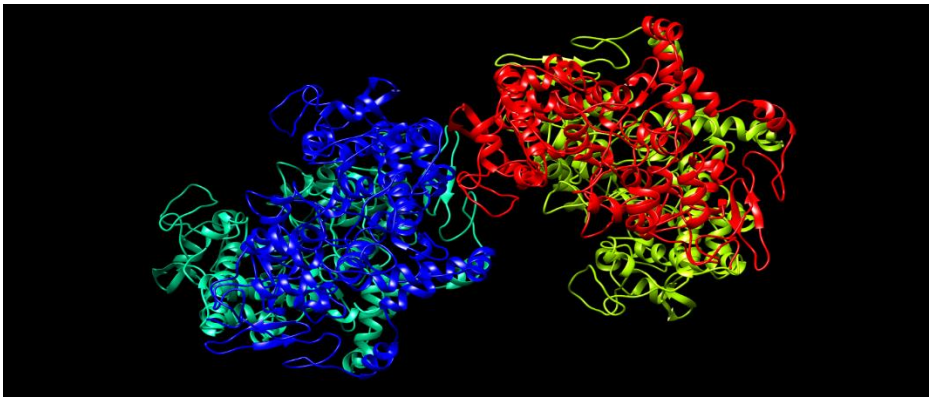

Figure 12: Cyclooxygenase (COX)-2 enzyme

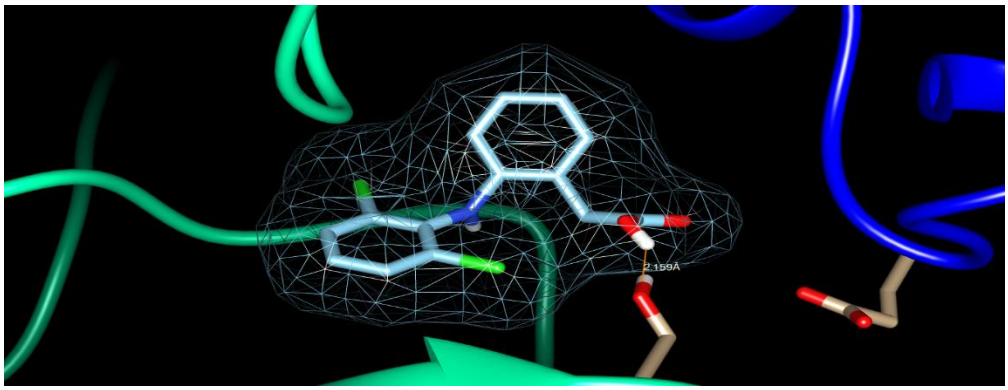

Figure 13: Docking image of diclofenac with COX-2

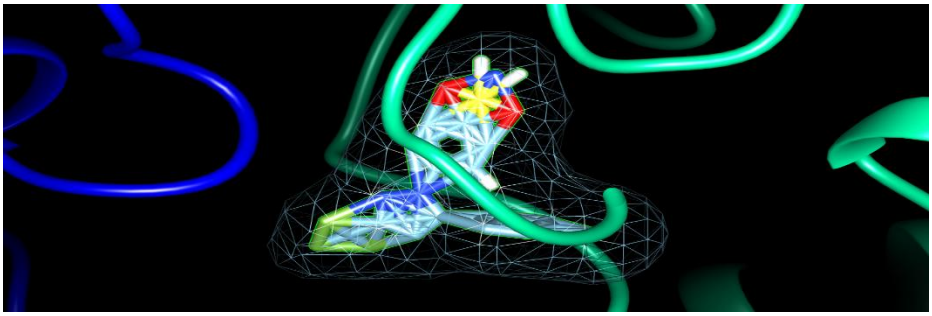

Figure 14: Docking image of celecoxib with COX-2

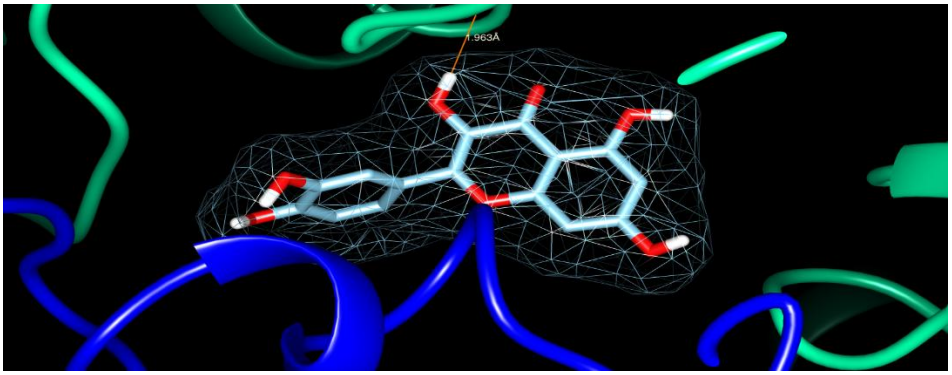

Figure 15: Docking image of quercetin with COX-2

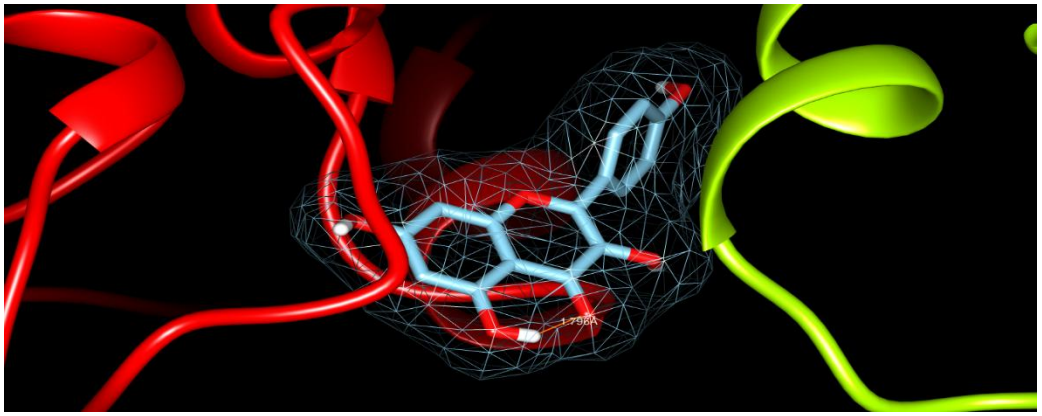

Figure 16: Docking image of kaempferol with COX-2

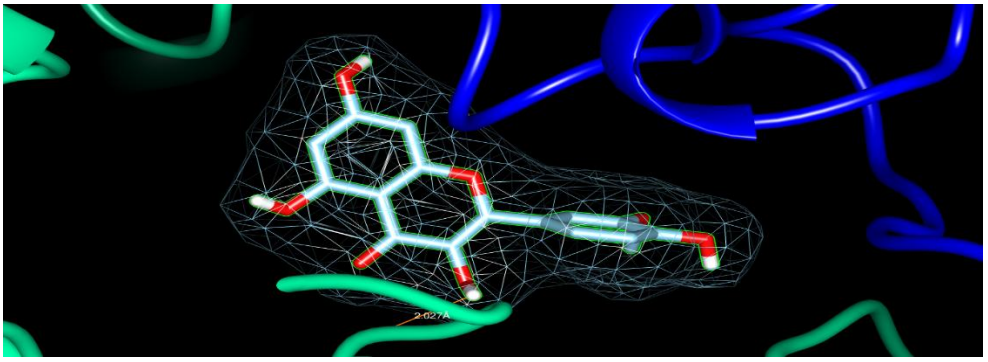

Figure 17: Docking image of isorhamnetin with COX-2

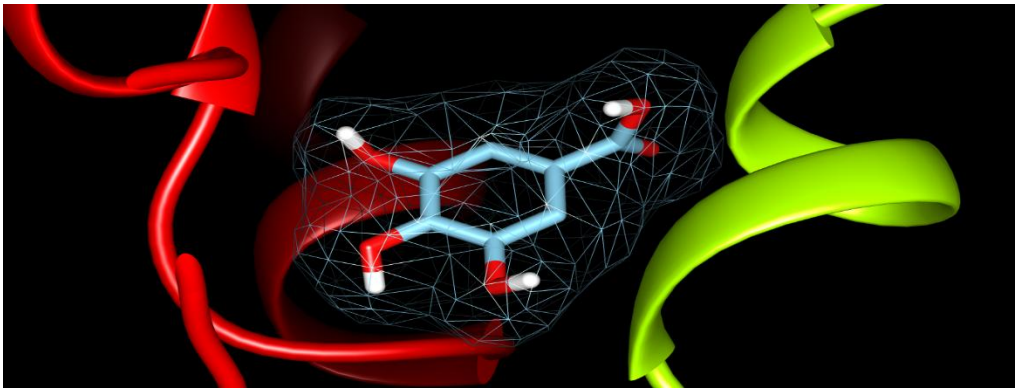

Figure 18: Docking image of gallic acid with COX-2

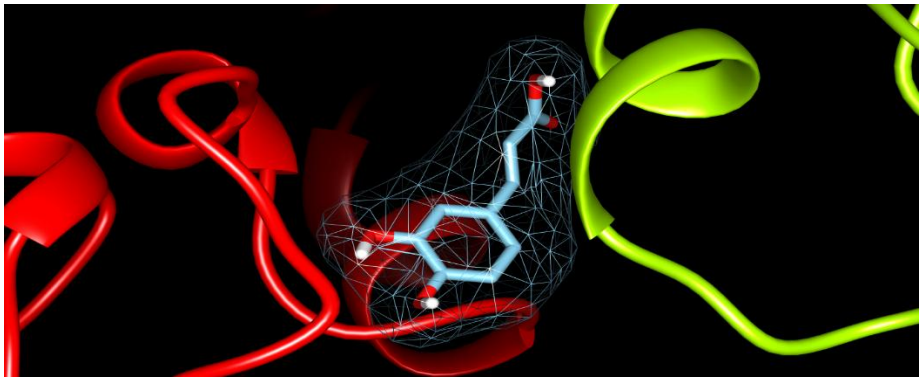

Figure 19: Docking image of caffeic acid with COX-2

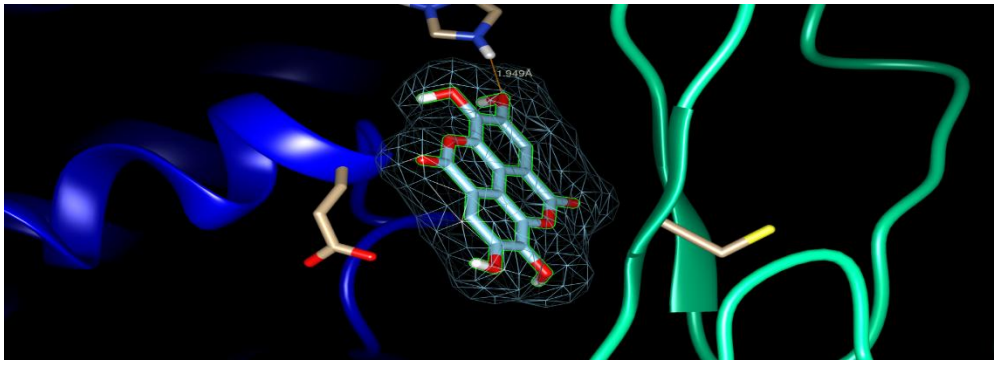

Figure 20: Docking image of ellagic acid with COX-2

Table 4: Hydrogen bond interaction and binding affinity of phytocompounds with COX-2

CLASS	PHYTOCOMPOUNDS	NUMBER OF HYDROGEN BOND INTERACTIONS	BINDING AFFINITY (Kcal/mol)
Standard	Celecoxib (selective cox-2 inhibitor)	3	-10.1
	Diclofenac	1	-6.3
Flavonoids	Quercetin	1	-8.1
	Kaempferol	2	-8.2
	Isorhamnetin	1	-7.7
Phenolic acids	Gallic acid	3	-6.4
	Caffeic acid	2	-6.5

	Ellagic acid	1	-8.6
Carotenoids	β-carotene	-	-
	Lycopene	-	-
Triterpenoids	Ursolic acid	-	-
	Oleonolic acid	-	-
Fatty acids and sterols	Palmitoleic acid	-	-
	β-sitosterol	-	-

“ - ” indicates no docking

In-vitro anti-inflammatory activity assay:

Egg albumin denaturation assay:

The inhibitory rate of denaturation of proteins for ethanolic fruit extract of *Hippophae rhamnoides*.L., increased progressively as concentration increased. In this study ethanolic fruit extract of *Hippophae rhamnoides*.L., showed maximum inhibition, 79% at 50 µg/ml. Standard anti-inflammatory drug diclofenac demonstrated its greatest degree of inhibition, 81% at 50 g/ml concentration. The percentage inhibition value at different concentration are given in table-5

Table-5: Effect of *Hippophae rhamnoides*.L., ethanolic fruit extract and diclofenac in egg albumin denaturation assay:

Table 5.1

CONCENTRATION (µg/ ml)	**ABSORBANCE**		**PERCENTAGE INHIBITION**
	CONTROL	**STANDARD**	
10 **µg/ ml**	0.34	0.1462	57%
20 **µg/ ml**		0.119	65%
30 **µg/ ml**		0.102	70%
40 **µg/ ml**		0.088	74%
50 **µg/ ml**		0.064	81%

Standard – Diclofenac

Table 5.2

CONCENTRATION (µg/ ml)	**ABSORBANCE**		**PERCENTAGE INHIBITION**	**INTERPRETATION**
	CONTROL	**SAMPLE**		
10 **µg/ ml**		0.153	55%	Moderate activity
20 **µg/ ml**		0.129	62%	Moderate activity

30 **µg/ ml**	0.34	0.108	68%	Moderate activity
40 **µg/ ml**		0.095	72%	Strong activity
50 **µg/ ml**		0.071	79%	Strong activity

Sample - ethanolic fruit extract of *Hippophae rhamnoides*.L.,

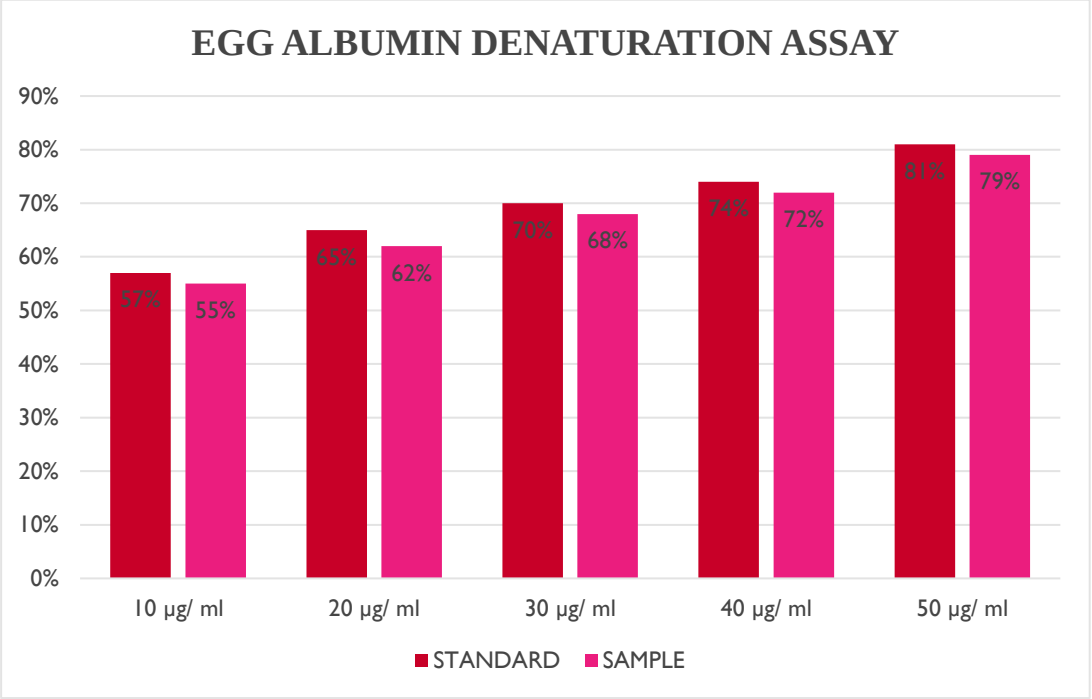

Figure 21- Effect of *Hippophae rhamnoides*.L., ethanolic fruit extract and diclofenac in egg albumin denaturation assay

HRBC Membrane stabilization assay:

The human red blood cell (HRBC) membrane stabilization rate of ethanolic fruit extract of *Hippophae rhamnoides*.L., increased progressively as concentration increased. In this study ethanolic fruit extract of *Hippophae rhamnoides*.L., showed maximum stabilization, 85% at 50 µg/ml. Standard anti-inflammatory drug diclofenac demonstrated its greatest degree of inhibition, 88% at 50 g/ml concentration. The percentage stabilization value at different concentration are given in table-6

Table 6

Effect of *Hippophae rhamnoides*.L., ethanolic fruit extract and diclofenac in HRBC membrane stabilization:

Table 6.1

CONCENTRATION (µg/ ml)	**ABSORBANCE**		**PERCENTAGE HEMOLYSIS**	**PERCENTAGE INHIBITION**
	CONTROL	**STANDARD**		
10 **µg/ ml**	0.68	0.290	43%	57%
20 **µg/ ml**		0.254	37%	63%
30 **µg/ ml**		0.211	31%	69%
40 **µg/ ml**		0.142	20%	80%
50 **µg/ ml**		0.081	12%	88%

Standard – diclofenac

Table 6.2

CONCENTRATION (µg/ ml)	ABSORBANCE		PERCENTAGE HEMOLYSIS	PERCENTAGE INHIBITION
	CONTROL	SAMPLE		
10 **µg/ ml**	0.68	0.308	45%	55%
20 **µg/ ml**		0.285	41%	59%
30 **µg/ ml**		0.244	36%	64%
40 **µg/ ml**		0.175	26%	74%
50 **µg/ ml**		0.114	17%	83%

Sample - ethanolic fruit extract of *Hippophae rhamnoides*.L.,

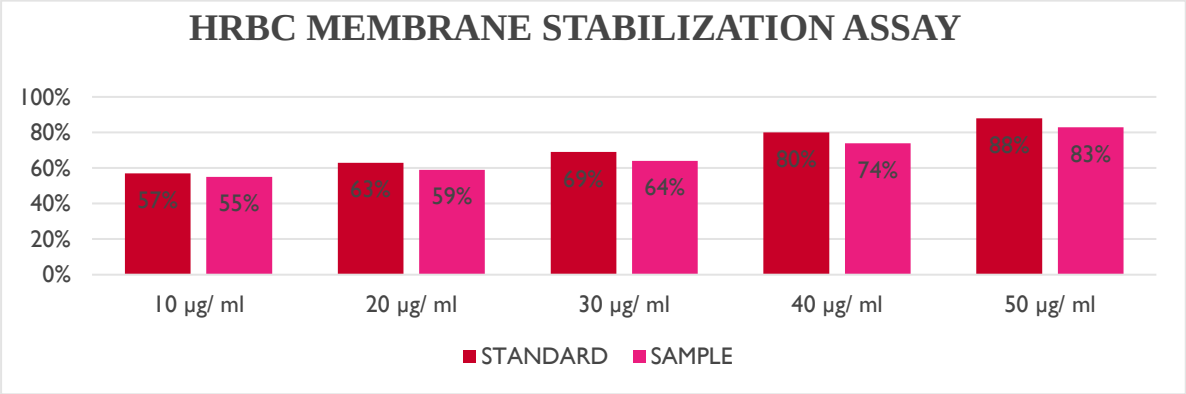

Figure 22 - Effect of *Hippophae rhamnoides*.L., ethanolic fruit extract and diclofenac in HRBC membrane stabilization assay.

In-vitro cyclooxygenase-2 enzyme inhibition assay:

The COX enzymes is crucial to the cyclooxygenase process of inflammation. COX-1 is required for a number of physiological processes. Therefore, there is a particular need for substances that selectively inhibit COX-2 without harming COX-1. From the results of in-silico molecular docking analysis, the phytocompounds in ethanolic fruit extract of *Hippophae rhamnoides.L.*, had strong anti-inflammatory potential with possible selectivity towards COX-2 inhibition. In the present investigation, ethanolic fruit extract of *Hippophae rhamnoides.L.*, were analyzed for cyclooxygenase 2 enzyme inhibitory activity using *in vitro* assay models. The results revealed that the extract showed inhibition of cyclooxygenase-2 enzyme compared with standard celecoxib and also the **IC_{50} - 40 µg/ ml** value of ethanolic fruit extract of *Hippophae rhamnoides.L.*, confirmed that the extract can act as a **potential anti-inflammatory** by selective inhibition of COX-2 enzyme.

Table 7 COX-2 inhibitory activity of ethanolic fruit extract of *Hippophae rhamnoides*.L.,

SAMPLE	CONCENTRATION **(µg/ ml)**	INHIBITION PERCENTAGE	IC_{50}
CELECOXIB	50 **µg/ ml**	55.2±3.2	16 **µg/ ml**
	100 **µg/ ml**	72.1±5.5	
	200 **µg/ ml**	89.8±4.3*	
ETHANOLIC FRUIT EXTRACT OF *Hippophae rhamnoides*.L.,	50 **µg/ ml**	49.3±5.7	40 **µg/ ml**
	100 **µg/ ml**	67.2±4.1	
	200 **µg/ ml**	83.5±5.6*	

Note: The values are mean ± S.E.M, * p<0.05.

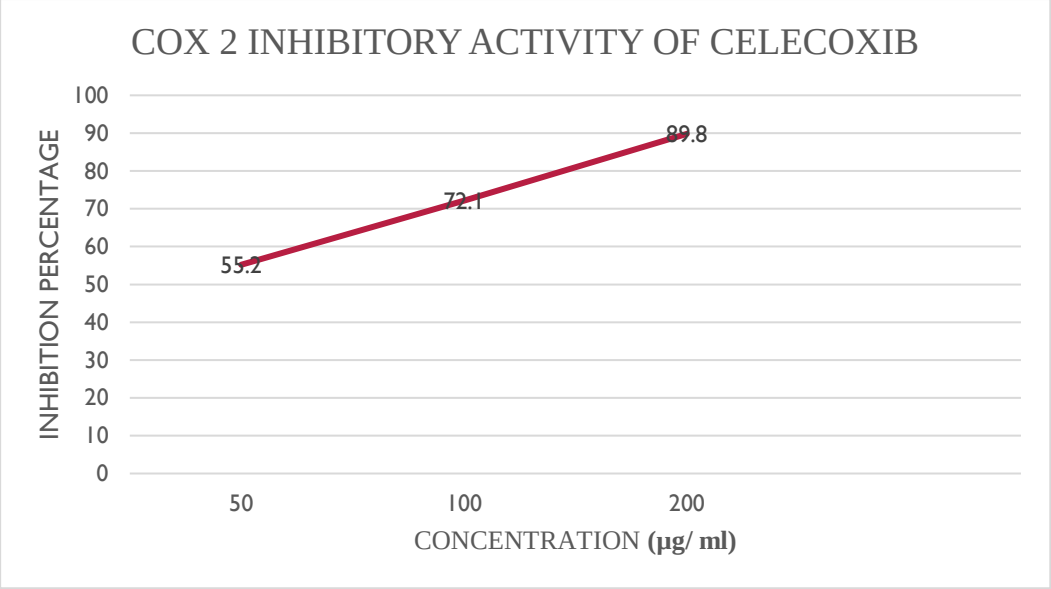

Figure 23: Cox-2 inhibitory activity of Celecoxib

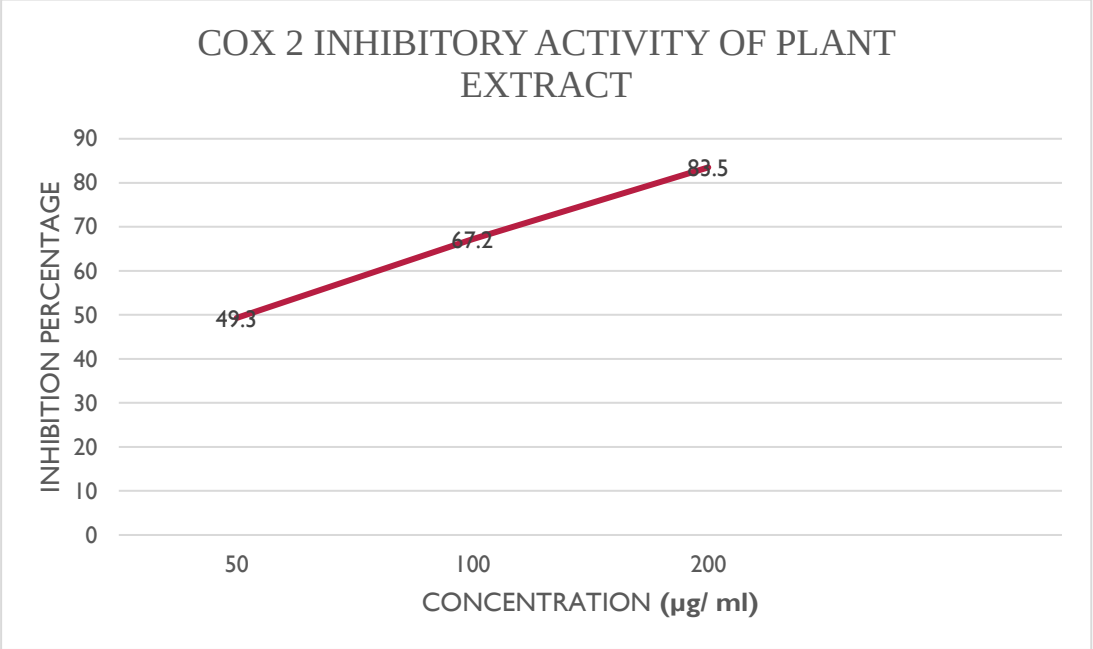

Figure 24: Cox-2 inhibitory activity of ethanolic fruit extract of *Hippophae rhamnoides.L.*,

Table 8 In-vivo anti-inflammatory activity assay: (Carrageenan-Induced paw edema model)

Groups	**Mean values of paw edema (ml) ± SEM**		
	1h	**2h**	**4h**
Control	0.29 ±0.06	0.30 ±0.06	0.33 ±0.06
Diclofenac sodium (10mg/kg orally)	0.18 ±0.08**	0.16 ±0.08***	0.14 ±0.08***
Ethanolic extract (250mg/kg)	0.20±0.07**	0.19 ±0.07*	0.17±0.07***
Ethanolic extract (500mg/kg)	0.19 ±0.04***	0.17 ±0.04**	0.15±0.04***

n=6, The percentage inhibition for each group was calculated by comparison with the control group. Values indicates mean ± S.E.M (One-way ANOVA followed by Dunnett's *t*-test). Significance variation against control at, *p<0.05, **p<0.01,***p<0.001

Table 9 Percentage inhibition of carrageenan induced paw edema

Groups	% inhibition			Average % inhibition
	1h	2h	4h	
Diclofenac sodium (10mg/kg orally)	37.93%	46.67%	57.57%	47.39%
Ethanolic extract (250mg/kg)	31.03%	36.67%	48.48%	38.72%
Ethanolic extract (500mg/kg)	34.48%	43.3%	54.54%	44.11%

The anti-inflammatory effects of the ethanolic fruit extract of *Hippophae rhamnoides.L.*,on carrageenan-induced edema in rat's hind paws were presented in Table 8 & 9 and Figure 24. The anti-inflammatory activity of test extract was found to have effect in dose-dependent manner. There was a gradual increase in edema paw volume of albino rats in the control group. However, in the test groups, ethanolic extract (250 & 500 mg/kg) showed a significant reduction in the edema paw volume. After 4 hours, Diclofenac sodium showed a paw edema volume of **0.14 ± 0.08 ml**, while the ethanolic extract at 250 mg/kg and 500 mg/kg showed volumes of **0.17 ± 0.07 ml** and **0.15 ± 0.04 ml**, respectively. The percentage inhibition of edema after 4 hours was 57.57% for Diclofenac, 48.48% for the 250 mg/kg extract, and 54.54% for the 500 mg/kg extract.

Figure 25: Carrageenan induced acute inflammatory paw edema in albino rats.

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