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Molecular docking, anti-inflammatory, antimicrobial and antioxidant evaluation of *Pterospermum rubiginosum* B. Heyne

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ABSTRACT

Pterospermum rubiginosum B.Heyne ex G.Don (PR) is a traditional medicinal plant used by the tribal people of the Western Ghats to treat bone fractures, inflammation, and sprain. Being an under-explored medicinal plant, the mechanism behind the anti-inflammatory activity of PR is unknown to the scientific community. FTIR analysis was done to recognize the functional groups in PR bark extract, and LCMS elucidated the phytochemical characterization. Molecular docking studies showed an excellent ligand and protein (inflammatory mediators) binding and their interactive mechanism. Total phenolic and total flavonoid contents were found to be 46.7 and 37.4 g/100 g for PRME. PRME showed hydrogen peroxide and DPPH (1,1diphenyl-2-picryl hydrazyl) radical scavenging activity with IC_{50} values of 48.33 and 42.70 µg/mL, respectively. Gene expression study of COX-1 and COX-2 enzymes confirmed the anti-inflammatory activity of PRME. The present study justifies the potential use of *PR* in the traditional medicine as an anti-inflammatory agent.

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1. Introduction

Traditional knowledge about the healing potential of tribal medicines and medicinal plant extracts has been communicated from one generation to another as an ancestral saying and usually as the outcome of careful and continuous trial-and-error experimentation over hundreds of years. Due to the lack of proper written documents, the most precious literature in every known ancient civilization is not currently available (Shrivastava et al., 2010). Plant extracts, as herbal formulations, along with standardized decoctions or purified products provide enormous possibilities for novel alternative therapeutic agents in the pharmaceutical industry. This is mainly due to diverse functional groups in phytochemicals with interactive adaptation in biological systems (Katiyar et al., 2012). The scientific world is now eagerly looking for alternate plant-derived products as novel anti-inflammatory agents with significant biological potency, availability, and affordability due to the severe side effects of generic and synthetic drugs (Wankhar et al., 2015).

Phytochemical quantification is a simple and inexpensive technique that helps to categorize different natural compounds constituting groups in the plant extracts. The biological evaluation of plant extracts could be optimized to enhance their therapeutic applications (Vaou et al., 2022). In search of novel bioactive molecules, natural product chemists have been working to purify and characterize the herbal formulations and decoctions used by indigenous populations worldwide for a couple of decades. However, the characterization and standardization of these formulations remain a time-consuming challenge for the scientists responsible for popularizing phytomedicines around the globe. As a result, drug discovery using natural products has remained a crucial checkpoint for pharmaceutical

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researchers (Atanasov et al., 2021). Many natural product-derived compounds such as curcumin, genistein, luteolin, and quercetin have been shown to serve as potent and safe therapeutic agents in treating chronic inflammatory diseases due to their efficacy and biocompatibility (Talib et al., 2020).

Inflammation could be defined as a complex defense mechanism in living beings coordinated through various intermediates, cytokines, and growth factors in a homeostatic manner without harmful effects. Any minute fluctuations in these pathways will release inflammatory mediators, associated enzymes, and cytokines into cellular space resulting in acute inflammatory disorders. If these mediator syntheses exceed the equilibrium without checkpoints, this may lead to several health issues and severe systemic function loss (Ansar and Ghosh, 2016). In addition, any compound that inhibits the inflammatory mediators without harming living beings can be considered a prospective candidate for developing potential anti-inflammatory agents. Therefore, it seems rational that these types of compounds will be subjected to computational analysis and target docking studies to understand the druggability, cellular permeability and affinity, bonding energy, as well as interactions between various inflammatory proteins and ligand molecules.

Pterospermum rubiginosum B. Heyne (PR), belonging to the Malvaceae family, is an indigenous tree with high therapeutic values found in the cleanest and pollution-free environments of the Western Ghats, India. The bark decoction of this herbal plant is commonly used by tribal practitioners for bone fracture healing, relieving pain and inflammation (Anish et al., 2021a). It is an evergreen tropical tree with a height of 25-28 m, a pink hard and wood having a bark thickness of 5-6 mm with outer exfoliating and characteristic reddish bark. This herbal plant species can be identified by its typical leaf color, the upper side of the leaves which is green, and the lower part that is pale reddish. The stem bark of the *P. rubiginosum* has been traditionally used for treating bone fracture and inflammation by tribals of Agasthyavanam region and in Wayanad settlements (Anish et al., 2021a; Nayar et al., 2006). Based on the traditional uses of the plant and the lack of enough valid scientific documentation, the present study was designed to assess its pharmacological effects.

This report deals with the phytochemical quantification of PR bark, including detailed FTIR analysis, molecular docking, antioxidant and antimicrobial screening as well as an *in vitro* assessment being conducted to determine cell viability after treatment with different concentrations of PR bark extract in a dose-dependent manner using RAW 264.7 cell lines along with anti-inflammatory assays and a gene expression evaluation.

2. Experimental

2.1. Plant material

After removing the exfoliated outer bark, the inner bark of PR was collected from the Kottur forest range in the Thiruvananthapuram district of Kerala with the help of tribal people during the December-January 2023. The geographical coordinates of the collection site were 77° 1979′ east longitude and 8° 8036′ north latitude at an altitude of 2500-3000 feet above sea level. The plant specimen was authenticated by Dr. G. Valsaladevi, Curator, Department of Botany, University of Kerala. The plant has been listed in the book "Flowering Plants of Kerala" written by Nayar et al. (2006). Plant voucher specimen KUBH 6189 has been deposited at the herbarium of the Department of Botany, University of Kerala, Thiruvananthapuram, India. The bark was cut into small pieces and shade dried for 20-30 days. The dried bark was powdered, and 1 kg of the powdered material was extracted with methanol at 66 °C using a Soxhlet apparatus. The total methanol bark extract (65 g) was then concentrated with the help of a rotary evaporator.

The crude methanol bark extract was partially purified and fractionated by sequential column chromatography using organic solvents, *e.g.*, *n*-hexane, ethyl acetate, acetone, and methanol based on their polarity, with silica powder as the stationary phase. 900-1000 g of silica was activated and loaded in the main column, and 40-45 g of the plant sample was subsequently added to the activated silica. The eluting solvent systems used were first *n*-hexane which its concentration was gradually increased to 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%. By maintaining the *n*-hexane as the base solvent, the concentration of ethyl acetate was gradually increased from 90% *n*-hexane-10% ethyl acetate to 100% ethyl acetate; acetone; from 90% *n*-hexane-10% acetone to 100% acetone and finally methanol, from 90% *n*-hexane-10% methanol to 100% methanol by 10% to 100% gradient. The organic solvent fractions were collected up to 250-500 mL and sub-fractions of 100-200 mL. Based on phytochemical screening results of all 40 fractions collected, all the 10 methanol sub-fractions were pooled together (10%-100%) as *P. rubiginosum* methanolic bark extract (PRME) and used for further analysis.

2.2. Phytochemical quantification

2.2.1. Total phenolic content

Total phenolic content (TPC) of PRME was determined using the Folin-Ciocalteau method with slight modifications (McDonald et al., 2001). Accordingly, the corresponding TPC values were calculated and then expressed as milligram gallic acid equivalent per gram PRME (mg GAE/g PRME).

2.2.2. Total flavonoid content

The total flavonoid content (TFC) of PRME was evaluated by the aluminium chloride colorimetric method described by Chang et al. (2002) with slight modifications. TFC was expressed as milligram quercetin equivalent per gram PRME (mg QCE/g PRME).

2.2.3. Total alkaloid content

The total alkaloid content (TAC) of PRME was studied using Dragendoff's reagent by spectrophotometric

method reported by Sreevidya and Mehrotra (2003). TAC was expressed as milligram caffeine equivalent per gram PRME (mg CAE/g PRME).

2.2.3. Antioxidant assays

2.2.3.1. DPPH (1,1-diphenyl-2-picryl hydrazyl) assay

The DPPH [TCI Chemicals (India) Pvt. Ltd] assay was performed calorimetrically by the method described by Chang et al. (2001).

2.2.3.2. Hydrogen peroxide scavenging assay

The hydrogen peroxide scavenging activity of PRME was determined following the method of Ruch et al. (1989).

2.4. Antibacterial and antifungal screening

Antibacterial activity of PRME was tested against the following microorganisms: *Escherichia coli* (ATCC-25922), *Staphylococcus aureus* (ATCC-25923), *Klebsiella pneumoniae* (ATCC-13883), *Pseudomonas aeruginosa* (ATCC-27853), and *Streptococcus mutans* (ATCC-25175), initially procured from HiMedia, Maharashtra, India and re-cultured by the agar well diffusion method in our lab (Yagoub, 2008). After performing morphological screening and biochemical tests to identify the selected microorganisms, they were maintained in nutrient agar and stored at 4 °C for further studies. All the tested bacterial strains were grown in Mueller Hinton broth for 18-24 hrs, and the bacterial suspension was further checked to a turbidity equivalent of 0.5 McFarland solutions (1-2×10^8 CFU/mL) with the addition of sterile saline. A well cutter was used to bore 10 mm wells. Samples containing 25, 50, and 100 µg/mL concentrations were added to the wells and kept at 37 °C for 24 hrs. The antibacterial activity was assayed by measuring the diameter of the inhibition zone and compared with streptomycin as a standard drug.

Antifungal screening of PRME was also performed using the agar well diffusion method. In this sense, the potato dextrose agar plates were point inoculated with fungal cultures of one week growth; *Aspergillus niger* and *Candida albicans* recultured in our lab were also used. Using a well cutter comb, approximately 10 mm width wells were cut and bark extracts with different concentrations of 25, 50, and 100 µg/mL were added and incubated at 37 °C for 72 hrs. The zone of inhibition was measured in mm and compared with the standard clotrimazole as an antimycotic agent (Bhalodia and Shukla, 2011).

2.5. Fourier-transform infrared spectroscopy (FT-IR)

An FTIR instrument (Perkin Elmer LS-55- Luminescence spectrometer, USA) was used to analyze the diversity of functional groups present in PRME. To obtain the relevant FTIR spectra using the KBr pellet method, 0.1 mg of the crude extract powder and 100 mg of KBr were ground together and the resulting mixture was made to a pellet which was then pressed through a hydraulic press to produce discs which were used to get FTIR spectra in the mid-IR range of 4000-400 cm^{-1}. FTIR fingerprint spectrum of PRME was compared with a reference IR spectrum (Pednekar and Raman, 2013).

2.6. Liquid chromatography mass spectrometry (LCMS)

LCMS (Shimadzu triple quadrapole LCMS/MS-8045) equipped with a heated ESI probe was used for investigating the detailed mass spectrum of phytoconstituents present in the PRME. The mobile phase comprised 0.1% formic acid in water (A) and methanol (B) at a flow rate of 0.4 mL/min. A linear gradient protocol was employed using a flow rate of 0.4 mL/min. The initial combination of the mobile phase was 5% B. The volume percentage of the solvent B was increased to 90% over 1-2 min and maintained at this concentration for up to 4 min, followed by a 4 min re-equilibration. Electrospray ionization was conducted in the positive mode, the nebulizer and sheath gas flows were adjusted at 10 and 10 L/min, respectively. The gas temperature was 350 °C, and the voltage was adjusted to 3.5 kV. The chromatographic separation of the plant compounds was carried out on the column1.9 microlitre C18 (150 × 2.1 mm i.d., particle size 2.2 µm, Shimadzu, Japan). The LCMS-8045 was able to provide a scan speed of (30,000 u/sec) and a polarity switching speed (5 msec), which can attain ultra-high-speed and high-sensitivity analysis. 0.5 g of PRME was diluted with methanol and filtered using a 0.22 µm nylon filter. The column temperature was set at 40 °C, and 5 µL of the sample was injected into the analytical column. The LCMS unit is directly connected with Software Lab solutions version 5.91 for analysis. The detected peaks and the corresponding compounds were identified using the available database for organic compounds and by a detailed literature survey (Dogan et al., 2022; Vignesh et al., 2022).

2.7. Molecular docking study

The molecular interaction study between the targets and ligands was performed using Biovia Discovery Studio v.20. Primarily, the binding sites of the proteins were predicted using the 'define and edit binding site' option in the software based on the PDB site records. In the molecular docking study regarding LibDock protocol, a reliable docking algorithm was used to detect ligand conformations in the protein active site based on polar interaction sites (hotspots). CHARMM was the force field applied that uses positional relationships between atoms to determine the energy and forces acting on each system particle. The binding pose associated with the lowest docked energy and better LibDock score was selected for post-docking analysis.

The three-dimensional structure of ovine cyclooxygenase-1 complexed with Ibuprofen and cyclooxygenase-2 (prostaglandin synthase-2) complexed with a selective inhibitor, SC-558 (1-phenylsulfonamide-3-trifluoromethyl-5-*para* bromophenylpyrazole) was downloaded from PDB database with ID: 1EQG and 1CX2 and having a crystallographic resolution of 2.61

A°and 3.00 A°, respectively. The protein 1EQG consists of two polypeptide chains, A and B, with 1102 amino acids and has a molecular weight of 126827 Daltons. The protein (1CX2) contains four polypeptide chains, A, B, C, and D, with 2208 amino acids, having a molecular weight of 253619 Daltons. The active site of the protein interacting with the standardized ligand molecules was selected as the binding site, 8 poses in the case of 1EQG and 17 poses (1CX2) of the selected ligands in the docked complexes were generated for docking studies (Sanner, 1999).

2.8. Cell culture and viability

In vitro cytotoxicity of PRME was evaluated by MTT assay (Bahuguna et al., 2017). RAW 264.7 cells were obtained from the National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecco's modified Eagle's medium, DMEM (obtained from Sigma-Aldrich, USA). The cell lines were cultured in a 25 cm^2 tissue culture flask with DMEM supplemented with FBS (10%), L-glutamine, sodium bicarbonate (Merck, Germany) and an antibiotic solution containing penicillin (100 µg/mL), streptomycin (100 µg/mL) and amphotericin B (2.5 µg/mL). Cultured cell lines were kept at 37 °C in a humidified CO_2 (5%) incubator (NBS Eppendorf, Germany). For the determination of the cellular viability, the RAW 264.7 cells with confluent growth at a density ($1x10^5$ cells/well) were added to 48-well plates and kept overnight. After incubation, the cells were stimulated with LPS administration, treated with PRME at different concentrations (12.5, 25, 50, 100 µg/mL) and the viability was determined by MTT assay through microscopic evaluation of cells using a Carl Zeiss microscope.

2.8.1. Intracellular ROS generation by fluorescent microscopy

The RAW 264.7 cells with confluent growth at a density of $1 x 10^5$ cells/well were added to 48 well plates and kept overnight to adhere to the plates. The cells were then washed with PBS buffer treated with 100 µM DCFH-DA (diluted in DMEM+1% FBS) for 1 h (37 °C, 5% CO_2) (Sekhar et al., 2015). 2',7'-Dichlorodihydrofluorescein diacetate (H_2DCFDA) is a chemically reduced form of fluorescein used as an indicator for reactive oxygen species (ROS) in cells, which is a fluorogenic dye that measures hydroxyl, peroxyl, and other ROS activity within the cell. After diffusion in the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound which is later oxidized by ROS into 2',7'-dichlorofluorescin (DCF) emitting green fluorescence that is subsequently monitored.

2.8.2. Anti-inflammatory assays

For anti-inflammatory analysis, RAW 264.7 cells were treated with LPS at a concentration of 1 µg/mL to activate the rat macrophages, and then sample PRME was added at different concentrations (12.5, 25 and 50 µg/mL) along with the standard anti-inflammatory drug (diclofenac sodium) and incubated for 24 hrs. After incubation, the cell lysate was used for inflammatory enzymatic studies. The range of cyclooxygenase enzyme activity was determined by the use of Walker and Gierse method with slight modifications (Bindu et al., 2016). The 5-lipoxygenase (5-LOX) activity was estimated by the method reported by Axelrod et al. (1981). In addition, the percentage of iNOS inhibition in PRME was estimated by the method described by Salter et al. (2002).

2.8.3. Isolation of Total RNA (Trizol method)

Total RNA was isolated using the RNA isolation kit (Invitrogen). The addition of Trizol solution causes the disruption of cells and the release of RNA. Chloroform extraction following centrifugation is performed exclusively in the aqueous phase, whereas proteins are collected in the interphase and organic phase. On mixing with isopropanol, RNA gets precipitated as a white pellet on the side and bottom of the tube. 1 mL of Trizol reagent was added to the cell lysate and homogenized until it formed a fine paste. In the next step, 200 µL of chloroform was added and shaken vigorously for 15 s and incubated at room temperature for 2-3 min. Then, the sample (PRME) treated wells were centrifuged for 15 min at 4 °C at $1.4 x 10^3$ rpm. The aqueous layer was collected in 500 µL of isopropanol and kept for 10 min at room temperature. The supernatant was discarded, the pellet was collected and washed with 75% ethanol, centrifuged for 5 min at 4 °C at $1 x 10^4$ rpm in a cooling centrifuge and finally dissolved in Tris-EDTA (TE) buffer (Rio et al., 2010).

2.8.4. Gene expression of cyclooxygenase enzyme in RAW 264.7 cell lines

Reverse transcription-polymerase chain reaction (RT-PCR) generated multiple copies of the desired DNA. Gene expression of cyclooxygenase enzyme was determined through an RT-PCR assay, and GAPDH was used as a housekeeping gene. The cDNA synthesis was performed using a verso cDNA synthesis kit. 5 µL of the isolated total RNA was mixed with Verso enzyme mix (1 µL), RT enhancer (2.5 µL), forward primer, and reverse primer (2 µL) in an RNAse free tube. To this mixture, 25 µL of primer RT PCR premix was added, and the total reaction mixture was 50 µL using sterile distilled water that mixed well. Using the Eppendorf master cycler, cDNA synthesis and amplification was initiated. The first step is DNA synthesis, followed by denaturation and extension step at 72 °C for 1 min, repeated for 35 cycles, and the final extension at 72 °C for 5 mins. After amplification, the PCR product was separated by agarose gel electrophoresis. It was also used to check the purity of isolated mRNA. The stained gel was visualized using a gel documentation system (E gel imager, Invitrogen). The gene expression was evaluated using the gel documentation system, and the gene expression was compared with GAPDH. oligonucleotide primer for PCR amplification of COX-1 forward 5'-AGTGCGGTCCAACCTTATCC-3' and reverse 5'-GGAGAGACTATCAAGATAGT-3'; COX-2 forward (5'-TGAGTACCGCAAACGCTTCTC-3') and reverse

(5′-TGGACGAGGTTTTTCCACCAG-3′) and GAPDH forward (5′-AGGGCTGCTTTTAACTCTGGT-3′); reverse (5′-CCCCACTTGATTTTGGAGGGA-3′) were considered, respectively.

2.9. Statistical analysis

The statistical analysis of antioxidants and cell viability assays were performed using GraphPad Prism 5 software (GraphPad Software Inc.). The standard deviation, two-way analysis of variance (ANOVA), and IC_{50} values were calculated. Pearson correlation coefficients and *p* values < 0.05 were considered significant. Values expressed are means of three replicate determinations ± standard deviation. SPSS version 17 (SPSS, Inc., Chicago, IL, USA) was used to perform one-way ANOVA. Duncan's post hoc multiple-comparison tests were used to determine significant differences among groups. In all of these statistical evaluations, $p < 0.05$ was considered a criterion to be significant.

3. Results and Discussion

Due to the diverse functional groups and chemical bonding in phytochemicals, natural product characterization, and biological evaluations are extremely interesting and hectic in the literature. One of the quite challenging tasks, especially in the pharmaceutical industry, is resolving the diverse phytoconstituents and exploring the wide possibilities of these novel compounds and their applications. The bioactivity of medicinal plant extracts is symbiotically regulated and influenced by various physiochemical and environmental factors (Borges et al., 2017). A phytochemical based screening report indicated that methanolic bark extracts contain various phytocompounds with diverse chemical groups and antioxidant activity (Anish et al., 2021a; 2021b). The compounds such as methyl protocatechuate, vanillic acid, protocatechuic acid (1,3,4) and β-sitosterol-3-*O*-β-D-glucoside were isolated from *Pterospermum acerifolium* (L.) Willd (Selim et al., 2006). GC-MS analysis of petroleum ether leaf extract of *Pterospermum canescens* Roxb. revealed the presence of α-sitosterol, 3,7,11,15-tetramethyl-2-hexa decane-1-ol, ricinoleic acid, vitamin-E, phytol, α-tocopherol, diethyl phthalate, squalene, benzhydrazide-3-mthoxy-N2-(4-phenylcyclo hexylideno, benzoic acid,4-heptyl-4-cyanophenyl ester and *n*-hexadecanoic acid (Jaiganesh et al., 2012). Detailed pharmacological findings of both aqueous and alcoholic extracts of the *Pterospermum acerifolium* (L.) Willd fruits exhibited their analgesic, antipyretic, anti-hyperglycemic, acute anti-hyperglycemic and anti-inflammatory activities (Selim et al., 2006). The *Pterospermum acerifolium* ethanolic bark extract on carrageenan-induced and arachidonic acid-induced rat paw edema showed significant inhibition of acetic acid and tail clip-induced analgesia (Chatterjee et al., 2012). The PRME were rich in flavonoids, phenolic acids, triterpenes, alkaloids, glycosides, and saponins which are among the naturally-occurring phytochemicals responsible for self-protection and defense mechanism of plants. These compounds are frequently isolated from medicinal plants and are commonly exploited by humans as pharmaceutical alternatives due to their potential biological properties (Bansal and Priyadarsini, 2021). More recently, Mohammadhosseini et al. (2019a; 2019b; 2022) have comprehensively reviewed the phytochemical composition of some valuable plant genera along with their promising pharmaceutical, biological and medicinal impacts. Some traditional aspects related to their relevant herbal and medicinal plants have been also covered in detail in these reviews from ethnobotanical point of view. It is evident that our life is highly dependent on the presence of the medicinal plants and these materials could be considered potential therapeutic agents for the treatment of a variety of hard diseases that endanger the life of the human beings (Chimi Fotso et al., 2021; Mouthe Kemayou et al., 2021; Olaoluwa et al., 2022). It also seems logical that the existence of bioactive phytochemicals is one of the most reliable criteria for justifying the observed and reported biological evaluation.

The current study was proposed for evaluating the biological efficacy of PRME based on its detailed phtyochemical quantification and characterization. In this relation, the antioxidant and anti-inflammatory mechanisms of PRME to eliminate free radical-induced inflammation and associated consequences have been evaluated.

3.1. Phytochemical and antioxidant evaluation

DPPH and hydrogen peroxide assays have been commonly used to evaluate the antioxidant capacity of PRME. Accordingly, PRME showed excellent scavenging activity when compared to the standard ascorbic acid in a linear manner at a concentration range of 6.25-100 µg/mL and the obtained results are shown in Fig. 1. The total phenolic, alkaloid, and flavonoid contents of PRME were evaluated quantitatively and found to be 46.7, 37.4 and 7.21 g/100 g extract, respectively. The maximum yield of PR bark extract was obtained in methanol (12.5%), followed by acetone (10.73%) and ethyl acetate (7.45%) during the separation through the chromatographic column. The PRME also showed excellent inhibitory activity in free radical scavenging assays, probably due to its higher levels of polyphenols. Phenolic compounds are excellent antioxidants with different biological functions and can considerably eliminate reactive oxygen species (ROS) and chelate transition metals which play crucial roles in free radical initiation reactions. Several studies have reported the importance of phenolic content and its relative correlation with the antioxidant activity of plant extracts (Mayakrishnan et al., 2013).

DPPH is a stable organic free radical which can accept an electron or hydrogen radical to gain more stability giving rise to the discoloration of the test sample from purple to yellow. Free radical scavenging is one of the well-known mechanisms by which antioxidants inhibit lipid peroxidation and protect our bodies from further damage (Halliwell and Gutteridge, 2006). In our study, PRME showed excellent DPPH scavenging capacity in a dose-dependent manner with an IC_{50} value of 42.70 µg/mL ($p < 0.05$).

Hydrogen peroxide is available in the environment and

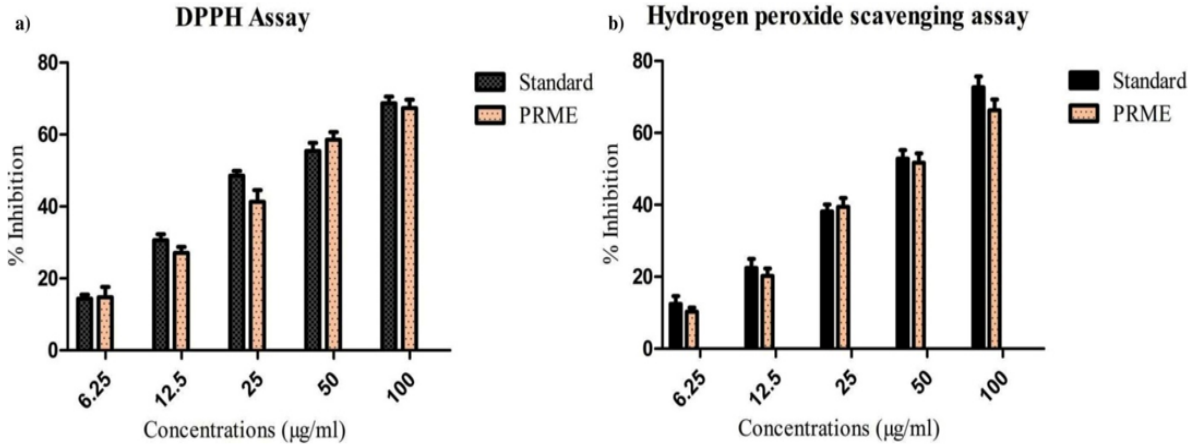

Fig. 1. DPPH and H_2O_2 assay of PRME (DPPH and hydrogen peroxide scavenging assay of PRME in a concentration-dependent manner. The PRME extract gave the significant IC_{50} values of 42.70 and 48.33 µg/mL for DPPH and H_2O_2 scavenging assay ($p < 0.05$). PRME: *Pterospermum rubiginosum* methanolic bark extract).

enters our bodies mainly through inhalation. It is not very reactive, but in the body, H_2O_2 rapidly undergoes a decomposition reaction and releases oxygen and water which even leads to the formation of a highly toxic hydroxyl radical having the cytotoxic ability to destroy biological membranes and capable of initiating lipid peroxidation and denaturation of DNA. The H_2O_2 scavenging mechanism by the plant extracts may be due to the presence of phenolic compounds capable of donating electrons to H_2O_2, thereby reducing it to water (Banerjee and Bonde, 2011). Thus, eliminating hydrogen peroxide radicals from the human body is crucial; PRME showed excellent dose-dependent effects on hydrogen peroxide scavenging with an IC_{50} of 48.33 µg/mL compared with standard ascorbic acid (47.30 µg/mL) (Fig. 2).

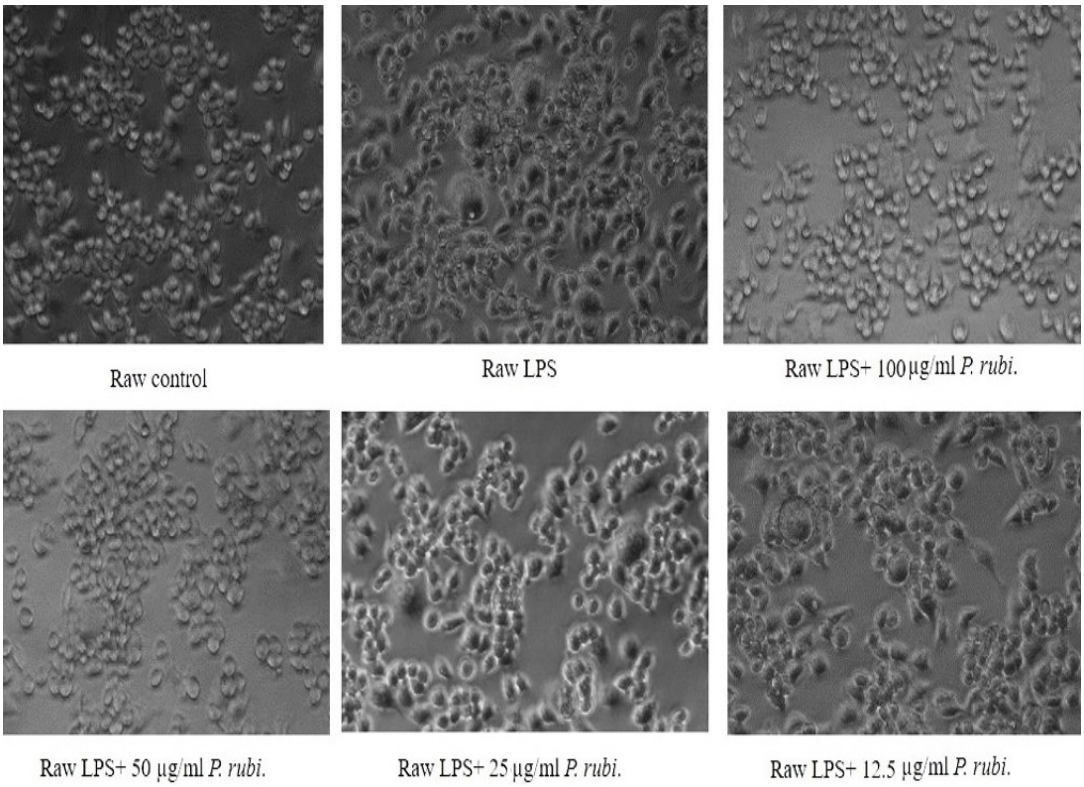

Fig. 2. *In vitro* MTT assay (phase contrast image of cells using Carl Zeiss microscope with 20X magnification); LPS: Lipopolysaccharides; PRME: *Pterospermum rubiginosum* methanolic bark extract.).

3.2. Antimicrobial screening

Antibacterial and antifungal screening of PRME was performed using standard bacterial and fungal culture for which the obtained zones of inhibition have been tabulated in Table 1. The antimicrobial inhibition zones generally have a uniform pattern, mostly circular due to the confluent growth of microbes and the diameter of inhibition zones are commonly measured in millimeters or centimeters (Erecevit and Kırbag, 2017). According to our experimental findings, PRME showed no growth inhibition up to a concentration of 100 µg/mL towards the entire Gram-positive, Gram-negative, and fungal test organisms. Thus, it was concluded that the PRME has no significant antimicrobial or antifungal activity.

The PRME also showed excellent antioxidant activity with significant amounts of phenolic acids, alkaloids and flavonoid. However, PRME did not show any significant antimicrobial activity against the organisms used in the present study. The ethanolic bark extracts of *Pterospermum acerifolium* are rich sources of alkaloids, flavonoids, phenolics and tannin and showed DPPH and ABTS radical scavenging activity (Rath et al., 2014). In another report, it has been shown that *Pterospermum canescens* ethanolic leaf extracts (ELE) possess alkaloids, flavonoids, phenolics and steroids. Moreover, in different concentrations, the ELE exhibits notable antimicrobial activity against the various fungi and bacterial strains (Jaiganesh and Arunachalam, 2011).

Table 1
Antibacterial and antifungal screening of PRME against the test microorganisms.

Strains	Test organisms	Diameter of zone of inhibition (cm)				
		PRME (25 µg/mL)	PRME (50 µg/mL)	PRME (100 µg/mL)	Streptomycin (10 µg/mL)	Clotimazole (10 µg/mL)
Gram negative	*Escherichia coli*	-	-	-	2.81 cm	-
	Klebsiella pneumonia	-	-	0.9 cm	2.85 cm	-
	Psuedomonas aeroginosa	-	-	-	2.79 cm	-
Gram positive	*Staphylococcus aureus*	-	-	1.03 cm	3.45 cm	-
	Streptococcus mutans	-	-	1.09 cm	3.41 cm	-
Fungal culture	*Aspergillus niger*	-	-	-	-	2.11 cm
	Candida albicans	-	-	-	-	1.91 cm

PRME: *Pterospermum rubiginosum* methanolic bark extract; µg/mL: *microgram per millilitre.*

3.3. FTIR spectra of PRME

FTIR spectroscopy requires only minimal sample preparation, but is a highly sensitive, a high-resolution analytical tool and a non-destructive technique that simultaneously determines different chemical bonds and functional moieties present in plant samples. The FTIR spectra of PRME showed the presence of diverse functional groups with peak values summarized in Table 2. In fact, FTIR spectra of pure compounds are unique and can be considered a molecular "fingerprint." Using the standard IR library, the spectrum of an unknown compound can be easily evaluated. The spectrum showed the absorption peak at 3289.93 cm^{-1}, corresponding to O-H stretching and alcohol functional groups. The peak appearing at 2926.59 cm^{-1} was assigned for the C-H stretching of aliphatic compounds, and 1517.48 cm^{-1} for N-O asymmetric stretch of nitro compounds. The functional groups such as alcohol, carboxylic acids, esters, ethers, alkyl halide, and aromatic compounds made the PRME spectra very complex. The observed complexity could be attributed to various phytochemical moieties in PRME that may influence the relevant biological activity significantly.

3.4. LCMS analysis of PRME

LCMS is an analytical technique widely used in the natural product and pharmaceutical-based disciplines. The LCMS data provides the molecular weight, possible formula and quantity of a molecule in PRME. On detailed analysis, nearly 40 peaks were detected, however only 21 molecules were identified and characterized (Fig. 3; Table 3) including kirenol derivative, cholorogenic acid, campesterol, vanillic acid, (*E*)-resveratrol, 4-*O*-methyl gallic acid, catechin, gallocatechin, betulin, betulinic acid, and hyperoside (flavonoid glycoside) (Garcia et al., 1993; Abd El-Razek, 2007; Prachayasittikul et al., 2009; Vijayalakshmi et al., 2015; Peng et al., 2017). Recently, some natural compounds, *e.g.*, 4-*O*-methyl gallic acid, β-sitosterol, β-sitosterol glucoside, (*E*)-resveratrol, 4′-*O*-methylgallocatechin and gallocatechin have been successfully isolated and characterized from PRME using nuclear magnetic resonance spectroscopy which also supported the corresponding LCMS analysis data (Anish et al., 2023).

3.5. Molecular docking

Docking studies are commonly used to predict the molecular interactions, binding sites of ligands to protein molecules and to understand the 3D orientation pattern of stable protein-ligand interactions. This is commonly exploited in pharmacology to determine the binding pockets of drugs during their interaction with target sites, especially proteins. The binding energy of

Table 2
The general details for FTIR spectra of PRME.

Sl Num.	Peak value (cm^{-1})	Bond stretching	Functional group
1	3289.93	O-H stretching vibration	Phenolic group
2	2926.59	Alkane (C-H), C-H stretching vibration	Alkanes, aliphatic compounds
3	1517.48	N-O asymmetric stretch	Nitro compounds
4	1607.47	C=O stretching vibration	Ketones
5	1442.83	C=C stretch	Aromatic
6	1336.53	C-F stretching vibration	Alkyl halide
7	1203.95	C-N stretching vibration	Aliphatic amines
8	1099.91	C-O stretching	Ether, Ester
9	1032.24	C-O stretching vibration	Alcohols, carboxylic acids, esters, ethers
10	989.6	C-O stretching	Ethers
11	923.42	P-O-C stretch,	Aromatic phosphates
12	819.44	N-H stretching	Proteins
13	772.3	-C-Cl stretching	Aliphatic chloro compounds
14	471.1	S-S stretch	Aryl disulfides
15	494.6	C-I bond	Halogen compounds
16	462.46	S-S stretching	Sulphur compounds

PRME: *Pterospermum rubiginosum* methanolic bark extract; wave number: cm^{-1}

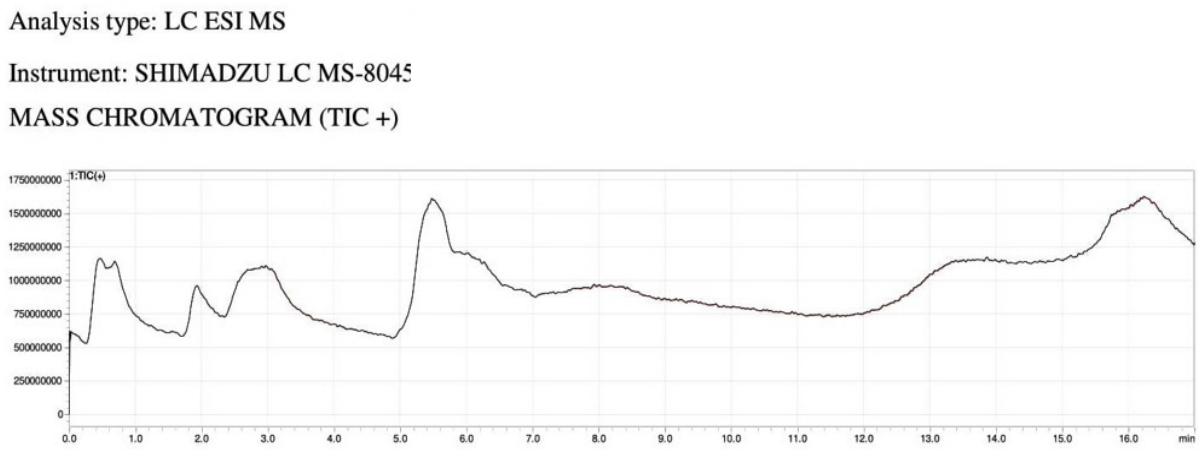

Fig. 3. PRME chromatogram by LCMS analysis (X-axis indicates the time; Y-axis represents relative signal intensity).

ligands against COX enzymes are summarized in Table 4. As seen, the docking studies with cyclooxygenase-1 (1EQG) revealed good binding affinity of vanillic acid ligand-89.5583 (kcal/mol) due to the protein-ligand interactions of COX-1 (A-chain) SER353, SER530 amino acid residues, and O2, O3, O4 moiety of vanillic acid through hydrogen bonding. The hydrophobic bond interactions of vanillic acid with COX-1 are also seen with amino acids LEU384, MET522, TRP387, VAL349, and LEU352 amino acids residues are shown in Fig. 4. Current study was carried out to explore non-bond interactions between the target and the ligand molecule. In addition, the 4-*O*-methylgallic acid ligand showed good binding interactions with COX-1 protein through hydrogen bonding with SER353 and SER530 residues. The ligand-protein plot indicates the hydrophilic interactions of the ligand with the residues like LEU384 (A), TYR385 (A), TRP387 (A), VAL349 (A), LEU352 (A), ALA527 (A), ILE523 (A). In a similar report, the docked complex of 1EQG with vanillic acid and standard ligand was analyzed to evaluate the non-bond interactions between the target and the ligand molecule (Bakhle, 1999), similar results were reported. The cyclooxygenase-2 (prostaglandin synthase-2) complexed with a selective inhibitor, SC-558 (1-phenylsulfonamide-3-trifluoromethyl-5-parabromophenylpyrazole) was used to study the interactions with various ligands present in PRME with COX-2. The binding affinity of-49.7518 (kcal/mol) corresponded to a vanillic acid ligand with different amino acid residues. ALA527 (A) is comparable and showed significant interactions with standard drug (diclofenac sodium) and VAL523 (A) of COX-2 through

Table 3
Compounds isolated from PRME using LCMS analysis.

Peak number	RT	Predicted molecule	Formula	Mass	*m/z*
1	0.449	3-Aminocaproic acid	$C_6H_{13}NO_2$	131.095	132.102
2	0.481	Octylamine	$C_8H_{19}N$	129.152	130.159
3	1.85	Gallic acid	$C_7H_6O_5$	170.122	171.550
4	1.931	3,5,6-Trihydroxy-5-(hydroxymethyl)-2-methoxy-2-cyclohexen-1-one	$C_8H_{12}O_6$	204.062	205.404
5	2.892	Vanillic acid	$C_8H_8O_4$	168.902	170.203
6	3.105	4-*O*-methyl gallic acid	$C_8H_8O^5$	184.037	185.335
7	4.331	E-resveratrol	$C_{14}H_{12}O_3$	228.804	229.450
8	5.591	Catechin	$C_{15}H_{14}O_6$	290.805	292.107
9	5.715	Gallocatechin	$C_{15}H_{14}O_7$	306.64	307.450
10	13.757	Kirenol derivative	$C_{20}H_{34}O_4$	338.141	339.202
11	13.953	Cholorogenic acid	$C_{16}H_{18}O_9$	354.151	355.051
12	14.051	Campesterol	$C_{28}H^{48}O$	400.263	401.207
13	14.233	β-sitosterol	$C_{29}H_{50}O$	414.378	415.205
14	15.607	Betulin	$C_{30}H_{50}O_2$	442.100	443.254
15	15.573	Betulinic acid (pentacyclic triterpenoid)	$C_{30}H_{48}O_3$	456.456	457.253
16	15.833	Hyperoside (flavonoid glycoside)	$C_{21}H_{20}O_{12}$	464.551	465.302
17	16.013	β-sitosterol glucoside	$C_{35}H_{60}O_6$	576.85	578.121
18	8.225	2-(4-Methyl-5-thiazolyl)ethyl formate	$C_7H_9NO_2S$	171.035	172.042
19	11.211	L-Tryptophanamide	$C_{11}H_{12}N_2O_2$	204.103	205.107
20	4.554	L-Pyridosine	$C_{12}H_{18}N_2O_4$	254.128	255.107
21	5.011	(2*E*)-Piperamide-C5:1	$C_{16}H_{19}NO_3$	273.136	274.133

PRME: *Pterospermum rubiginosum* methanolic bark extract; *m/z-mass-to-charge ratio*; RT: retention time; LCMS analysis library search results*.

Table 4
Binding energy of various phytochemicals isolated from PRME against COX enzymes.

Protein	Ligands	Binding energy (kcal/mol)
COX-1	Vanillic acid	-89.558
COX-1	4-O-Methylgallic acid	-16.039
COX-1	Diclofenac sodium	40.958
COX-2	Vanillic acid	-49.752
COX-2	4-*O*-Methylgallic acid	27.383
COX-2	*E*-resveratrol	68.686
COX-2	Catechin	77.025
COX-2	Diclofenac sodium	368. 26

PRME: *Pterospermum rubiginosum* methanolic bark extract; COX-1-Cyclooxygenase-1; COX-2-Cyclooxygenase-2 enzyme, respectively; Diclofenac sodium-standard drug; kcal/mol; *kilocalorie* per *mole.*

hydrogen bonding. As shown in Fig. 5, the docked complex of 1CX2 with vanillic acid and standard ligand was analyzed to assess non-bond interactions between the target protein and the ligand molecule. The O3 moieties of the vanillic acid ligand interact with PHE518 (A) of 1CX2 via an electrostatic bond of interaction. Dhorajiwala et al. (2019) reported that unfavourable bonds might affect the stability and activity of drugs-ligand complex interaction. The formation of any kind of unfavourable bond between protein-ligand complexes may reduce the stability of the complex due to the steric repulsion force occurring between protein-ligand interactions. However, the unfavourable bond interactions and their effects can be confirmed only

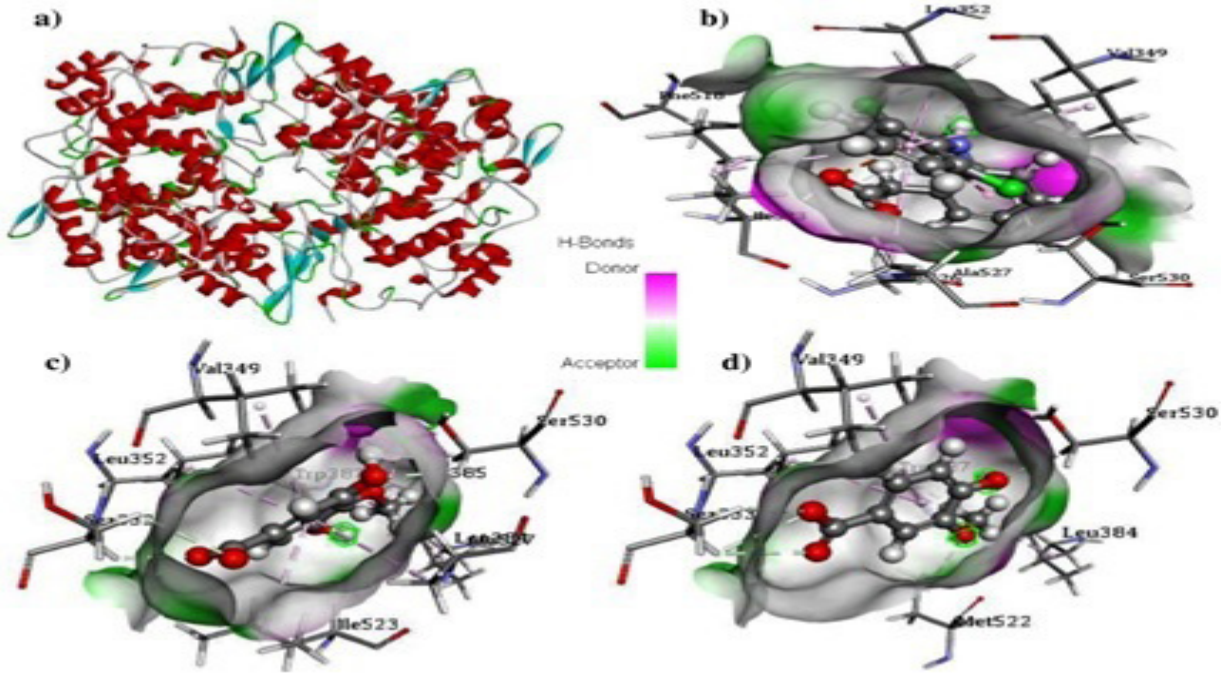

Fig. 4. Cox-1 protein interactions with the ligand molecule isolated from PRME (a). Crystallographic structure of COX-1 alpha from PDB database, the secondary structures of proteins are colour-coded as red-helix; grey-coil; green-turn; blue-sheet; b), c) and d) represents the surface view interactions of COX-1 protein with diclofenac sodium, 4-*O*-methyl gallic acid and vanillic acid, respectively).

after wet lab experiments.

The second ligand, 4-*O*-methylgallic acid, also showed good hydrogen bond and electrostatic interactions with ARG513 (A) and the O4 moiety; ASP515 (A) and ARG513 (A) also revealed hydrogen bonding with different oxygen molecules of the ligand. The hydrophobic bonding was seen with the interactions of ASP515 (A), PRO514 (A), ALA516 (A) and ligand residues. The *E*-resveratrol ligand also exhibited hydrogen bond interactions with ARG513, hydrogen and electrostatic bonding with ARG120 and ARG513, hydrophobic bonding with SER353, VAL523, VAL349, ALA527 along with LEU531 of the A-chain of COX-2 with the ligand. The catechin ligand (73160) also showed a hydrogen bond of interaction with the amino acid residues of protein molecules like TYR385, HIS90, and VAL523. Hydrophobic bonding also revealed good bonding with some amino acids such as GLY526, VAL349, LEU352, VAL523, and ALA527 of the A-chain. The molecular docking analysis showed the excellent interactions of vanillic acid ligand isolated from PRME with COX protein. The presence of vanillic acid in bark extract may be the factor behind the excellent anti-inflammatory activity of PRME.

3.6. MTT assay

The MTT assay was used to investigate the cytotoxicity of PRME on murine macrophages RAW 264.7 cells. The whole plate was observed after 24 hrs of treatment using an inverted phase contrast tissue culture microscope for microscopic cellular evaluation. Observations were recorded as images, and no significant changes in the morphology, granulation, or vacuolization in the cytoplasm of the cells were observed (Fig. 2) (Zahri et al., 2009). During the plant material biological screening, preliminary quantifying the cellular toxicity in cell lines is a crucial step before moving to higher levels of molecular studies. The MTT assay showed good cellular viability of PRME up to a concentration of 100 µg/mL. The IC_{50} value was found to be 107.615 µg/mL.

3.7. Intracellular ROS generation by fluorescent microscopy

ROS are normally generated during mitochondrial oxidative metabolism as well as in cellular response to LPS stimulation which results in an imbalance of oxidant-antioxidant equilibrium and leads to severe cellular stress and enhanced inflammatory mediators. As shown in Fig. 6, the PRME-treated groups exhibited a significant reduction in ROS production when compared to LPS stimulated cells. ROS can irreversibly damage and degrade the genetic materials of living beings and act as a signalling molecule that further aids the progression of inflammatory episodes and disorders (Mittal et al., 2014). ROS measurement using DCFDA staining showed an elevated fluorescence in LPS-stimulated cells (36517.25 FIU) compared to normal control which enhances oxidative stress in culture conditions. These stress mechanisms can directly activate various transcription factors including

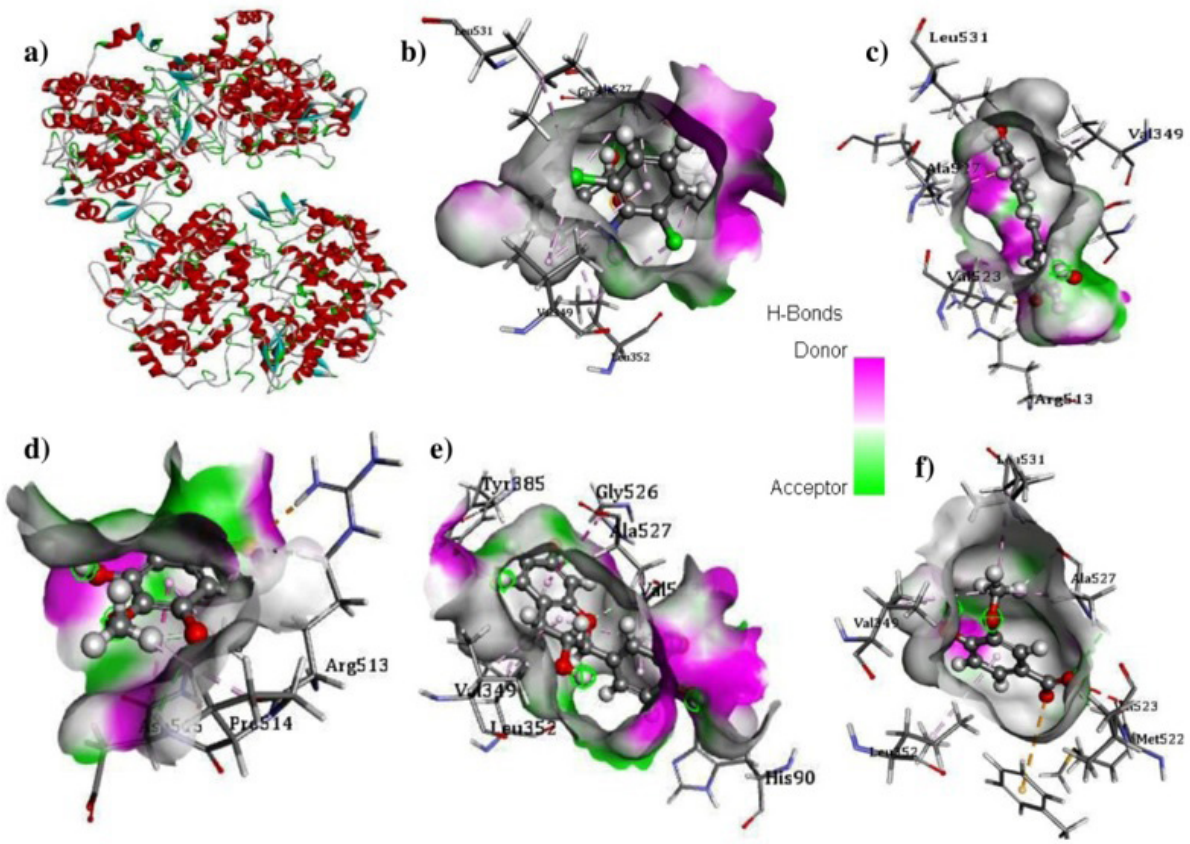

Fig. 5. Cox-2 protein interaction with the ligand molecules isolated from PRME (a) Crystallographic structure of COX-2 alpha from PDB database, the secondary structures of proteins are color-coded as red-helix; grey-coil; green-turn; blue-sheet; b), c), d), e) and f) represents the surface view of COX-2 protein interactions with diclofenac sodium, (*E*)-resveratrol, 4-*O*-methyl gallic acid, catechin and vanillic acid, respectively; PRME: *Pterospermum rubiginosum* methanolic bark extract).

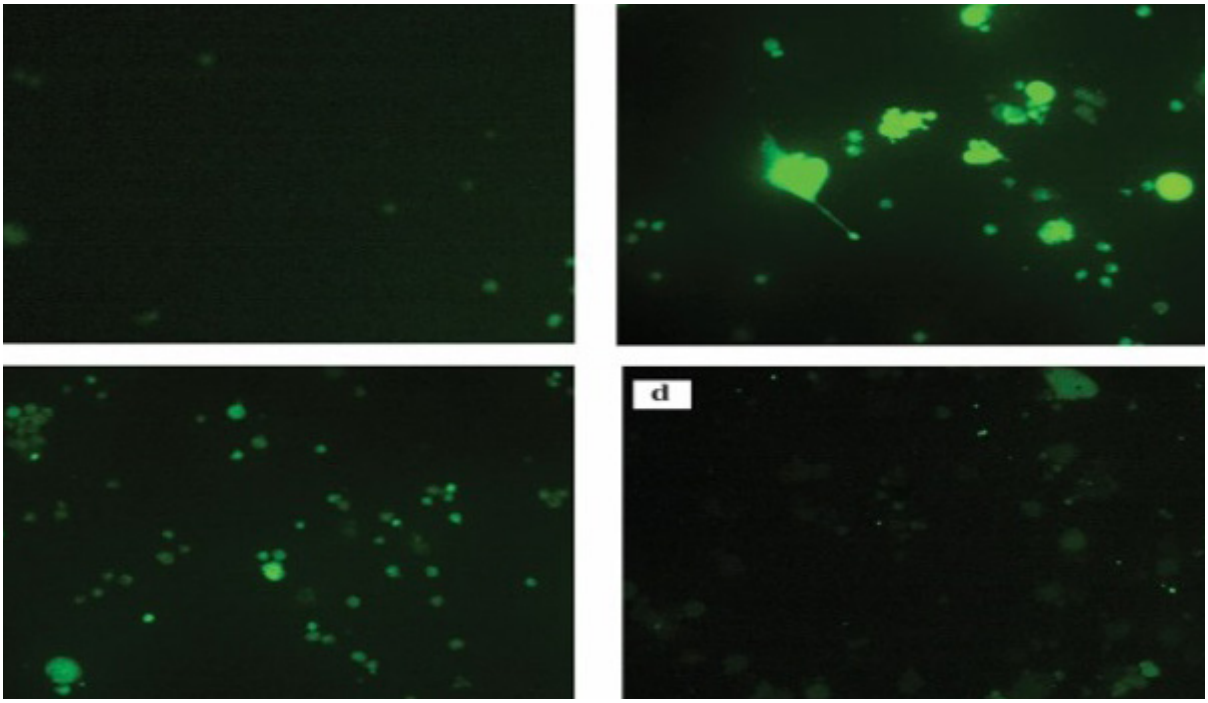

Fig. 6. DCFDA staining (DCFDA, a fluorogenic dye that measures the level of oxidative stress within the cell (a. Control; b. LPS stimulated group; c. LPS+PRME; d. LPS+diclofenac sodium); where LPS: Lipopolysaccharides; PRME: *Pterospermum rubiginosum* methanolic bark extract.).

NF-kB in the chronic inflammatory pathway and release numerous mediator molecules. The results showed a significant reduction in ROS generation of cells treated with PRME after LPS stimulation (16253.86 FIU), compared with the standard drug (diclofenac sodium-9184.33 FIU) most probably due to the antioxidant potential of phenolic acids and flavonoids in PRME.

3.8. Anti-inflammatory assay

Regarding the obtained results of our *in vitro* anti-inflammatory study, PRME administered groups that significantly reduced the inflammatory enzymes such as cyclooxygenase (COX), lipoxygenase (LOX), and iNOS (see Table 5). Nitric oxide is one of the early markers of inflammation (Liu et al., 2017) and is considered a pro-inflammatory mediator that induces inflammation due to the overproduction of abnormal mediators during cellular stress. During LPS activation, macrophages upregulate NO synthesis leading to cellular damage (Kim et al., 2009). After PRME treatment (50 µg/mL), a significant reduction in iNOS levels was observed as 70.55 ± 1.36%. The reduction of inflammatory markers, pro-inflammatory cytokines and iNOS levels may be due to the phytochemical diversity in PRME. Aquino et al. (2017) reported the interactive equilibrium between NO production and prostaglandin levels as well as the inhibition of NO synthesis that can directly reduce prostaglandins and COX-2 enzyme. The ability of the PRME to inhibit NO overproduction partly confirms its anti-inflammatory potential and supports the hypothesis of prostaglandin synthesis modulation in inflammatory pathways.

After LPS stimulation, stimulated macrophages increased the release of cyclooxygenase enzymes. During the PRME treatment at concentrations of 25 and 50 µg/mL, a notable reduction in COX enzyme was observed, and the percentage of inhibition was respectively as 52.82 ± 2.59% and 70.89 ± 2.45%. Specific inhibitors of COX-2 act as therapeutic agents in pain treatment particularly in patients with osteoarthritis or rheumatoid arthritis (Dinarello, 2010). COX-1 is present in almost all cells and regulates cellular homeostasis. Any pharmaceutical agent which acts as dual inhibitors of COX and LOX enzymes can be considered a good anti-inflammatory agent because the molecule can indirectly inhibit the formation of prostaglandins, leukotrienes, and other biologically active mediators of inflammation (Nguyen et al., 2020). The lipoxygenase (LOX) activity of PRME at a concentration of 50 µg/mL showed good inhibition of 65.05 ± 1.70% followed by the standard diclofenacsodium (97.19 ± 1.32%). The current study revealed that PRME administration significantly reduced the activity of LOX, COX, and iNOS in LPS stimulated RAW 264.7 cells.

3.9. Gene expression study of COX-1 and COX-2 enzymes

Gene expression of inflammatory enzyme markers COX-1 and COX-2 were studied to understand the mechanism of PRME behind the inflammatory pathways. LPS treatment stimulated the RAW 264.7 macrophages and increased the release of cyclooxygenase enzymes, COX-1, and COX-2 expression up to a range of 1.2 to 1.4 arbitrary units, a three-fold increase when compared to the control group (Fig. 7). During PRME treatment, the phytochemicals present in the PRME can tolerate inflammation and down-regulated gene expression by nearly 0.6 arbitrary units compared to the standard (diclofenac sodium) treated group.

Table 5
Inflammatory enzyme assay in RAW 264.7 cells during PRME treatment.

Sample Concentration (µg/mL)	Cox assay	Lox assay			iNOS assay	
	PRME	Standard	PRME	Standard	PRME	Standard
12.5	31.14±2.06	41.49±3.15	24.66±1.42	49.82±1.78	29.93±2.73	40.19±1.33
25	52.82±2.59	66.81± 3.89	49.91±1.89	76.71±2.89	57.55±1.64	62.48±2.19
50	70.89±2.45	81.41±3.13	65.05±1.70	97.19±1.32	70.55±1.36	86.64±1.46

Values are expressed as mean±standard deviation (n=3). Where, µg/mL: *microgram/millilitre;* COX-Cyclooxygenase; *LOX*-Lipoxygenases; iNOS: Inducible nitric oxide synthase; Standard-diclofenac sodium; PRME: *Pterospermum rubiginosum* methanolic bark extract.

4. Concluding remarks

Total flavonoid and phenolic content along with H_2O_2 and DPPH assay showed the antioxidant activity of PRME. FTIR analysis revealed the diverse chemical bonds and functional groups in PRME. Structural characterization of PRME using LCMS analysis identified 21 various phytoconstituents. On LPS treatment, the macrophages activate and initiate the release of cytokines and associated inflammatory mediators to promote the inflammatory process. After PRME administration, LOX, COX, and iNOS were significantly reduced. Molecular docking data and gene expression studies support the anti-inflammatory activity of PRME. Thus, it can be concluded that PRME can specifically suppress LPS-activated macrophages and be considered an alternative anti-inflammatory candidate for detailed *in vivo* and clinical studies. Current findings are crucial in the case of anti-inflammatory drugs which have been commonly administered for over 100 years in the

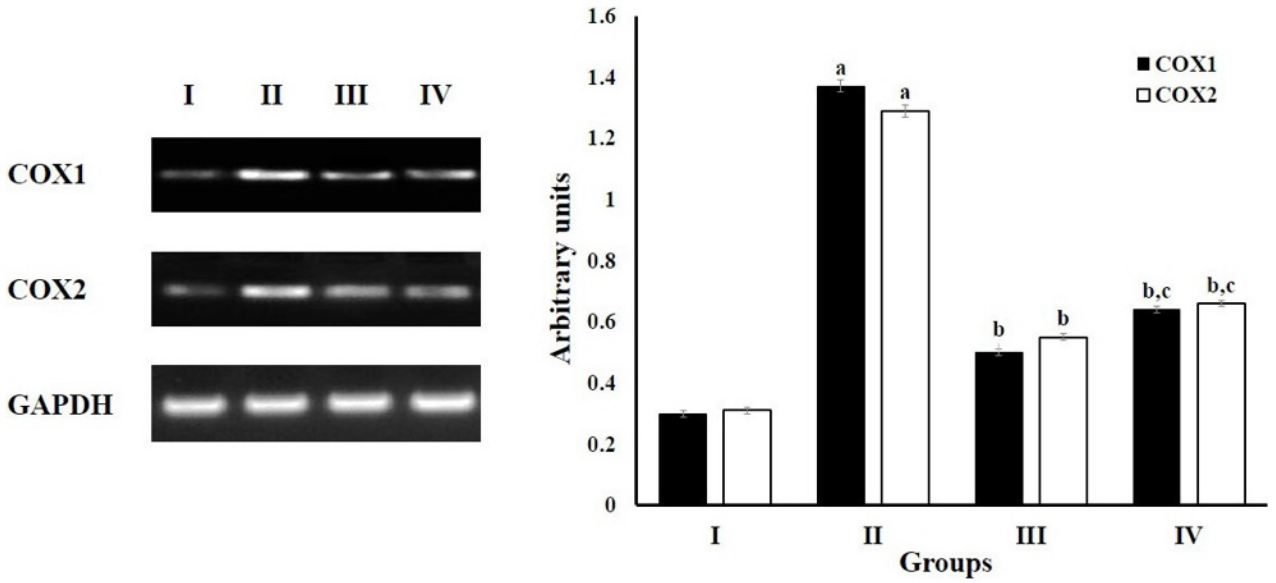

Fig. 7. Graphical and photographic representations of mRNA expressions of COX-1and COX-2 on RAW 264.7 macrophages treated with PRME. (The results were presented as mean±SD, n = 4 with $p \leq 0.05$. Group: I-control; group: II-LPS stimulated; group: III-LPS + PRME; Group: IV-LPS + diclofenac sodium. I has been compared with II ('a' indicates values were significantly different from I), II is compared with III and IV ('b' indicates values were significantly different from II) and III is compared with IV ('c' indicates values were significantly different from III). LPS: Lipopolysaccharide and PRME: *Pterospermum rubiginosum* methanolic bark extract.

therapeutic practice in tribal and traditional medical systems. These findings would enhance the significance of *P. rubiginosum* in scientific research and clinical trials as an alternative anti-inflammatory drug and support its use in the traditional medicine in India.

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Conflicts of Interest

The authors declare no conflict of interest.

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