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In vitro ANTI-INFLAMMATORY AND ANTIOXIDANT EVALUATION OF AN INDIGENOUS MEDICINAL PLANT – *Pterospermum rubiginosum*

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ABSTRACT

The current study was carried out to determine the antioxidant potential, anti-inflammatory activity, and cellular viability of *Pterospermum rubiginosum* (PR), a tropical tree endemic to the Western Ghats. The antioxidant activities of the PR bark methanolic (PRME) and aqueous extract (PRAQ) were tested using ABTS as well as superoxide, nitric oxide, and hydroxyl radical assays. Total antioxidant activity was evaluated by adopting the colorimetric method and correlation with their antioxidant activities was derived by Pearson co-efficient analysis. The PRME showed the highest ABTS radical scavenging activity, EC₅₀ (46.09µg/ml) followed by PRAQ (52.08µg/ml). Furthermore, the PRME exhibited the highest scavenging activity against superoxide, nitric oxide, and hydroxyl radicals. The MTT assay results revealed good cellular viability up to a concentration of 100µg/ml with an EC₅₀ (106.869µg/ml). The inflammatory mediators such as Cox-2, IL-1β, IL-6, and NF-kB were reduced during the treatment of PRME in LPS stimulated RAW cells. The stress marker in rat liver cells such as glutathione reductase (GR), glutathione peroxidase (GPx), and reduced glutathione (GSH) levels was found in normal levels when compared to the untreated group of rats. The antioxidant enzyme superoxide dismutase and catalase also exhibited notable bioactivity in PRME treated groups up to a concentration of 1000µg/ml. The present study showed excellent *In vitro* and *In vivo* antioxidant activity; the potent anti-inflammatory ability of PRME in reducing the LPS induced inflammation in cell culture conditions.

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1 Introduction

Reactive oxygen species (ROS) is a biologically harmful group of free radicals formed in our body during cellular metabolism, under the influence of various secondary metabolites and coenzymes (Pizzino et al., 2017). Any change in metabolic equilibrium will lead to the excess production of free radicals, including ROS, which is a sensitive trigger for oxidative stress mechanism. Various food materials are a rich source of natural antioxidants and are found to be very effective for scavenging unwanted radicals, thereby minimizing the complications related to degenerative diseases and aging (Shields et al., 2021). The oxidation induced by ROS such as nitric oxide radical, superoxide radical, hydroxyl radical, hydrogen peroxide (H_2O_2), and singlet oxygen (O_2), resulting in cell membrane disintegration, protein damage, and mutation, which may further lead to diseases such as diabetes, cancer, liver injury and cardiovascular disease (Srivastava et al., 2017). During mitochondrial respiratory chain reaction, superoxide anion is produced as natural by-products of cellular metabolism. The main sources of extracellular ROS are UV and other ionizing radiation and pollutants (Pizzino et al., 2017).

Oxidative stress occurs when the critical balance between ROS production and the endogenous antioxidant defense gets altered due to severe oxidative damage. For maintaining the oxidant status and to repair the redox equilibrium, cells undergo a self-adjustment to homeostatic changes; otherwise, we have to supplement antioxidants (Kurutas, 2015). Antioxidants have a synthetic or natural origin that reacts with free radicals and reduces their lethal effects in our body by neutralizing them. The imbalance between oxidants and antioxidants in the body leads to the damage of macromolecules such as enzymes and proteins in various regulatory and metabolic pathways and diverse health problems including lifestyle disorders. Therefore, homeostasis between antioxidants and oxidants is considered to be a crucial mechanism that reduces ROS and helps to maintain a healthy biological status (Hasanuzzaman et al., 2020).

Due to the limited availability of natural antioxidants, several synthetic molecules have been used as an alternative in the pharmaceutical industry, but long-term use of these chemicals can result in severe side effects including DNA damage, induce premature senescence, and other related complications (Kornienko et al., 2019). Due to ease of availability, synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly used in food preservation and recent animal toxicity (*In vivo*) studies have validated that, these compounds are highly lethal and can induce apoptosis and promote tumor growth (Xu et al., 2021). Strict implementation of dietary restrictions is necessary to minimize systemic disorders, by European Food Safety Authority (EFSA) & Food and Drug Administration (FDA) (Lourenço et al., 2019). So finding novel

safe antioxidants from natural sources, especially plant derivatives, is of great interest in food and pharmaceutical applications. Plant extracts have been widely used as effective remedies for the prevention and treatment of multiple health issues for centuries by almost every known culture. The traditional medicinal system of India, Ayurveda has been practiced for more than 5,000 years and decoctions and extracts from medicinal plants remain an essential component of its practice (Subhose et al., 2005; Babich et al., 2020). There is a diverse variety of secondary metabolites isolated from different medicinal plants exhibiting excellent anti-inflammatory, anticancer, analgesic, antibacterial, antiviral activities and it is mainly due to the presence of novel antioxidants (Tungmunnithum et al., 2018).

Pterospermum rubiginosum (Sterculiaceae), “Ellooti” bark, or “Ellooripatta” is a tropical tree endemic to the Western Ghats, Kerala, and has been previously identified by Kanikaran tribes to exert beneficial effects on bone fractures preventing bone loss and promoting bone health (Anish et al., 2021; Vijayan et al., 2007). There is still a lack of knowledge regarding the exact mechanism behind the bioactivity of *P. rubiginosum* and it requires further study for potential therapeutic applications. Other than traditional knowledge, there have been no detailed reports on the antioxidant activities of this plant; hence the present work investigates the complete antioxidant free radical scavenging profile of methanolic and aqueous fractions from *P. rubiginosum* bark. Best of our knowledge, there has been no previous literature being available on the anti-inflammatory and animal experimental studies of *P. rubiginosum* methanolic bark extract.

2 Materials and Methods

2.1 Plant Materials and reagents

The bark of *P. rubiginosum* was collected from the Kottur forest range, Thiruvananthapuram district of Kerala with the help of tribals. The plant voucher specimen is kept in the herbarium of the Department of Botany, University of Kerala, India with a voucher number of KUBH 6189. All the reagents, methanol, and organic solvents (Merck Millipore, Germany) were analytical grade. The experiment was conducted using cell culture media, antibiotics (Sigma Aldrich, USA) & inflammatory markers (Santa Cruz, USA).

2.2 Sample extraction

After removing exfoliated outer bark, the inner bark of *P. rubiginosum* was shade dried for 3-4 weeks. The dried bark was powdered, and 500g of the powdered material was extracted with methanol in a soxhlet apparatus for 72 hrs. The extract was then concentrated with the help of a rotary evaporator and qualitative analysis of their active ingredients was carried out using standard

procedures (Azmin & Nor, 2020). For water extraction, 100g powdered bark was mixed with 1L distilled water and extracted with the help of a magnetic stirrer.

2.3 Total Antioxidant Activity

The total antioxidant activity (TAA) of both methanolic and aqueous extracts of *P. rubiginosum* was determined by adapting the method used by Subhasree et al. (2009) with slight modifications. Different concentrations of both the extracts (12.5-500µg/ml) from a stock concentration of 1mg/ml were mixed with 3ml of reagent solution (0.6M H₂SO₄, 28mM sodium phosphate, and 4mM ammonium molybdate) in capped test tubes. The tube containing the reaction solutions was incubated in a boiling water bath at 95°C for 90 minutes. After cooling to room temperature, the absorbance of the solution was measured at 695nm against a blank (0.3ml of methanol was used in place of PRME). TAA was determined based on the number of gram equivalents of ascorbic acid.

$$\% \text{ Inhibition} = \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100$$

2.4 ABTS Assay

The radical mono-cation of 2, 2'-azino-bis (3-ethyl benzothiazoline 6-sulfonic acid (ABTS) was generated by oxidation of ABTS with potassium persulfate (a blue chromogen) and is reduced in the presence of hydrogen donating antioxidants, following the method of Re et al (1999). ABTS (20 mM) and potassium persulfate (17 mM) were prepared as the stock solution and kept overnight at room temperature in the dark. The stock solution was diluted by mixing 1 mL of ABTS solution with 60% methanol to obtain an absorbance of 0.708±0.001 units at 734nm. 1ml of distilled water was added to 0.2ml of 5 different concentrations of the sample (5-250µg/ml from a stock concentration of 2mg/ml) and 0.16ml of ABTS solution was added and made up to the final volume of 1.36 ml. A tube with an equivalent amount of distilled water but without the test sample served as a control. The decrease in absorbance was taken after 20min at 734nm; against ascorbic acid as standard. The following formula was used to calculate the percentage inhibition:

$$\% \text{ Inhibition} = \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100$$

2.5 Nitric Oxide Scavenging Activity

In aqueous solutions at physiological pH, sodium nitroprusside spontaneously produces nitric oxide, which interacts with oxygen to create nitrite ions, which can then be measured with the Griess reagent (Patel et al., 2010). A solution of sodium nitroprusside (5mmolL⁻¹) was mixed with different concentrations of extracts (5-250µg/ml) from a stock concentration of 2mg/ml and incubated at 25°C for 30 minutes. A control without test compound, but an equivalent amount of distilled water was taken. The incubated

solution (after 30 minutes) was diluted with Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% N-1-naphthyl ethylene diamine dihydrochloride). The chromophore generated in this study was measured at 546nm and with reference standards of gallic acid was quantified in terms of its scavenging activity.

$$\% \text{ Inhibition} = \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100$$

2.6 Super Oxide Scavenging Assay

NADH oxidation generates a superoxide anion in the riboflavin-NADH system, which is detected by reducing NBT to blue formazan products (Valento et al., 2002). From the stock solution of 2mg/ml, 5 different concentrations of the sample (5 -250µg/ml) mixed with 0.05 ml of riboflavin (0.12 mM), 0.2 ml of EDTA solution (0.1 mM), and 0.1 ml of NBT solution (1.5 mM) were taken in test tubes, reaction mixtures were mixed with phosphate buffer (0.067M), a control without test compound, but distilled water in an equivalent amount. Triggering of reaction was done by illuminating the above solutions for 5mins using a fluorescent lamp. After 30 minutes, the absorbance was measured at 560 nm using a UV-visible spectrophotometer, percentage of scavenging superoxide anion generation was calculated as

$$\% \text{ Inhibition} = \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100$$

2.7 Hydroxyl Radical Scavenging Activity

The assay was based on a method to quantify ribose degradation by condensation with TBA (Elizabeth & Rao, 1990). Hydroxyl radical was generated by the Fe³⁺ - ascorbate - EDTA - H₂O₂ system (Fenton reaction). From the stock concentration of 2mg/ml, 5 different concentration of extracts were mixed with 500µl reaction mixture {2 deoxy 2 ribose (2.8mM), FeCl₃ (100µM), EDTA (100µM), H₂O₂ (1.0mM), ascorbic acid (100µM) in KH₂PO₄ - KOH buffer (20 mM; pH 7.4)} was made up to a final volume of 1 ml. Control was taken without a test sample but in an equivalent amount of distilled water. After incubation for one hour at 37°C, 2.8% TCA and 1ml of 1% aqueous TBA were added and reacted at 90°C for 15 minutes for color development. The absorbance of the cooling solution was measured against a blank solution at 532nm. Hydroxyl radical scavenging was calculated as:

$$\% \text{ Inhibition} = \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100$$

2.8 Cell culture

Mouse monocyte macrophage (Raw264.7) cells were initially procured from National Centre for Cell Sciences (NCCS), Pune, India, and maintained Dulbecco's modified Eagles medium, DMEM (Sigma Aldrich, USA). The cell lines were grown in DMEM with 10% FBS, L-glutamine, sodium bicarbonate, and the antibiotic solution containing Penicillin (100U/ml), Streptomycin

(100g/ml), and Amphotericin B (2.5g/ml). The cultured cell lines were kept at 37°C in a humidified 5% CO₂ incubator (NBS Eppendorf, Germany). Two days old confluent monolayers of cells were trypsinized, suspended in a 10% growth medium, seeded in 96 wells of a tissue culture plate at 37°C in a humidified 5% CO₂ incubator, and then incubated for 24 hours. 1mg of bark extract was dissolved in 1mL DMEM using a cyclomixer (stock sample), and then the sample was filtered using a 0.22 µm Millipore syringe filter to ensure sterility. After incubation, growth medium was discarded and freshly prepared stock samples in 5% DMEM were serially diluted (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of 5% DMEM). 100µl of diluted samples were added to corresponding wells (in triplicates) and incubated in a humidified 5% CO₂ incubator at 37°C. The cell viability was examined using an inverted phase-contrast microscope (Olympus CKX41 camera equipped with an Optika Pro5 CCD), followed by MTT assays. Microplate readers were used to measure absorbance values at a wavelength of 570 nm. The formula was used to calculate the percentage of growth inhibition,

$$\% \text{ cell viability} = \frac{\text{Mean OD Samples}}{\text{Mean OD of control}} \times 100$$

2.9 ELISA estimation of inflammatory mediators

The ELISA estimation is normally performed to quantify the level of different inflammatory markers (antigens) using a specific antibody (Engvall & Perlman, 1971). The confluent raw 246.7 cells were treated with a sample (PRME) at a stock concentration of 1mg/ml and kept at 37°C for 24 hrs in a CO₂ incubator. The supernatant was collected for protein estimation and transferred 100µL to 96 well plates and incubated at 37°C overnight for cell lysate coating. The cell lysate coated wells were blocked using blocking buffer containing 0.2% gelatin in 0.05% Tween 20 in PBS and incubated for 1 hour at room temperature, washed twice with PBS TWEEN20 and 100µl of Cox-2, IL1-β, IL-6, and NF-kB primary antibodies (Santa Cruz, USA) was added and left for 2 hours at room temperature, washed again with PBS TWEEN (2 times). 100µL of secondary antibody (HRP Conjugate, Santa Cruz, USA) was added and kept at room temperature for 1 hour, washed with PBS TWEEN two times, and added 200µL chromogen; O-dianizidine hydrochloride, incubated at room temperature for 30 minutes. The reaction was terminated by adding 50 µl 5N HCl and the absorbance was read at 450 nm after 5-30 minutes in an ELISA reader (Islam & Jones, 1988).

$$\text{Activity of Antibody} = \frac{\text{OD Value}}{\text{Protein concentration}}$$

2.10 Animal studies

Healthy female Sprague Dawley rats of 2-3month age and 200-250g body weight, procured from the animal house, Department of Biochemistry, University of Kerala were used for the study.

Animals were treated as per CPCSEA guidelines and the experimental protocol was approved by the Institutional animal ethical committee order number as follows; IAEC-2-KU-01/2018-19-BCH-AAR and IAEC-KU-09/2018-19-BCH-AAR; the dose-dependent toxicity study was sanctioned to be conducted in agreement with OECD guidelines. Rats were grouped into five groups of six animals each, group: I - control rats (normal saline) and group (II, III, IV, V) were administered with different doses of PRME 50, 300, 500, 1000mg/kg/day respectively and kept under observation for 14 days. Animals were scarified on the 15th day; blood was collected for biochemical investigations and stress marker studies. SOD activity was determined by the method described by Kakkar et al. (1984). The catalase activity was determined by the method of Maehly & Chance (1954). Glutathione reductase was assayed by the method of David & Richard (1983). The glutathione peroxidase was estimated by the method of Lawrence & Burk (1976) as modified by Agergaard & Jense (1982). The glutathione content of the tissues were determined by the procedure described by Patterson & Lazarow (1955).

2.11 Statistical analysis

Graph Pad Prism 5 software (GraphPad Software Inc.,) was used to calculate the Standard deviation, Two-way analysis of variance (ANOVA), and EC₅₀ values of different antioxidant assays. The Pearson correlation coefficient and p values < 0.05 were regarded as significant. Values expressed are means of three replicate determinations ± standard deviation.

3 Results and discussion

With the help of a single assay, we cannot understand the complete antioxidant profile of any natural product because the bio-potentiality of plant extracts is influenced by various physicochemical factors in a symbiotic pattern and most of the natural antioxidants are multifunctional due to the presence of abundant phytochemicals. So it is very essential to study the complete antioxidant profiling, to understand the antioxidant potential and its interactive mechanisms to eliminate free radical initiated toxicity in various disease conditions and also in metabolic pathways (Gan et al., 2010).

3.1 Antioxidant activity by ABTS and Total antioxidant assay

The ABTS radical scavenging and total antioxidant activity of PRME and PRAQ were estimated and compared with standard (ascorbic acid). The highest scavenging activity was exhibited by PRME followed by PRAQ (Figure 1). Being simple and reliable antioxidant measurement, the total antioxidant assay has been commonly used to evaluate the antioxidant capacity of plant extracts. The method is based on the reduction of Mo⁶⁺ to Mo⁵⁺ by

the antioxidants present in natural products and results in the formation of green phosphomolybdenum complex with the maximum absorption being calorimetrically measured at 695nm. The transfer of electrons from antioxidants to neutralize toxic radicals depends on the structure and binding moieties of antioxidant molecules (Lobo et al., 2010). The PRME showed a higher reduction (61.09g AA/100g extract) followed by PRAQ (57.96g AA/100g extract). The antioxidant activity confirmed the medicinal importance of both plant extracts as naturally occurring antioxidants.

ABTS is a protonated radical having a characteristic maximum absorbance at 734 nm and decreases with the scavenging of the $ABTS^+$ radicals. The plant extract possessing ABTS free radical scavenging activity indicated that it can act as a hydrogen donor, which converts free radicals to more stable products and thereby terminating the oxidation process (Ilyasov et al., 2020). The high molecular weight phenolics have an excellent capacity to scavenge $ABTS^+$ free radicals. Since both the extracts PRME and PRAQ can quench free radicals, thereby lowering the lipid oxidation process via chain-breaking reaction, these molecules can be considered as

novel antioxidants based on detailed biological studies. This might be due to the presence of hydroxyl groups in ortho, meta, and para positions of the phenolic compounds present in these extracts (Mathew et al., 2015). The studies on the antioxidant activity of different medicinal plants have indicated that the phenolic compounds are key players to scavenge ABTS radicals. The results showed that PRME with an EC_{50} value of 46.09 μ g/ml followed by PRAQ fractions (52.08 μ g/ml) showed good ABTS scavenging capacity, which can lower the oxidation process by reducing free radicals in a dose-dependent manner ($p < 0.05$). The correlation analysis showed good Pearson co-efficient relation between standard AA ($r=0.8988$) and PRME ($r=0.8585$) and PRAQ with r value 0.9160 respective.

3.2 Antioxidant activity by Nitric oxide and Superoxide radical scavenging assay

The nitric oxide and superoxide radical scavenging ability of both methanolic and aqueous bark extracts were compared with the ascorbic acid standard. The PRME showed better radical scavenging activity than PRAQ and is shown in figure 2. Nitric

Figure 1 The PRME extract gave the highest TAA with EC_{50} value 81.85 μ g/ml followed by aqueous EC_{50} value 86.26 μ g/ml. ($p < 0.05$). Values are expressed as mean $\pm$ standard deviation ($n=3$).

Figure 2 Shows the Nitric oxide and Superoxide radical scavenging activity of PRME and PRAQ ($p < 0.05$). Values are expressed as mean $\pm$ standard deviation ($n=3$).

oxide is an important chemical mediator involved in inflammatory mechanisms. Reactive nitrogen species (RNS) are free radicals that are formed due to the interaction of NO with oxygen, which leads to the production of different intermediates such as stable nitrates, NO₂, N₂O₄, and N₃O₄ (Ozcan & Ogun, 2015). The singlet oxygen (O₂) and NO radicals are directly involved in the lipid peroxidation process and chronic inflammatory cascades (Valko et al., 2007). The results showed a dose-response increase in the capacity to quench hydroxyl radicals for all the concentrations studied (Figure 2). A concentration-dependent scavenging of NO radicals by the PRME was significant at $p < 0.05$ ($r = 0.9786$, $p = 0.01$), followed by PRAQ ($r = 0.9778$) on correlation analysis. The PRME fraction showed the highest OH scavenging potential (EC₅₀ value of 177.21 µg/ml) in a concentration-dependent manner followed by PRAQ fraction 197.82 µg/ml.

The superoxide scavenging ability as measured by the riboflavin-NBT-light system indicates that the anion radicals produced due to the photochemical reduction of riboflavin lead to a decrease in blue formazan solution absorbance at 560 nm. The O₂ anion radical was inhibited in a dose-dependent manner as shown in figure 2. The EC₅₀ values of PRME and PRAQ were 76.24 µg/ml & 82.98 µg/ml respectively. These results indicated that PR bark fractions, especially methanolic fractions showed significant inhibition of superoxide radical, compared to positive control ascorbic acid (EC₅₀ values 75.18 µg/ml).

3.3 Hydroxyl radical scavenging assay

The hydroxyl radical scavenging capacity of both PRME and PRAQ, compared with standard gallic acid, is summarised in table 1. Hydroxyl radical is one of the dangerous reactive oxygen species in a biological system having the capacity to damage most of the biomolecules in our body. There is no specific enzyme or molecules available in the human body to eliminate or minimize their harmful effects (He et al., 2017). This free radical can combine with unwanted DNA nucleotides, resulting in a mismatch of base sequences and leading to ageing, genetic damages, and carcinogenesis (Sharifi-Rad et al., 2020). Hence eliminating the hydroxyl radical is a very crucial defence mechanism to keep our body healthy and the ability of PRME to reduce hydroxyl radical is considered to be good for preventing the lipid peroxidation process. The results showed a dose-response increase in the capacity to quench hydroxyl radicals for all the concentrations studied (Table 1). Inhibition of hydroxyl radicals by the methanol extract was found to be significant at $p < 0.05$ ($r = 0.9009$, $p = 0.01$) with gallic acid standard ($r = 0.9161$), followed by aqueous ($r = 0.8615$) on correlation analysis. The PRME fraction showed the highest OH scavenging potential (EC₅₀ value of 95.11 µg/ml) in a concentration-dependent manner followed by PRAQ fraction 181.29 µg/ml.

3.4 Antioxidant assays by correlation analysis

The results of the different antioxidant assays were compared, correlated with each other, and summarized in table 2. Significant correlations (p -value < 0.001) were observed for all the antioxidant assays. The TAA assay values showed good correlation coefficient 'r' values for ascorbic acid standard, PRME, and PRAQ with 0.9509, 0.9522, and 0.9560 respectively, Superoxide radical assay with ascorbic acid ($r = 0.9150$), PRME ($r = 0.9107$), and PRAQ ($r = 0.9032$). In hydroxyl radical scavenging assay also standard ($r = 0.9161$) showed close value to PRME ($r = 0.9009$). The correlation values fall in a range of ($r = 0.8585$) to ($r = 0.9786$), and this is due to the antioxidant activity of plants extracts which are influenced by various compounds like alkaloids, flavonoids, phenolics & glycosides (Tungmunnithum et al., 2018).

Table 1 Hydroxyl radical scavenging activity of PRME and PRAQ

Concentrations (µg/ml)	Gallic acid	PRME	PRAQ
25	16.50±1.43	17.16±1.06	13.50±1.46
50	31.36±1.53	34.05±1.34	33.24±1.57
100	40.70±1.67	43.59±1.73	38.62±1.43
200	56.58±2.46	52.73±1.95	45.81±1.81
400	65.66±2.35	63.11±2.47	55.16±2.85

Values are expressed as mean±standard deviation (n=3).

Table 2 Comparative correlation analysis of different antioxidant assays

Antioxidant assays	Pearson co-efficient values (r)		
	Standard	PRAQ	PRME
Total antioxidants assay	0.9509	0.9560	0.9522
Superoxide radical assay	0.9150	0.9032	0.9107
Nitric oxide radical assay	0.9427	0.9786	0.9778
Hydroxyl radical assay	0.9161	0.9009	0.8615
ABTS radical assay	0.8988	0.9160	0.8585

Values are expressed as mean±standard deviation (n=3).

3.5 MTT assay

The MTT assay is used to evaluate the cellular toxicity of any drug or plant material in *In vitro* culture. During plant material characterization, preliminary quantifying the toxicity in cell lines is a crucial step before moving to higher level molecular studies. The MTT assay showed good cellular viability of PRME up to a concentration of 100 µg/ml (Figure 3). The anti-inflammatory effect of PRME on the LPS-induced inflammation in RAW 264.7 cells (murine macrophage cell line) was assessed using MTT assay and the entire plate was observed after 24 hours of treatment in an inverted phase contrast tissue culture microscope. The morphological changes in cells including shrinkage, rounding,

granulation, and cytoplasmic vacuolization were regarded as the symptoms of cytotoxicity. The results showed that PRME does not influence the viability of RAW 264.7 cells and the cellular competency was selected for detailed study. LC50 Value of PRME was found to be 106.869 μ g/ml and the LC50 concentration was selected for evaluating the inflammatory mediators (Figure 3).

Figure 3 Effect of PRME on RAW cells. The graph represents the percentage cell viability on treatment with different concentrations of the extract. The values are represented as mean $\pm$ standard of three experiments.

3.6 Inflammatory marker study

The anti-inflammatory activity of PRME was evaluated using the ELISA method. On LPS treatment, the RAW cells shows the increased activity of inflammatory mediators, after the addition of PRME significantly reduced the activity of cyclooxygenase-2, interleukins - 1 β and 6, and nuclear factor-kB (Table 3). The LPS treated wells showed the antibody activity of Cox-2, IL-1 β , IL-6, and NF-kB in the order respectively 1.34996, 1.569258, 1.520547, and 1.624434, and this activity is significantly reduced after PRME treatment and incubation period for 24hrs as 1.15089, 1.084416, 1.03618 and 1.102227 (Table 3). During LPS treatment, the macrophages get activated and various inflammatory mediators and cytokines (Cox-2, IL-1 β , IL-6) are synthesized, which enhance the microenvironment for inflammation and further aggravate the conditions. The interleukins IL-1 β , IL-6 plays an important role in enhancing autoimmune diseases, bacterial infections, metabolic syndromes, malignant disorders, acute and chronic inflammation (Kany et al., 2019). These mediators along with other subsidiary factors activate the transcription factor NF-kB, a crucial checkpoint of inflammatory pathways because NF-kB controls nearly 150 genes involved in inflammation pathways, immune defense as well as in cell proliferation and differentiation mechanisms (Panday et al., 2016). During the LPS activation, NF-kB translocates from the cytoplasm to the nuclear region and acts

as a transcription factor for the synthesis of inflammatory enzymes and cytokines. Any agents which can inhibit or reduce the level of inflammatory mediators are considered good anti-inflammatory agents. The result demonstrated that PRME can inhibit the activity of inflammatory enzyme Cox-2 along with the interleukins, thereby reducing the production of pro-inflammatory cytokines, including IL-6 and IL-1 β in RAW 264.7 cells stimulated by LPS; which directly or indirectly suppress the activation of transcription factor NF-kB. These results suggest that PRME significantly improves LPS induced inflammatory conditions in RAW cells by inhibiting the overproduction of inflammatory mediators.

Table 3 Inflammatory marker study

Inflammatory markers	Activity Units/mg protein	
	Control	Sample
Cox-2	1.34996	1.15089
IL-1 β	1.569258	1.084416
IL-6	1.520547	1.03618
Nf-Kb	1.624434	1.102227

Values are expressed as mean $\pm$ standard deviation (n=3).

The antioxidant enzymes and stress markers are investigated from the liver tissue obtained from animal acute toxicity study for 14 days. After the PRME treatment, the animals were scarified and quantified the levels of different stress mediators in liver tissue are summarised in table 4. The first line antioxidant enzyme defense mechanism is from superoxide dismutase (SOD), catalase, and glutathione peroxidize, which convert the toxic superoxide and hydrogen peroxide radicals into water (Ighodaro & Akinloye, 2018). The PRME treated animals up to a concentration of 1000 μ g/ml did not show any significant variation in antioxidant enzymes when compared to normal rats. The enzyme catalase and SOD of the treated group showed good linearity with control rats, the glutathione peroxidize values exhibited a constant increase in the antioxidant activity in PRME treated animals in the range, (35.71 $\pm$ 1.05 to 39.73 $\pm$ 1.72) from table 4, which indicated the potent antioxidant activity of PRME, and it may be due to the presence of high phenolic content in bark extracts. Manna et al. (2009) reported that the bark extract of *P. acerifolium* significantly reduced alcohol-induced ulceration on oxidative damages in adult Charles foster rats, pre-treatment elevated the enzymes SOD, CAT, and GSH content when compared to the control group animal.

The oxidoreductase enzyme, glutathione reductase (GR) enhances the reduction of oxidized glutathione to reduced glutathione (GSH) in the presence of NADPH as a co-factor. The antioxidant enzyme (GR) and cellular thiol antioxidant (GSH) play a major role in cellular defense against drug and toxin-induced hepatotoxicity, associated with oxidative stress (Espinosa-Diez et al., 2015). Being a sensitive indicator of toxicity, both the values of GSH and GR in PRME treated groups were compared with normal control rats, the

Table 4 Stress marker study in liver tissue of rats

Parameters	Group I	Group II	Group III	Group IV	Group V
GPx(U/mg protein)	32.56 ± 1.38	32.69 ± 1.34	34.88 ± 1.34	34.80 ± 2.02	33.85 ± 1.79
GSH (U/mg protein)	82.86 ± 1.86	82.93 ± 2.07	82.65 ± 1.91	84.033 ± 1.95	83.78 ± 2.06
GRd(μmole of glutathione reduced/ min/mg protein)	144.67 ± 4.23	143.61 ± 3.31	146.62 ± 4.84	145.06 ± 4.49	143.22 ± 4.12
SOD (U/mg protein)	2.41 ± 0.19	2.45 ± 0.123	2.48 ± 0.12	2.57 ± 0.18	2.51 ± 0.26
Catalase (μmol of H ₂ O ₂ consumed/ min/mg protein)	7.43 ± 0.38	7.57 ± 0.25	7.53 ± 0.21	7.59 ± 0.28	7.56 ± 0.237

Values are expressed as mean ± standard deviation (n=6).

PRME treated group showed a slight increase in the values of GSH, a strong non-enzymatic antioxidant in 500 and 1000μg/ml treated groups, at the same time the levels of GR are comparatively high in all treated groups when compared to control rats 147.65 ± 4.08 (Table 4). From this study, we concluded that the PRME is rich in antioxidants, compared to PRAQ, and showed excellent antioxidant activity under *In vitro* condition; stress marker studies in rat liver cells confirmed the non-toxicity of PRME up to a concentration of 1000μg/ml for 14 days of treatment. MTT assay and inflammatory marker studies revealed the anti-inflammatory activity of PRME in reducing the LPS induced inflammation in RAW246.7 cells.

Conclusion

The present study revealed that PRME possesses excellent antioxidant and anti-inflammatory properties and could serve as free radical inhibitors, scavengers, or primary antioxidants. A positive correlation was detected for phenolic content and free radical scavenging activity of PRME. Toxicity evaluation studies on female Spargue Dawley rats showed that PRME up to a concentration of 1000μg/ml for 14 days is not harmful, indicating the prospects for more advanced investigations to better understand PRME's mechanism as a pharmacological agent.

Author Contributions

The authors contributed to this work as follows; extraction, antioxidant assays, anti-inflammatory analyses, and animal experiments were performed by R J Anish under the supervision of Arun R Rauf. Both the authors contributed to statistical and data analysis, manuscript preparation, reviewed the article, edited, and approved the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Abbreviations

PRME: *P. rubiginosum* methanolic extract; PRAQ: *P. rubiginosum* aqueous extract; ROS: reactive oxygen species; ABTS: 2, 2'-azino bis 3 ethyl benzothiazoline 6- sulfonic acid; TAA: total antioxidant activity; (MTT) : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; cox-2: cyclooxygenase-2; IL-1β: interleukins-1β; IL-6: interleukins-6; NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells; GR: glutathione reductase; GPx: glutathione peroxidase; GSH: reduced glutathione.

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