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Research Article

# Potential Investigation of *In Vitro* Antioxidant, Anti-Inflammatory and Anti-Haemolytic Activities from Polar Solvent Extracts of *Pterocarpus marsupium*

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**ABSTRACT**

*Pterocarpus marsupium* is a well known plant in ayurvedic system of medicine. The plant has been found to possess diverse number of biological activities and is thus commercially exploited. In the present study, methanol and aqueous extracts of *P.marsupium* bark were used to evaluate *in vitro* antioxidant, anti-inflammatory and anti-haemolytic activities. *In vitro* antioxidant activity was carried out by DPPH, ABTS, phosphomolybdenum, reducing power assays, anti- inflammatory activity by inhibition of protein denaturation and anti-haemolytic by hyposaline induced haemolysis. Total phenolic and flavonoid content was also estimated. Methanol extract showed an effective pharmacological activity in all assays when compared with their respective standards. The maximum phenolic and flavonoid contents were found to be (280 ±0.47mg/g) and (620±0.81mg/g) respectively in methanol extract of *Pterocarpus marsupium*. This study indicates the presence of active constituents which can be exploited for the treatment of various diseases and also could be used in pharmaceutical industry.

**Keywords:** *Pterocarpus marsupium* bark, antioxidant activity, anti-inflammatory activity, anti-haemolytic activity, total phenol and flavonoid content.

## INTRODUCTION

An individual cell generates excess free-radicals because of biochemical and pathophysiological processes in response to factors such as chemicals, environmental pollutants, radiation and toxins. This creates an imbalance between production of reactive oxygen species and defensive antioxidant molecules leading to oxidative stress in the physiological system, which results in numerous diseases such as cancer, arthritis, inflammation, infertility, acquired immunodeficiency syndrome and aging[1]. Excess production of pro-inflammatory molecules such as TNF-α and nitric oxide (NO) are known to cause cell death and tissue damage because NO can react with free radicals such as superoxides to produce peroxynitrite, that can lead to irreversible damage to cell membranes. Furthermore, erythrocytes are also highly susceptible to oxidative stress due to high cellular concentration of oxygen and haemoglobin, high polyunsaturated fatty acid content resulting in haemolysis[2]. Therefore, antioxidants are involved in the defense mechanisms of the organism against the pathologies associated to the attack of free radicals. Synthetic antioxidants play a major role in protecting biological systems against oxidative stress, but have been imposed restriction because of their remarkable side effects[3]. Consequently, natural antioxidants like crude extracts or their chemical constituents have considerably increased for use in food, cosmetic and pharmaceutical products which are safe and cost-effective. These natural antioxidants are composed of phytoconstituents such as flavonoids, carotenoids, terpenoids, alkaloids, polyphenols and phenolic compounds which exhibit antioxidant, anticancer activities and also act as excellent anti-inflammatory agents[4,5]. Thus, it is crucial to develop effective natural antioxidants and anti-inflammatory drugs that can protect the human body from free radicals and delay the growth of many chronic diseases.

*Pterocarpus marsupium* Roxb. is a large deciduous tree that occurs in parts of the Western Ghats in Karnataka-Kerala region. It is commonly known as Indian Kino or Vijayasar, belonging to family Fabaceae. The heart wood, bark, flowers and leaves have useful medicinal values. *P.marsupium* heartwood is astringent, bitter, anti-inflammatory, antihelmintic and anodyne[6]. Bark of this plant is considered as good source of natural antioxidants for free radical mediated ailments. The plant contains iso-flavonoids, terpenoids and phenolic compounds with strong antimicrobial, antioxidative, anti-inflammatory, antidiabetic and anticancer activities[7]. Numerous medicinal plants exhibit free radical scavenging properties but still there is a demand to find more information concerning the antioxidant potential of plant species[8]. In order to search for effective natural antioxidants and anti-inflammatory compounds, the current study is aimed on the exploration of *P.marsupium* bark extracts in different

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polar solvents to screen for potent antioxidant and anti-inflammatory activities.

## MATERIALS AND METHODS

*Chemicals*

Chemicals such as ABTS (2, 20-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), DPPH (1, 1 diphenyl-2-picrylhydrazyl), potassium persulfate, potassium ferricyanide and aluminium chloride were purchased from Sigma Chemicals (Steinheim, Germany). Gallic acid, Quercetin, Butylated hydroxytoluene (BHT), ascorbic acid and diclofenac sodium were purchased from HiMedia (India). All chemicals and solvents used in the study were of analytical grade.

*Plant material*

*Pterocarpus marsupium* Roxb. bark was collected from in and around the area of Kalaburagi district, Karnataka, India during the month of June, 2015. It was identified and authenticated by the Department of Botany, Gulbarga University, Kalaburagi, Karnataka.

*Preparation of plant extract*

Freshly collected bark of *Pterocarpus marsupium* Roxb. was washed thoroughly under running tap water and subsequently with distilled water, shade dried and then powdered to required particle size. The air dried bark powder (100g) was successively extracted by Hot Soxhlet extraction with polar solvents like methanol and water. The extract was heated at 40ºC till the solvent got evaporated. Dried extract was kept in refrigerator at 4ºC for future use.

*Total Phenolic Content*

The total soluble phenolic compounds in the methanolic and aqueous extracts of *Pterocarpus marsupium* bark were determined with Folin-Ciocalteu method[9].Gallic acid was used as a standard (100, 200, 400, 600, 800, 1000 µg/ml) and the calibration curve was plotted. Extracts and standard compound were diluted to the concentration of 1 mg/ml in respective solvents and was blend with 2.5 ml of Folin-Ciocalteu reagent and 2 ml of $NaHCO_3$ (7.5%). After 15 minutes at 45°C, the absorbance was measured at 765nm versus blank sample on UV-Visible Spectrophotometer. Distilled water was used as blank. Determination of total phenolic content was carried out with respect to Gallic acid standard curve. Measurements were carried out in triplicates. The results are expressed in term of milligrams of gallic acid per 100 g dried weight (mg GAE/100 g DW).

*Total flavonoid content*

Total flavonoid content was determined with aluminium chloride colorimetric assay[10]. Methanol and aqueous extracts of *Pterocarpus marsupium* bark and standard quercetin solution (1mg/ml) was positioned into test tubes, 4ml of distilled water and 0.3 ml of 5% sodium nitrite solution was piled up into each. After five minutes, 0.3 ml of 10 % aluminum chloride and at $6^{th}$ minute, 2 ml of 1M sodium hydroxide were added. Lastly, volume was made up to 10 ml with distilled water and orange yellowish color was developed. The absorbance was measured at 510nm spectrophotometer using UV-visible Spectrophotometer. The blank was prepared using distilled water. The samples were performed in triplicates. The calibration curve was plotted against standard quercetin and the results were expressed in term of milligrams of quercetin equivalents/ 100 g of dry weight.

*Antioxidant activity*

The free radical scavenging activity of the polar solvent extracts of *Pterocarpus marsupium* bark was determined by using various *in vitro* assays such as DPPH, phosphomolybdenum, ABTS and reducing power. All these procedures were done in triplicates.

*DPPH radical scavenging activity*

The antioxidant activity of methanol and aqueous extracts were evaluated on the basis of radical scavenging effect using stable DPPH assay[11].DPPH solution (0.004%,w/v) was prepared in 95% methanol and serially diluted the stock solution(10mg/ml) of different extracts. Different concentrations of extracts were mixed with DPPH solution (900µl), incubated in dark for 30 minutes and absorbance was measured at 517nm. DPPH solution as a reference; ascorbic acid and BHT as standard were used for this assay. The percentage of the DPPH radical scavenging is calculated using the equation and $IC_{50}$ value was calculated from the graph of inhibition percentage plotted against extract concentration

DPPH radical scavenging activity (%) = (Ac − As)/Ac × 100,

where, Ac is the absorbance of control and As is the absorbance of test samples.

*Phosphomolybdenum assay*

The antioxidant capacity can be calculated by the green phosphomolybdenum complex formed in the assay[12]. Different concentrations (5-100µg/ml) of various extracts are combined with 1mL of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tube is capped and incubated in a boiling water bath at 95˚C for 90 minutes. After cooling the sample to room temperature, the absorbance is measured at 695nm against blank in UV spectrophotometer. A typical blank solution contained 1mL of reagent solution and the appropriate volume of the same solvent used for the sample and it is incubated under same conditions as rest of the sample. Ascorbic acid and BHT are used as standard reference.

*ABTS radical scavenging activity*

ABTS radical cation decolorization activity was assayed with slight modification[13].ABTS radical cations were generated by reacting 7mM ABTS with 2.45mM potassium persulfate (1:1). The mixture was left to stand for 16hr in the dark at room temperature. The ABTS radical cation solution (100µL) was then diluted with ethanol (3.9ml) to give an absorbance of 0.700 ±0.02 at 734nm. Different concentrations (5-100µg/ml) of the polar extracts were mixed with diluted ABTS radical cation solution (1ml) and incubated at room temperature for 6 minutes. The absorbance was measured at 734nm using a UV-visible spectrophotometer. Ascorbic acid and BHT at the same concentration were used as standard reference. The radical scavenging activity was calculated as follows:

ABTS radical scavenging activity % = (Ac − As)/Ac × 100,

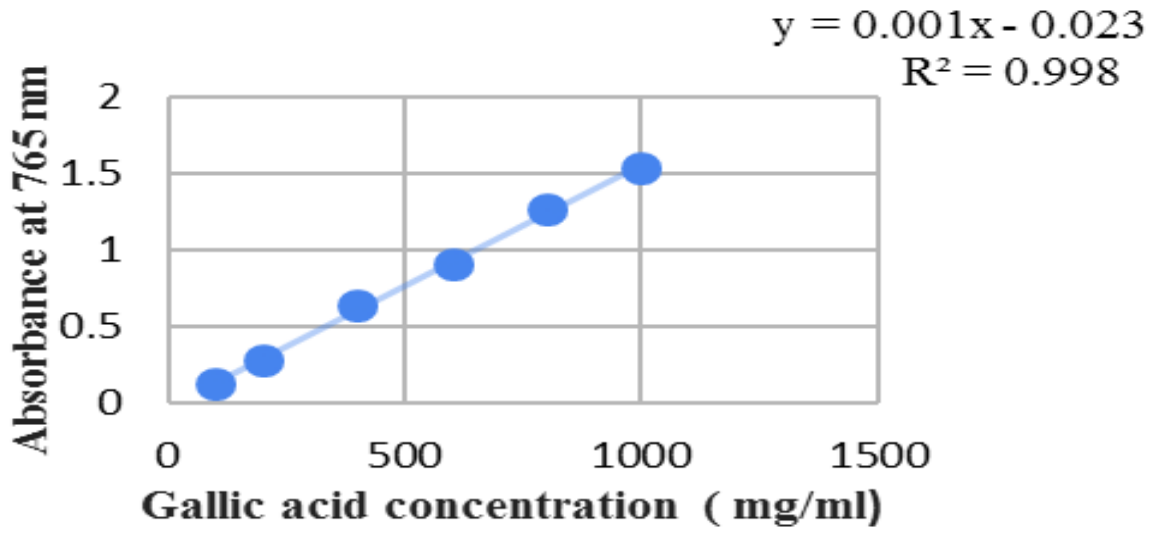


Figure 1: Standard Gallic acid Curve

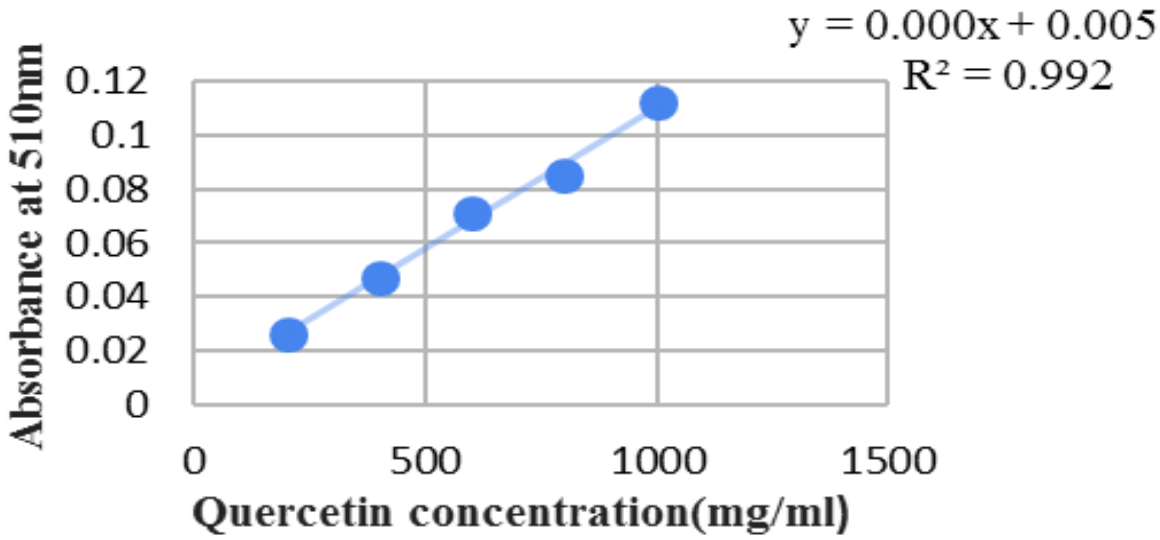


Figure 2: Standard Quercetin Curve

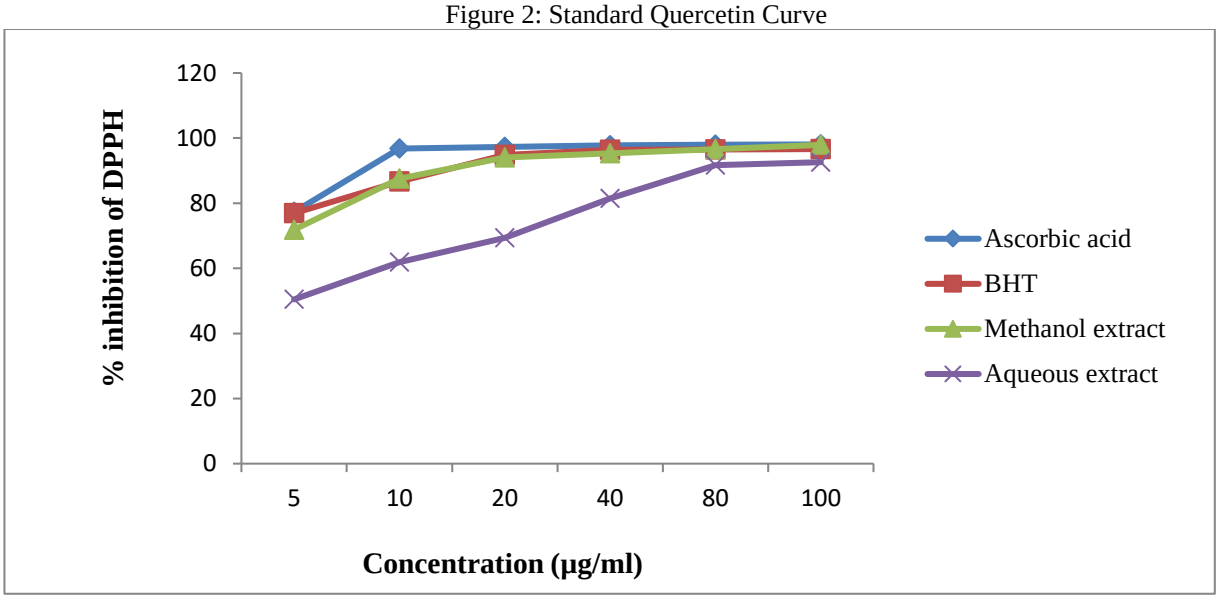


Figure 3: DPPH radical scavenging activity of ascorbic acid, BHT, methanol and aqueous extracts. Values are Means ± SD (n = 3)

where Ac is the absorbance of ABTS mixture and As is the absorbance of ABTS mixture with test samples.

*Reducing power assay*

The reducing power of methanol and aqueous extracts was determined with minor modifications[14].Various concentrations (5-100 µg/ml) of different extracts were mixed with 0.25ml phosphate buffer (0.2mM, pH 6.6) and 1% potassium ferricyanide (0.25ml). The reaction mixture was incubated at 50°C for 20 minutes. After the incubation period, 0.25ml of 10% trichloroacetic acid was added to the mixture, which was then centrifuged at 5000rpm for 5mins.The supernatant (0.5mL) was mixed with an equal volume of distilled water, and 0.1mL of ferric chloride solution (0.1%, w/v) and absorbance was measured at 700nm.The results are compared with Ascorbic acid, and BHT as a standard reference and the readings are expressed as the mean absorbance value.

*Anti-inflammatory activity*

*Protein denaturation method*

*In vitro* anti-inflammatory activity of different extracts of *Pterocarpus marsupium* bark was tested by the above method with slight modification[15]. The reaction mixture (5mL) consisted of 0.2mL of egg albumin (from fresh hen's egg), 2.8mL of phosphate buffered saline (PBS, pH 6.4) and 2mL of varying concentrations of the different extracts (final concentrations 62.5, 125, 250, 500, and 1000 µg/mL). An equal volume of double-distilled water served as a control. The mixtures were incubated at 37±2°C in an incubator for 15 minutes and then heated at 70°C for 5 minutes. After cooling, their absorbance was measured at 660nm (UV-1800 spectrophotometer, Shimadzu), with the vehicle used as a blank. Diclofenac sodium was treated similarly for determination of absorbance and used as a standard at the final concentrations of 62.5, 125, 250, 500, and 1000 µg/mL. The percentage inhibition of protein denaturation was calculated by using the following formula:

% Inhibition = (Vc -Vt/ Vc) × 100,

where Vt = absorbance of the test sample, Vc = absorbance of positive control.

The extract/drug concentration giving 50% inhibition ($IC_{50}$) was determined by plotting the percentage of inhibition relative to the control against treatment concentrations.

*Anti-haemolytic activity*

*Hyposaline induced haemolysis*

The hyposaline induced haemolysis was evaluated for *Pterocarpus marsupium* extracts[16]. Blood sample (5-10ml) was collected from healthy adult human volunteers in sterile Alsever's solution and used within 5hours of its blood collection. The blood samples were centrifuged at 3000rpm for 15minutes, and the packed cells obtained were washed with Phosphate Buffer Saline (PBS, pH 7.4) 3 times and 10% (v/v) RBC suspension was made with PBS. Assay mixture contained different concentrations of methanol and aqueous extracts, 1ml of PBS, 2ml of hyposaline (0.36%) and 0.5ml of 10% RBC suspension in various test tubes. Hyposaline was served as positive control. All the tubes were incubated at $37^0$C for 30 minutes and centrifuged. The colour of the supernatant due to haemoglobin release was measured at 540nm and the percentage of prevention of haemolysis of the extract was calculated using the relation

$$\text{Percentage prevention of haemolysis} = \frac{\text{Absorbance of treated sample} \times 100}{\text{Absorbance of control}}$$

*Statistical analysis*

Data was expressed as mean ± SD. Analyses of variance (ANOVA) were done for the comparison of results using Fischer's test. Statistical significance was set at $p<0.05$.

## RESULTS AND DISCUSSION

*Total Phenolic Content*

Based on the result obtained from phytochemical screening, the methanol and aqueous extracts are further subjected to quantitative estimation of phenols and flavonoids. Gallic acid was used as a standard compound and the total phenols were expressed as mg/g gallic acid equivalent using the standard curve equation: $y = 0.001x - 0.023$, $R^2 = 0.998$, Where y is absorbance at 765 nm and x is total phenolic content in the methanol and aqueous extracts of *Pterocarpus marsupium* expressed in mg/gm as shown in Figure-1.The maximum phenolic content was found to be 280±0.47mg/g in the methanol extract, followed by aqueous extract to be 200±1.24mg/g.

*Total Flavonoid Content*

The quercetin solution of concentration (1mg/ml) conformed to Beer's Law at 510nm with a regression co-efficient ($R^2$) = 0.992. The plot has a slope (m) = 0.000x and intercept = 0.005. The equation of standard curve is y = 0.000x + 0.005 as shown in Figure-2.The maximum flavonoid content was found to be 620±0.81 mg/g in the methanol extract, followed by aqueous extract to be 280±1.69 mg/g. Earlier reports also suggest that the phenol and flavonoid contents are in much higher concentrations in the methanol extract[17].

*Antioxidant activity*

*DPPH radical scavenging activity*

DPPH radical is a classical model to investigate the free radical scavenging activities of various plant extracts, which when encounters proton donors such as anti-oxidants, gets quenched and converts it to α,α-diphenyl-β-picryl hydrazine. The degree of discoloration indicates the scavenging potential of the plant extracts. The DPPH radical scavenging abilities of methanol extract was increased in a concentration-dependent manner similar to ascorbic acid and BHT followed by aqueous extract as plotted in Figure-3. $IC_{50}$ values were calculated from graph and were found to be methanol extract (5±0.04µg/mL), aqueous extract(9±0.21µg/mL), BHT(5±0.16µg/mL) and ascorbic acid (3±0.08µg/mL) respectively. These overall results reveals that the methanol extract is having a potential free radical trapping ability by donating protons and can serve as a source of free radical scavengers. Previous findings in our laboratory suggest that the methanol extract of *Amoora rohituka* bark has showed a pronounced DPPH radical scavenging activity comparable to ascorbic acid, a standard antioxidant drug[18].

*Phosphomolybdenum assay*

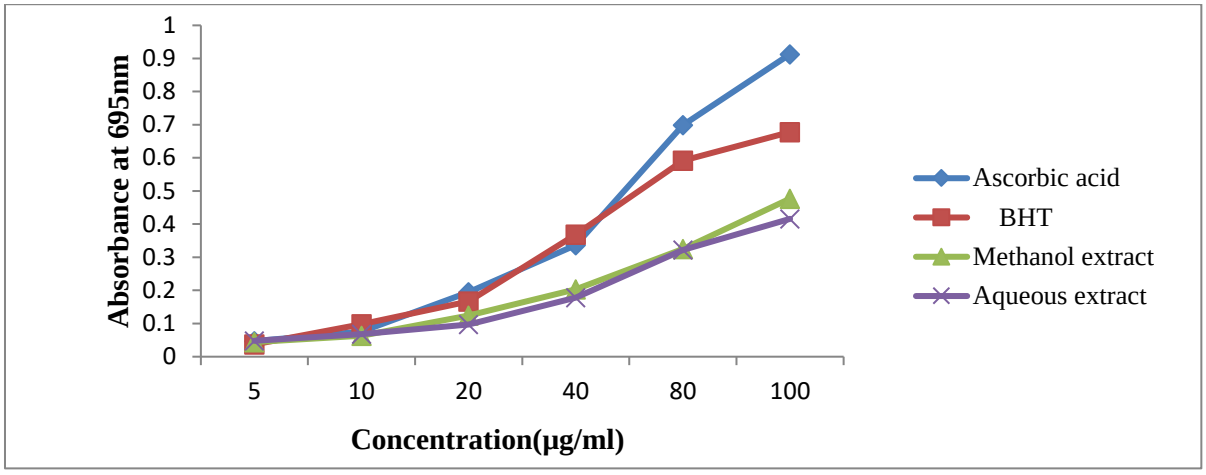


Figure 4: Phosphomolybdenum assay of ascorbic acid, BHT, methanol and aqueous extracts. Values are Means ± SD (n = 3).

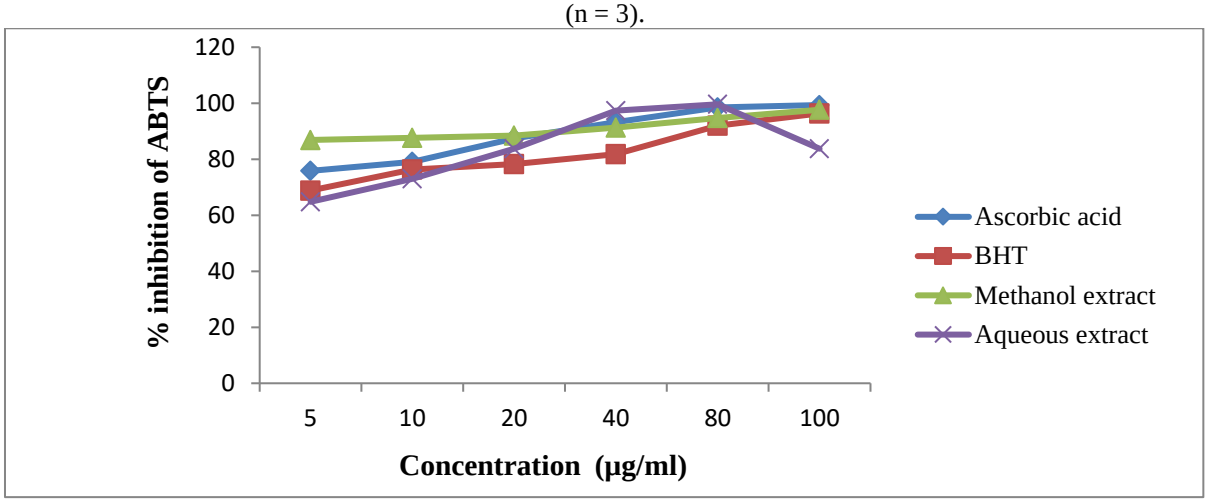


Figure 5: ABTS radical scavenging activity of ascorbic acid, BHT, methanol and aqueous extracts. Values are Means ± SD (n = 3).

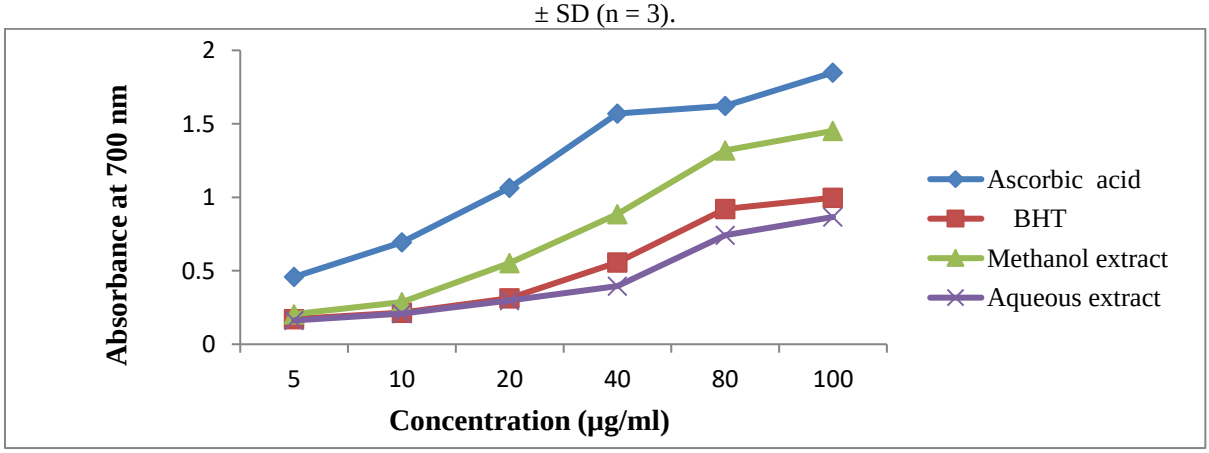


Figure 6: Reducing power of ascorbic acid, BHT, methanol and aqueous extracts. Values are Means ± SD (n = 3).

The assay is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and subsequent formation of a green phosphate Mo (V) complex at acidic pH, which is measured at 695nm. The reducing activity of methanol extract, aqueous extract, ascorbic acid and BHT are presented in Figure-4 which certainly represents a dose dependent curve. Phosphomolybdenum reduction assay increases with increase in concentration of methanol extract of *Hypericum hookerianum* leaves, which was in accordance with our results[19].

*ABTS radical scavenging activity*

The ABTS cation radical is formed by the loss of an electron by the nitrogen atom of ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) which absorbs at 743nm giving a bluish-green colour in the presence of antioxidant, yielding the solution decolorization[20]. Result of present study reveals that the methanol extract possesses

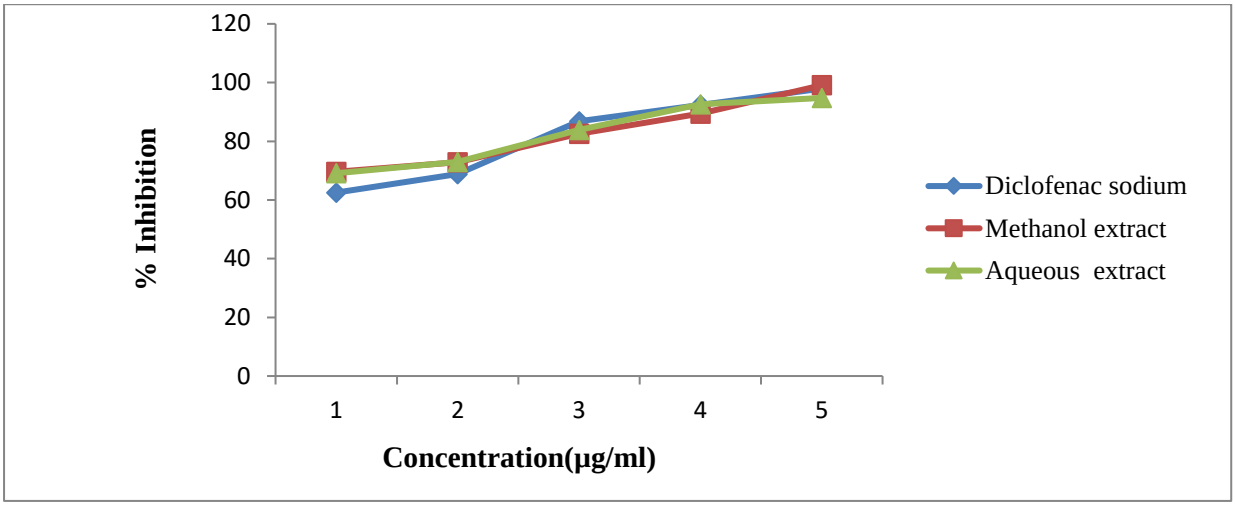


Figure 7: Percentage inhibition of diclofenac sodium, methanol and aqueous extracts against denaturation of protein. Values are means ± SD (n = 3).

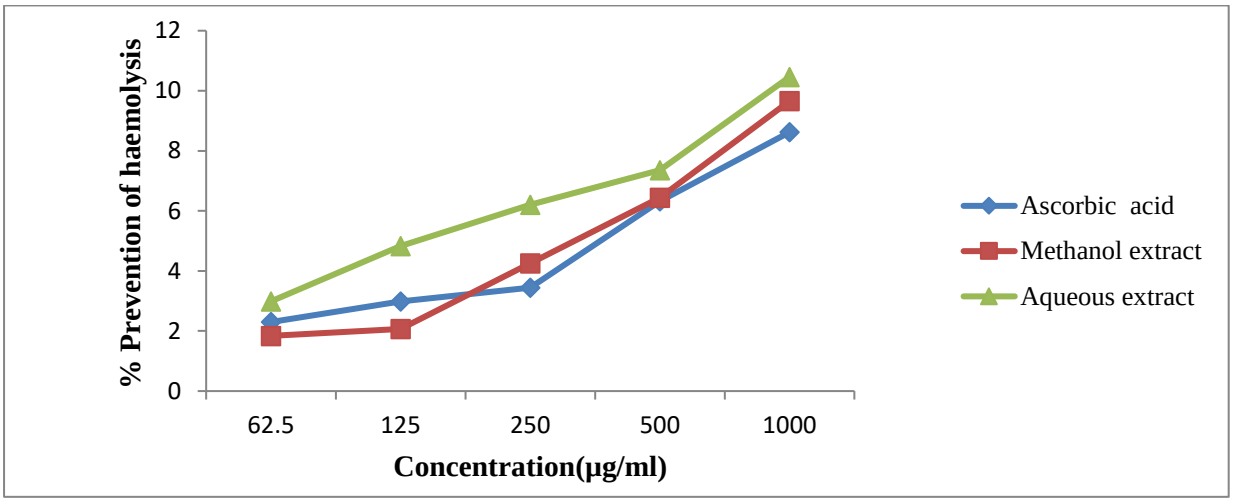


Figure 8: Percentage prevention of haemolysis of ascorbic acid, methanol and aqueous extracts of *Pterocarpus marsupium*. Values are means ± SD (n = 3).

superior antioxidant activity which is higher than standards, ascorbic acid and BHT as depicted in Figure-5.The $IC_{50}$ values of the methanol extract showed lower $IC_{50}$ value (3±0.38µg/mL) than aqueous extract(4±0.29µg/mL) when comparable with standards ascorbic acid and BHT (3±0.21 and 4±0.09µg/mL respectively) which is clearly indicating the percentage inhibition of ABTS radical scavenging activity was concentration-dependent with increased in the reaction mixture for the extracts and the standards. Similar results were observed in the methanolic extract of *Adiantum caudatum* leaves where it has exhibited radical scavenging activity in a dose-dependent manner[21].

*Reducing power assay*

The reducing ability of a compound generally depends on the presence of reductants which have been exhibiting antioxidative potential by breaking the free radical chain and donating a hydrogen atom[22]. The presence of reductants in the extracts causes the reduction of the Fe3+/ferricyanide complex to the ferrous form, monitored by measuring the formation of Prussian blue at 700nm. As shown in Figure-6, a higher absorbance value indicates a stronger reducing power of ascorbic acid and methanol extract exhibiting concentration-dependent reaction followed by BHT and aqueous extract. This concentration-dependent activity pattern was also followed by the methanol extract of *Syzygium cymosum* bark[23].

*Anti- inflammatory activity*

*Protein denaturation method*

Denaturation of tissue proteins is one of the well-documented causes of inflammatory and arthritic diseases. Production of auto antigens in certain arthritic diseases is because of in vivo protein denaturation. Thus, any plant material that prevents protein denaturation could be an effective anti-inflammatory agent[24]. The results are summarised in Figure-7, suggests that the methanol and aqueous extracts showed similar anti-inflammatory activity in a concentration dependent manner with standard anti-inflammatory drug diclofenac sodium. The difference was further confirmed by comparing the respective $IC_{50}$ values. The methanol and aqueous extracts showed an $IC_{50}$ value of 45±1.6/mL,45±0.94µg/mL, whereas that of diclofenac sodium was found to be 55±0.24µg/mL. Therefore, it can be concluded that *P.marsupium* extracts

have a distinct anti-inflammatory effect against inhibition of protein denaturation *in vitro*.

*Anti-haemolytic activity*

*Hyposaline induced haemolysis*

The study of erythrocytes is a good model for direct indication of toxicity of injectable formulation and membrane, thus extensively used for oxidative stress studies. Exposure of red blood cell to hypotonic medium leads to membrane lysis and oxidation of haemoglobin which results in secondary damage through free radical induced lipid peroxidation[25]. In the current study, the protective effect of *Pterocarpus marsupium* bark extracts was investigated. The vulnerability of erythrocytes to haemolysis in presence of methanol extract was lower than aqueous extract that was comparable with standard ascorbic acid as shown in Figure-8, suggesting concentration dependent activity. This discovery was equivalent to the earlier study on methanolic extract of *Costus speciosus* leaves which showed the concentration dependent anti-haemolysis of RBCs[26].

## CONCLUSION

From the results of present investigation, it can be concluded that the methanol extract of *P.marsupium possesses* a strong antioxidant, anti-inflammatory and anti-haemolytic potentials that is due to the contribution of its phenolics and flavonoid contents. These findings also suggest that this plant have a potential source of natural pharmacological agents which might be helpful in preventing the progress of various chronic diseases. However, further studies are needed for the isolation and identification of bioactive compounds and its evaluation in *in vivo* models to understand their exact mechanism of action as an antioxidant and anti-inflammatory agents.

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