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Comparative screening of *in-vitro* free radical scavenging, anti-inflammatory and anti-haemolytic activities from non-polar solvent extracts of *Pterocarpus marsupium*

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Abstract

Pterocarpus marsupium is widely used as 'Rasayana' in ayurvedic system for curing several medical ailments. In view of this, petroleum ether and chloroform extracts of *Pterocarpus marsupium* bark was scrutinized to unfold free radical scavenging by measuring their capability for scavenging DPPH radical, ABTS, phosphomolybdenum assay as well as reducing power capacity assessment and anti- inflammatory activity by inhibition of protein denaturation. Additionally, RBC'S were also used to explore the potentiality of *Pterocarpus marsupium* different extracts to shield RBC in oxidative stress condition. Chloroform extract showed an effective pharmacological activity in all assays when compared with petroleum ether extract and their respective standards. This reveals the use of *Pterocarpus marsupium* as a potent source of antioxidant, anti-inflammatory, anti-haemolytic agent and would provide an ample opportunity for further investigation. Isolation of active compounds from this plant responsible for producing such bioactivities is under process.

Keywords: *Pterocarpus marsupium,* antioxidant activity, anti-inflammatory activity, anti-haemolytic activity, petroleum ether extract, chloroform extract

Introduction

Free radicals are regarded as fundamental biochemistry which forms an important part of aerobic metabolism. They are also important for maintaining normal physiological functions such as signaling molecules, regulation of signal transduction and gene expression, activation of receptor and nuclear transduction [1]. Imbalance of this metabolism due to metabolic and other environmental factors generates excessive ROS that give rise to oxidative stress. This results in the damage of various biomolecules like DNA, lipids and proteins which is affiliated with several illnesses like cancer, neurodegenerative diseases, atherosclerosis, infertility, hepatitis and rheumatoid arthritis [2]. It also results in an inflammatory pathological state characterized by increased expression of pro-inflammatory mediators, chemokines i.e.TNF-alpha, cytokines, recruitment of adhesion molecules and caspases which are the crucial factor in many human diseases [3].To overcome these hazards, human body has their own antioxidant defense mechanism that is synthesized within the body or taken in diet form. Accordingly, antioxidants act as free-radical scavengers by safeguarding living entity from destruction generated by abnormal ROS production. In recent years, the quest for natural antioxidants is gaining importance in food processing and pharmaceutical industry due to their presumed safety, nutritional and therapeutic values that restitute unnatural antioxidants which are restrained for their carcinogenicity [4]. Scientific research on medicinal plants has implied antioxidant presence such as tannins, phenolics, flavonoids and its consumption reduced morbidity, mortality caused from degenerative disorders [5]. In addition to these biological properties, plants have reconnoitred for anti-hemolytic activity to cure anemia-related disorders. RBC's are found in ample amount in an individual, where oxidation reduces membrane protein components and disrupts micro-constituents that lead to their lysis, liberating haemoglobin, which can be quantified by a spectrophotometer [6]. Hence, substantial attention has been focused towards credentials of plants with antioxidant, anti-inflammatory and anti-haemolytic abilities.

Pterocarpus marsupium is a huge tree, generally known as Vijayasar or Indian Kino. It is belonging to family Fabaceae, grown in India, Nepal and Srilanka. The heartwood of this plant are used as an anti-inflammatory ,anti-helmintic, astringent and treating diarrhoea, skin diseases, diabetes, asthma, bronchitis etc [7]. Different pieces of bark are used as antacid, anti-diarrheal, for

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treatment of toothache and diabetes [8]. A collection of flavonoids, their derivatives is isolated from various portion of plant and are also a opulent source of polyphenolic compounds [9].Thus, considering its traditional claims, chemical constituents and pharmacological activities, the current research work is designed.

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).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 Kalaburagi district, Karnataka, in midst 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 beneath running faucet water and later through distilled water, shade dried and then powdered to required particle size. The bark powder (100g) was successively extracted by Hot Soxhlet extraction with non polar solvents like petroleum ether and chloroform. The extract was heated at 30-40ºC in hot air oven till the solvent got evaporated. Dried extract was kept in refrigerator at 4ºC for future use.

Antioxidant activity

The antioxidant activity of non polar solvent extracts was determined by different *in vitro* assays such as DPPH, phosphomolybdenum, ABTS and reducing power. The experiments were carried out in triplicates.

DPPH radical scavenging activity

The free radical scavenging activity of bark extracts against stable DPPH was performed using the method [10]. Briefly, 0.004%w/v of DPPH radical solution was prepared in methanol; 900µl of this solution was mixed with varying concentration of extracts or standard ascorbic acid solution and kept in dark place for 30 minutes. The absorbance was measured at 517nm. Scavenging capacity of DPPH radicals (%Inhibition) was measured by the following formula and finally calculated 50% inhibition concentration (IC_{50}) 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 activity of samples was evaluated by the green phosphomolybdenum complex according to the method of Prieto et al [11]. Different concentrations (5-100µg/ml) of extracts are combined with 1mL of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate) and incubated in a boiling water bath at 95˚C for 90 minutes. After incubation, these tubes were normalized to room temperature for 20 minutes and the absorbance of the reaction mixture was measured at 695nm against a blank using Ascorbic acid and BHT as standard reference.

ABTS radical scavenging activity

ABTS radical cation decolorization activity was determined by the method of Izabela et al [12] with a slight modification. ABTS and potassium persulfate were dissolved in distilled water to a final concentration of 7mM and 2.45mM respectively. These two solutions were mixed and the mixture was allowed to stand in the dark at room temperature for 16h before use in order to produce ABTS radical (ABTS•+). The ABTS radical solution was diluted with ethanol to give an absorbance of 0.700±0.02 at 734nm. Different concentrations (5-100µg/ml) of non polar extracts were added to diluted ABTS radical cation solution (1ml) and the absorbance reading was taken 6 minutes after mixing using the spectrophotometer at 734nm. The ABTS scavenging capacity of the extract was compared with that of BHT and ascorbic acid and percentage inhibition calculated as ABTS radical scavenging activity.

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

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

Reducing power assay

The total reducing power of each extract was determined according to the method described by Parisa et al [13]. Different concentrations of the plant extracts in corresponding solvents were mixed with phosphate buffer (0.25ml) and potassium ferricyanide (0.25ml). This mixture was kept at 50ºC in water bath for 20 minutes. After cooling, 0.25ml of 10% trichloro acetic acid was added and centrifuged at 5000rpm for 10 minutes. The upper layer

of solution (0.5ml) was mixed with distilled water (0.5ml) and a freshly prepared ferric chloride solution (0.1ml). The absorbance was measured at 700nm. Control was prepared in similar manner excluding samples. Ascorbic acid, BHT at various concentrations was used as standard.

Anti-inflammatory activity

Protein denaturation method

The inhibition of albumin denaturation was assessed according to the modified method of Asha *et al* [14].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).The mixtures were incubated at 37 C for 15min and then heated at 70 C for 5min. After cooling, their absorbance was measured at 660 nm (UV-1800 spectrophotometer, Shimadzu), with double distilled water used as a blank. The diclofenac sodium was used as reference control. The turbidity was determined using UV-VIS spectrophotometer at 660 nm. The percentage inhibition of protein denaturation was determined by the formula and IC_{50} value was calculated.

% Inhibition = (Vc -Vt/ Vc) 100,

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

Anti-haemolytic activity

Preparation of human erythrocyte suspension and haemolysis assay

The human blood sample (5-10ml) were obtained from healthy volunteers in sterile Alsever's solution and centrifuged at 3000 rpm for 15minutes. The packed cells were washed with sterile Phosphate Buffer Saline (PBS) 3times and 10% erythrocyte suspension was prepared for haemolytic study. The haemolytic activity of the crude extract was tested under in vitro conditions [15]. To 0.5ml of freshly prepared human erythrocyte suspension, various concentrations (62.5–1000µg/ml) of extract were added with 2ml hyposaline solution. After 30 minutes incubation at room temperature, cells were centrifuged and the supernatant was used to measure the absorbance of the liberated haemoglobin at 540nm. Two controls were prepared without extracts; negative control received sterile phosphate buffer saline, while positive control received hyposaline. The average value was calculated from triplicate assays. Haemolysis percentage for each sample was calculated by the following equation

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

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

Antioxidant activity

DPPH radical scavenging activity

DPPH assay mainly depends on the hydrogen donating capacity to scavenge DPPH radicals. Antioxidants respond to the DPPH by reducing the DPPH molecules which is equivalent to their OH ions prevailing. The reduced capacity of the DPPH radical, when a DPPH electron binds to a radical scavenger is estimated in colour change from purple to yellow suggesting that the decrease of absorbance by antioxidants action is proportional to the number of residual DPPH [16].The free radical scavenging abilities of chloroform extract (73.77%) was increased in a concentration-dependent manner followed by petroleum ether extract (50.73%) compared with standards ascorbic acid(96.14%) and BHT(89.81%) is plotted in Figure-1.These findings were comparable to the previously reported chloroform extracts of *Sida acuta* that showed high inhibition at low concentration compared with standard reference [17].Further, DPPH result was also expressed as IC_{50} (half maximal inhibitory concentration) value and lower value indicates better antioxidant capacity as presented in Table-1.

Table-1. IC_{50} values of DPPH, ABTS and absorbance of Phosphomolybdenum, Reducing Power of ascorbic acid, BHT, petroleum ether and chloroform extracts of *Pterocarpus marsupium* bark

Samples	DPPH (µg/mL)	ABTS (µg/mL)	Phosphomolybdenum (Abs. at 695 nm)	Reducing power (Abs. at 700 nm)
Ascorbic acid	3±0.081	3±0.047	0.37±0.004	1.02±0.008
BHT	3.5±0.14	4±0.12	0.29±0.016	0.56±0.020
Petroleum ether extract	8±0.16	6±0.20	0.039±0.021	0.66±0.036
Chloroform extract	5±0.09	4±0.21	0.1±0.016	1.22±0.032

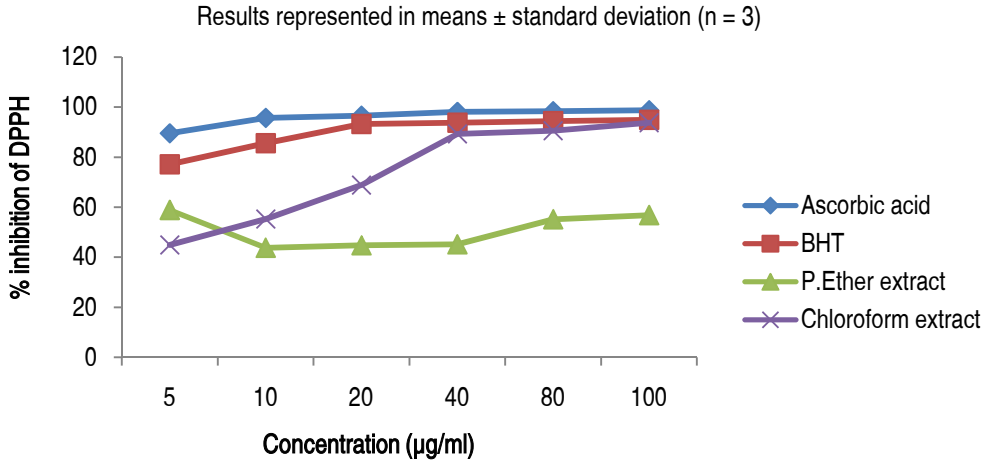

Figure-1.DPPH radical scavenging activities of ascorbic acid, BHT, petroleum ether and chloroform extracts. Values are Means ± SD (n = 3)

Phosphomolybdenum assay

This method is based on the reduction of phosphomolybdic acid to phosphomolybdenum blue complex by sodium sulfide. The obtained phosphomolybdenum blue complex is oxidized by the addition of nitrite and this causes a reduction in intensity of the blue colour. The antioxidant capacity of petroleum ether extract, chloroform extract, ascorbic acid and BHT are presented in Figure-2 and Table-1, which certainly indicates dose dependent curve. The chloroform extract of *Withania somnifera* has showed maximum antioxidant capacity in term of ascorbic acid equivalent with maximum phenol content that is in similarity with our results [18].

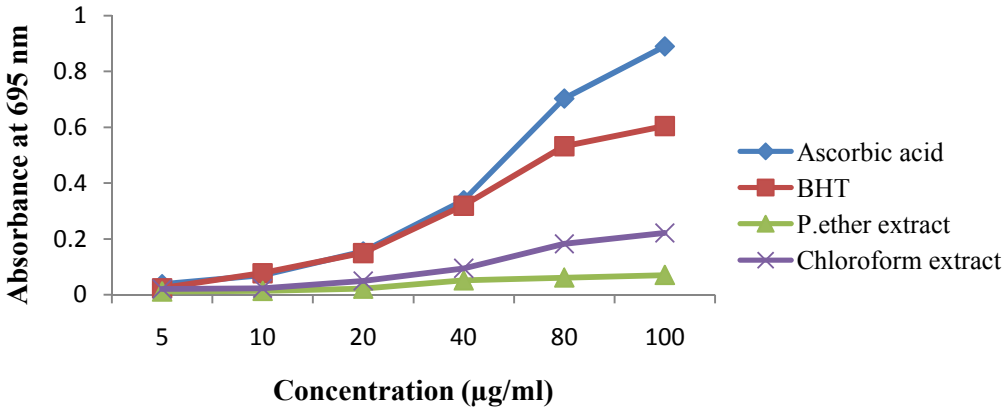

Figure-2. Phosphomolybdenum assay of ascorbic acid, BHT, petroleum ether and chloroform extracts. Values are Means ± SD (n = 3)

ABTS radical scavenging activity

ABTS (2-2`azinobis [3-ethylbenzthiazoline-6-sulphonic acid]) assay is a powerful assay used to determine the chain-breaking antioxidants in case of lipid peroxidation and antioxidant activity of hydrogen donating antioxidants. This assay involves the oxidation of ABTS to form an intensely-green colored nitrogen-centered ABTS•+ [19]. As showed in Figure-3, the chloroform extract of *Pterocarpus marsupium* exhibited the highest antioxidant activity (83.80%) whereas, petroleum ether extract of the plant has showed the lowest antioxidant activity (73.83%) clearly indicating chloroform extract acted as a potent antioxidant agent when compared with standards ascorbic acid (88.89%) and BHT (82.27%). A similar result was observed in the chloroform extracts of *Bryonopsis laciniosa* where it exhibited a highest antioxidant activity followed by ascorbic acid and BHT (90.45%, 68.83% and

55.05%, respectively) [20]. The IC_{50} values of each sample are presented in Table-1.

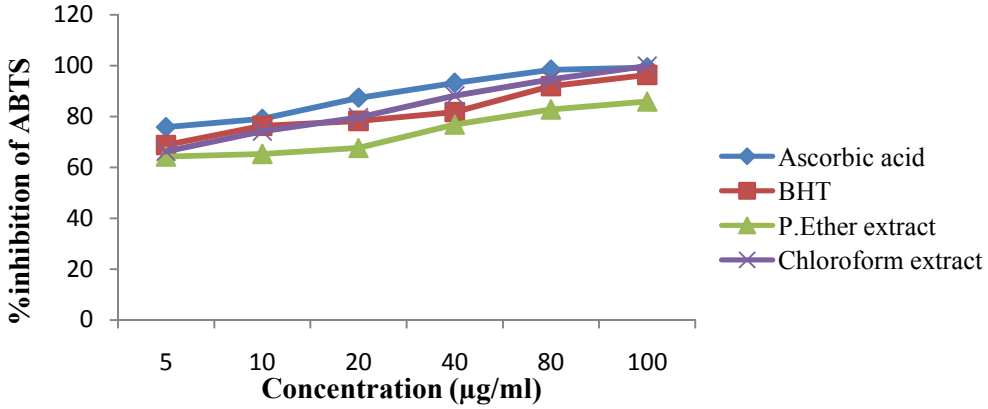

Figure-3.ABTS radical scavenging activities of ascorbic acid, BHT, petroleum ether and chloroform extracts. Values are Means ± SD (n = 3)

Reducing power assay

Reducing power assay involves the reduction of Fe^{3+}/ferricyanide complex to the Fe^{2+}/ferrous that can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increased absorbance of the reaction mixture indicates increase in reducing power [21]. As depicted in Figure-4 and Table-1, the chloroform extract had shown good reducing power than petroleum ether extract that was comparable with standard ascorbic acid and BHT which indicates the presence of reductants or antioxidants in extract which may be responsible for exerting its reducing power.These results are in agreement with the previous study where chloroform extract of *Thysanolaena maxima* revealed the reductive capabilities compared to ascorbic acid which had the highest reductive activity than the other extracts [22].

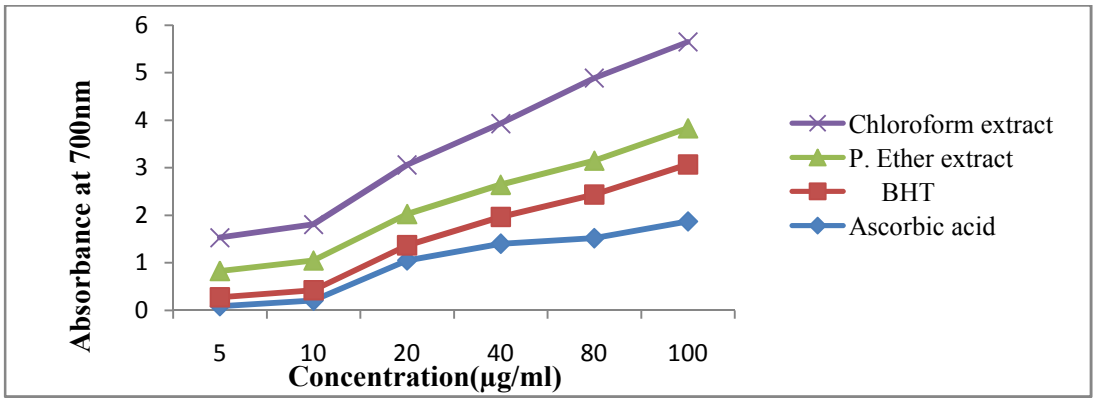

Figure-4. Reducing powers of petroleum ether extract, chloroform extract, BHT and ascorbic acid. Values are Means ± SD (n = 3)

Anti-inflammatory activity

Protein denaturation method

Denaturation of proteins is a well-documented cause of inflammation and arthritis, because of auto antigen production. Numerous non-steroidal anti-inflammatory drugs (NSAIDs) exhibited dose dependent ability to inhibit thermally induced protein denaturation, but the ability of plant material to bring down the protein denaturation could be more effective against inflammation diseases [13]. Hence, the *in vitro* anti-inflammatory effect of *P.marsupium* extracts were evaluated for its ability to prevent denaturation of egg albumin. As shown in Figure-5, petroleum ether and chloroform extracts have showed significant inhibition of egg albumin denaturation in a concentration dependent manner when compared with control diclofenac sodium. Further, petroleum ether and chloroform extracts have showed an IC_{50} value of 485±0.81µg /mL,150±0.94µg/mL respectively, whereas that of diclofenac sodium was found to be 195±1.24µg/mL. Therefore, it can be stated that the *P.marsupium* extracts are capable of controlling auto antigen production and thereby show remarkable anti-

inflammatory activity against inhibition of protein denaturation *in vitro*

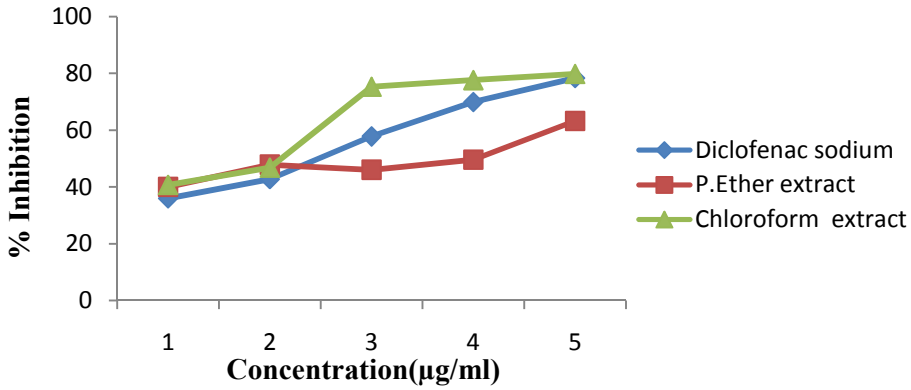

Figure-5. In vitro anti-inflammatory activity of petroleum ether and chloroform extracts comparison with Standard (Diclofenac sodium). Values are means ± SD (n = 3).

Anti-haemolytic activity

Preparation of human erythrocyte suspension and haemolysis assay

RBC haemolysis is a sensitive method for evaluating antihaemolytic properties of medicinal plants. Exposure of HRBC to injurious substances such as hypotonic medium, results in the membrane lysis accompanied by haemolysis, rendering the cell more susceptible to secondary damage through free radical-induced lipid peroxidation [24]. In this study, chloroform extract significantly stabilized red blood cell membrane, an indication of the extracts ability to prevent haemolysis in hypotonic-stress induced condition when compared with petroleum ether extract and standard ascorbic acid as shown in Figure-6. Membrane stabilizing profiles of various extracts of *Lantana camara* on bovine red blood cell exposed to both heat and hypotonic induced lyses were reported previously [25].

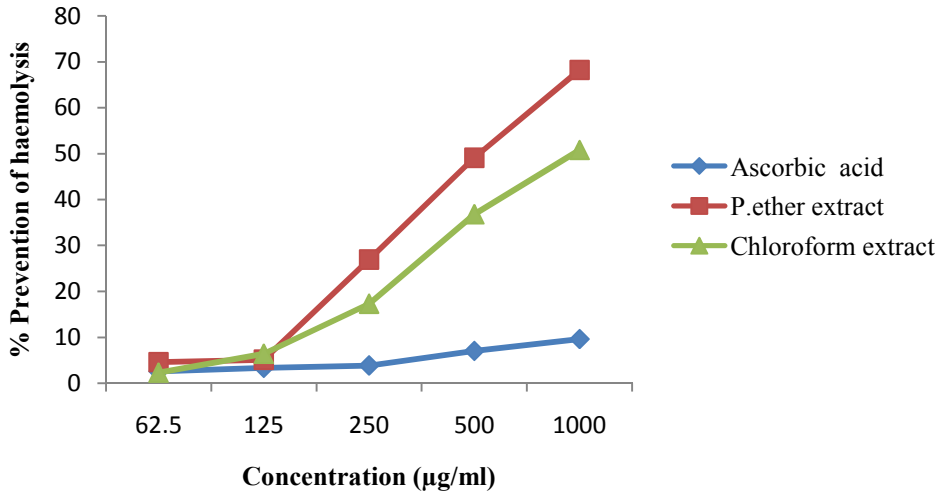

Figure-6. Percentage prevention of haemolysis of ascorbic acid, petroleum ether and chloroform extracts of *Pterocarpus marsupium*. Values are means ± SD (n = 3).

Conclusion

Based on the above investigation, *Pterocarpus marsupium* extracts exhibited significant antioxidant and anti-inflammatory properties,

besides safeguarding against RBC oxidative damage which contributed for the presence of phenolic and flavonoid compounds.

Thus this study suggests that the extracts could be of great importance in therapeutic drugs for the implications of human health. Hence, further work is under progress to isolate and elucidate the bioactive principles and to evaluate their mechanisms of action of specific bioactivities.

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