

## ORIGINAL ARTICLE



# *In vitro* antioxidant and anti-inflammatory activities of the chloroform extract of *Combretum punctatum* var *squamosum*

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The objective of this study was to investigate the antioxidant and anti-inflammatory activities of the chloroform extract of *Combretum punctatum* var *squamosum* by *in vitro* models. Antioxidant activity was determined by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, reducing power and lipid peroxidation assays. The phenolic and flavonoid contents of the extract were also assessed. The extract at different concentrations showed significant percent inhibition of DPPH and lipid peroxidation and high reducing power when compared with the standard. The extract also showed a significantly high content of phenolics and flavonoids. Anti-inflammatory activity was studied by inhibition of albumin denaturation and human red blood cell (HRBC) membrane stabilization methods. The extract at different concentrations showed significant percent inhibition of albumin denaturation and percent inhibition of haemolysis when compared with the standard. Thus, the result indicates that the chloroform extract exhibited significant potential on anti-inflammatory and antioxidant activities.

**Keywords:** Anti-inflammatory, antioxidant, *Combretum punctatum* var *squamosum*, chloroform, inhibition.

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## Introduction

Plants have played an important role in the treatment of human diseases since ancient times till today, particularly in the developing world.<sup>1</sup> They have always acted as important sources of therapeutically active compounds.<sup>2</sup> The utilisation of plants for curing illnesses was based only on experiences and no sufficient information is available on the usage of medicinal plants for the treatment of diseases. In time, discovery of the reasons for the usage of specific medicinal plants for treatment of certain diseases was achieved and thus, the medicinal plants' usages are gradually based on scientific data and facts.<sup>3</sup> There is a great challenge on research of the traditional medicinal plants for their constituents and therapeutic activities.

*Combretum punctatum* var. *squamosum* (Roxb. Ex. G. Don), belonging to the family Combretaceae, is a deciduous climber found mainly in South-east Asian countries like India, Myanmar, Bangladesh, Bhutan, Nepal, Philippines, Thailand, Vietnam and China. It is locally known as '*leihruisen*' in Mizo language.<sup>4</sup> Traditionally, the juice of the fresh leaves is applied to wounds and cuts to stop bleeding; the broths of boiled leaves are taken as medicine for diarrhoea and cholera.<sup>5</sup> However, no proper investigations on activity of the plant have been done. The present study aimed for *in vitro* evaluation of anti-inflammatory and antioxidant activity of the plant.

## Materials and Methods

### Materials

Soxhlet apparatus, petroleum ether, chloroform, simple distillation unit, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), ultraviolet-visible (UV-Vis) spectrophotometer, methanol, incubator, ascorbic acid, potassium ferricyanide, trichloroacetic acid, centrifuge, gallic acid, Folin-Ciocalteu reagent, sodium carbonate, quercetin, sodium nitrite (NaNO<sub>2</sub>), aluminium chloride (AlCl<sub>3</sub>), sodium hydroxide (NaOH), egg yolk, FeSO<sub>4</sub>, acetic acid, sodium dodecyl sulphate, butanol, diclofenac sodium, hydrochloric acid (HCl), bovine albumin, human red blood cell (HRBC), Alsevers solution, normal saline, hyposaline, test tubes, etc.

### Collection of plant material

The leaves of *Combretum punctatum* var. *squamosum* were collected from Khanpui village, Aizawl District, Mizoram, NE India during April, 2018. The plant was identified by Botanical Survey of India, Kolkata. A voucher specimen (IBTH-1) is being preserved in the Department of Pharmacy, RIPANS, for further reference. The leaves were cleaned and dried under shade to remove moisture with occasional shifting and then powdered with a mechanical grinder. It was then subjected to successive extraction of phytoconstituents using Soxhlet apparatus.

### Extraction of phytoconstituents

The dried powdered leaves of the plant were defatted with petroleum ether and the defatted powdered material thus obtained was further extracted with chloroform in a Soxhlet apparatus. The extraction was carried out exhaustively and the solvents were recovered by simple distillation. The extract was concentrated and dried to yield free flowing powder.

### DPPH radical-scavenging activity

The antioxidant activity of extract and the standard were estimated according to the method described by Blois (1958) with minor changes.<sup>6</sup> Butylated hydroxytoluene (BHT) was used as reference standard. A mixture consisting of 0.5 ml of DPPH solution in methanol (0.1 mM) and 3 ml of the methanol extract and 3 ml of standard prepared in various concentrations (0.1, 0.5, 1, 2, 3, 4, 5 µg/ml), respectively was prepared. All the mixtures were incubated for 30 minutes at 37°C. Absorbance was measured at 517 nm using ultraviolet-visible (UV-Vis) spectrophotometer. Control reading was also taken. The scavenging effect of DPPH free radical

was calculated using the following equation:

$$\% \text{ DPPH radical scavenging} = \frac{(\text{Abs of Control} - \text{Abs of extract})}{\text{Abs of Control}} \times 100$$

### Determination of reducing power

The reducing power of extract was determined by the method of Oyaizu.<sup>7</sup> Ascorbic acid was used as reference standard. 1 ml of the extract and 1 ml of the standard with various concentrations (10, 20, 40, 60, 80, and 100 µg/ml) were mixed with 2.5 ml of phosphate buffer (6.6 pH) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50°C for 30 minutes. The reaction was stopped by adding 2.5 ml of 10% trichloroacetic acid and the mixture was centrifuged at 3000 rpm for 10 minutes. 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water, and 0.5 ml of 0.1% ferric chloride solution and the absorbance was taken at 700 nm using UV-Vis spectrophotometer. The higher absorbance of the reaction mixture indicated that the reducing power is increased.

### Determination of total phenolic content

The total phenolic content of extract was determined using the method of Mc Donald *et al.*,<sup>8</sup> with slight modifications. A standard calibration curve was prepared by using gallic acid. 1 ml of methanolic solution of gallic acid (10, 20, 40, 60, 80, and 100 µg/ml) was mixed with 5 ml Folin-Ciocalteu reagent (diluted tenfold). After 3 minutes, 4 ml of sodium carbonate solution (0.7 M) was added, and the mixture was allowed to stand for 1 hr at room temperature. Absorbance was measured at 765 nm using UV-Vis spectrophotometer. 1 ml of the extract (50 mg/ml) was also mixed with the reagents above and after 1 hr the absorbance was measured to determine total plant phenolic content. From the calibration curve, the amount of phenolic compounds was determined and expressed as milligrams of gallic acid equivalent (GAE)/g of the dried extract.

### Determination of total flavonoids content

The total flavonoid content of the extract was determined by the aluminum chloride method.<sup>9</sup> A standard curve was prepared with quercetin at different concentrations (10, 20, 40, 60, 80, and 100 µg/ml). 1 ml of the extract (50 mg/ml) and 1 ml of quercetin were mixed with 2 ml of distilled water. After 5 minutes, 3 ml of 5% sodium nitrite (NaNO<sub>2</sub>) and 0.3 ml of 10% aluminium chloride (AlCl<sub>3</sub>) were added. After 6 minutes, 2 ml of NaOH (1 M) was added, and the volume was made up to 10 ml with distilled water. After 1 hr, absorbance reading was

taken at 510 nm. From the calibration curve of the reference standard, the total flavonoid content was determined and expressed as milligrams of quercetin equivalent (QE/g) of dried extract.

### Lipid peroxidation inhibition assay

In this assay, a modified thiobarbituric acid-reactive species (TBARS) was used to measure the lipid peroxide formed, and egg yolk homogenate was used as a lipid source.<sup>10</sup> The reaction mixture contains egg homogenate (0.5 ml of 10% v/v in distilled water) and 0.1 ml of extract in a test tube and made up to 1 ml with distilled water. 0.05 ml of FeSO<sub>4</sub> (0.07 M) was added to induce lipid peroxidation and incubated for 30 min. Then 1.5 ml of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 ml of 0.8% (w/v) TBA in 1.1% sodium dodecyl sulphate and 0.05 ml 20% TCA were added and the resulting mixture was vortexed and then heated at 95°C for 60 min. It was cooled, and 5.0 ml of butan-1-ol were added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm.<sup>11</sup> The percentage inhibition was calculated as follows:

$$\text{Percentage inhibition} = \frac{\text{Abs of Control} - \text{Abs of extract}}{\text{Abs of Control}} \times 100$$

### Anti-inflammatory activity by Inhibition of albumin denaturation

The anti-inflammatory activity of chloroform extract of *Combretum punctatum* var. *squamosum* was studied by using inhibition of albumin denaturation method according to Mizushima *et al.*,<sup>12</sup> and Sakat *et al.*,<sup>13</sup> with minor changes. Diclofenac sodium was used as the reference standard. The reaction mixture consists of test extract and standard prepared in various concentrations (100, 200, 300, 400, and 500 µg/ml) and 1% aqueous solution of bovine albumin fraction. The pH of the reaction mixture was adjusted using small amount of 1N HCl. The sample extracts were incubated at 37°C for 20 min and then heated to 51°C for 20 min. After cooling the samples, the turbidity was measured at 660 nm (UV-visible spectrophotometer). The percentage inhibition of protein denaturation was calculated as follows:

$$\text{Percentage inhibition} = \frac{\text{Abs of Control} - \text{Abs of Test}}{\text{Abs of Control}} \times 100$$

### HRBC membrane stabilization method

The anti-inflammatory activity of chloroform

extract of *Combretum punctatum* var. *squamosum* was studied by using stabilization method of human red blood cell (HRBC) membrane by hypotonicity induced membrane lysis<sup>14</sup>. Blood was collected (2 mL) from healthy volunteers and was mixed with equal volume of sterilized Alsevers solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl in distilled water) and centrifuged at 3,000 rpm. The packed cells were washed with isosaline solution and a 10% v/v suspension was prepared with normal saline and kept at 4°C undisturbed before use. Different concentrations of chloroform extract of *Combretum punctatum* var. *squamosum* in normal saline and diclofenac sodium as standard (50, 100, 200, 500 and 1,000 µg/0.5 mL) and control (distilled water instead of hypo saline to produce 100% hemolysis) were separately mixed with 1 mL of phosphate buffer, 2 mL of hyposaline and 0.5 mL of 10% HRBC suspension was added. All the assay mixtures were incubated at 37°C for 30 min and centrifuged at 3,000 rpm for 20 min and haemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm. The percentage of HRBC membrane stabilization or protection was calculated by using the following formula:

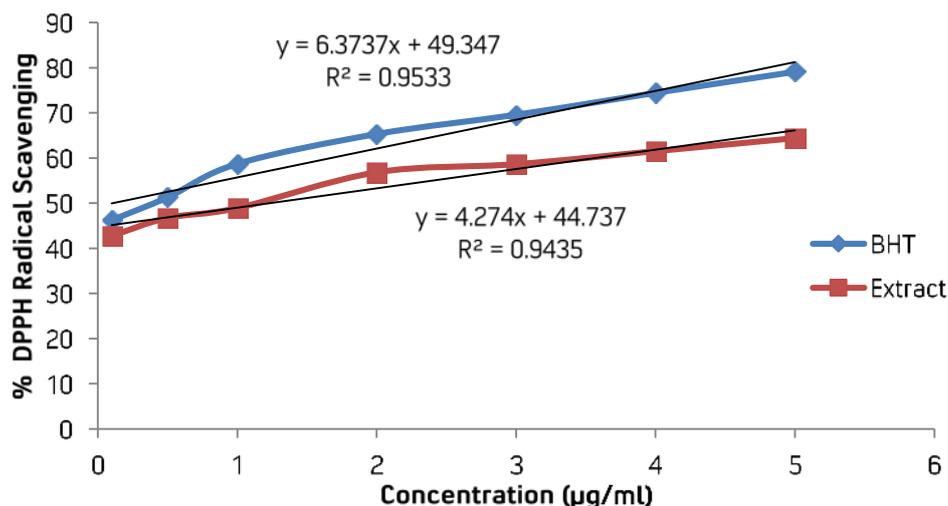
$$\text{Percentage inhibition} = \frac{\text{Abs of Control} - \text{Abs of Test}}{\text{Abs of Control}} \times 100$$

## Results and Discussion

DPPH radical is one of the most widely used free radicals for testing preliminary radical scavenging activity of the plant extract.<sup>15</sup> DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The hydrogen-donating ability of antioxidants is thought to be the reason of the main effect on DPPH radical scavenging.<sup>16</sup> DPPH radical scavenging activity of chloroform extract of *Combretum punctatum* var. *squamosum* and standard is shown in **Table 1** and **Figure 1**. In this study, the extract significantly

**Table 1 |** Effect of chloroform extract of *Combretum punctatum* var. *squamosum* on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity.

Sl. No	Concentration (µg/ml)	% Inhibition of standard (BHT)	% Inhibition
1	0.1	46.26	42.8
2	0.5	51.32	46.76
3	1	58.74	48.92
4	2	65.34	56.83
5	3	69.61	58.63
6	4	74.46	61.51
7	5	79.13	64.38



**Figure 1** | Graph representing % DPPH radical scavenging of standard (BHT) and chloroform extract of *Combretum punctatum* var. *squamosum*.

reduced the DPPH radical with increasing concentrations compared with the standard. The percentage inhibition of the DPPH radical by the chloroform extract and standard at 100 µg/ml was 64.38 % and 79.13% respectively.

Inhibition concentration ( $IC_{50}$ ) was determined for the interpretation of the results from DPPH method. It is defined as the concentration of the extract to scavenge 50% of the DPPH radical. The lower  $IC_{50}$  value, higher is the antiradical activity.<sup>17</sup> The percentage DPPH radical scavenging activity of the extract of the plant was plotted against standard butylated hydroxyl toluene (BHT) in a graph and shown in **Figure 1**. Antioxidant activity in the form of  $IC_{50}$  values of the plant methanol extract were calculated as 1.231 µg/ml compared to standard BHT, which exhibited 0.102 µg/ml.

The antioxidant potential of the chloroform extract of *Combretum punctatum* var. *squamosum* was also studied by determining its reducing capacity (**Table 2**). The ability of compounds to donate electron and to reduce the oxidized intermediates of lipid peroxidation processes are

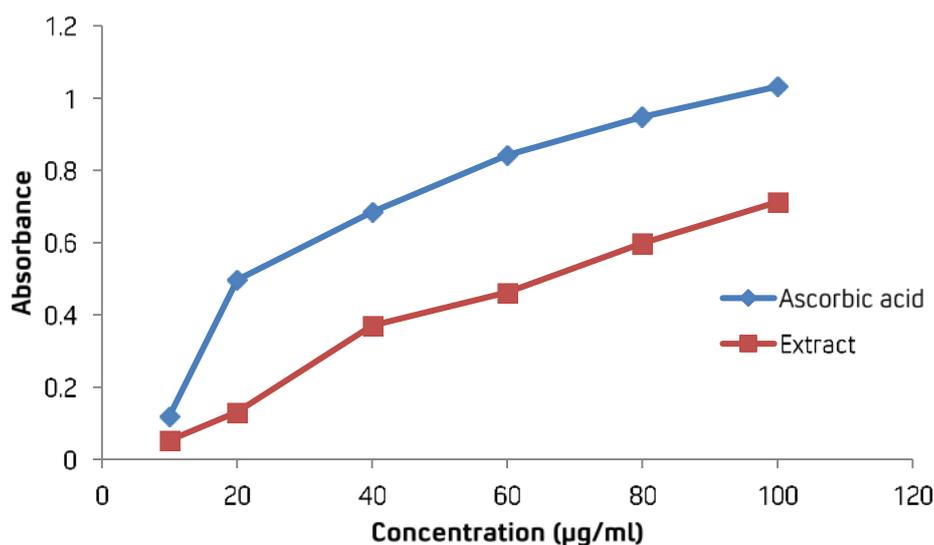
indications of reducing power and they can act as primary and secondary antioxidants. The presence of an antioxidant in the extract resulted in reduction of the ferric cyanide complex ( $Fe^{3+}$ ) to the ferrous cyanide form ( $Fe^{2+}$ ). Concentration of ferrous ions can be determined by absorbance measurement at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power of the extracts.<sup>18</sup> The increase in absorbance of the extract with increasing concentration was observed, and thus proved the reducing power even though the reducing power of the plant extract were lower than the standard ascorbic acid as shown in **Figure 2**.

Antioxidants, such as phenolic compounds including flavonoids are distributed in the plant kingdom and may prevent oxidative damage by scavenging reactive oxygen species (ROS).<sup>19</sup> The total phenolics and flavonoids content of the chloroform extract of *Combretum punctatum* var. *squamosum* were measured using F-C reagent and aluminium chloride methods respectively. The chloroform extract showed the total phenol contents of 634.34 mg of gallic acid equivalents per gram of dry extract. Total flavonoids were found to be 386.6 mg of quercetin equivalents per gram of dry extract respectively (**Figures 3 & 4**).

Lipid peroxidation is widely recognized as major toxicological event. A variety of sources including organic hydro peroxides, redox cycling compounds and iron-containing compounds caused the generation of free radicals leading to lipid peroxidation.<sup>20</sup> Biological membrane contains polyunsaturated lipid which are prone to oxidative reaction of free radicals, which lead to lipid peroxidation.<sup>21</sup> In this study, the lipid peroxidation inhibitory effect of chloroform extract of *Combretum punctatum* var. *squamosum* was assessed to determine the antioxidant activity. The inhibition of

**Table 2** | Effect of chloroform extract of *Combretum punctatum* var. *squamosum* on reducing power activity.

Sl. No	Concentration (µg/ml)	Absorbance of standard (ascorbic acid) at 700 nm	Absorbance at 700 nm
1	10	0.120	0.053
2	20	0.497	0.131
3	40	0.685	0.371
4	60	0.842	0.462
5	80	0.948	0.598
6	100	1.031	0.712



**Figure 2** | Graph representing reducing power of standard (ascorbic acid) and chloroform extract of *Combretum punctatum* var. *squamosum*.

lipid peroxidation of the extract and the standard were compared and shown in **Table 3** and **Figure 5**. Percentage inhibition of lipid peroxidation of the extract and standard at a concentration of 100 µg/ml were 74.86 and 86.21 respectively.

**Table 3** | Effect of chloroform extract of *Combretum punctatum* var. *squamosum* on lipid peroxidation.

Sl. No	Concentration (µg/ml)	% Inhibition of standard (α-tocopherol)	% Inhibition
1	10	59.18	23.78
2	20	65.67	31.89
3	40	71.62	45.40
4	60	77.83	59.72
5	80	81.62	62.97
6	100	86.21	74.86

Denaturation of proteins is a well-documented cause of inflammation.<sup>22</sup> When bovine serum albumin (BSA) is heated it undergoes denaturation and expresses antigens associated to type III hypersensitive reaction and which are related to many inflammatory diseases.<sup>23</sup> As part of the investigation on the anti-inflammation activity, ability of plant extract to inhibit protein denaturation of heat treated (immunogenic) bovine serum albumin (BSA) was studied. Maximum inhibition of 56.66% by the extract was observed at 500 µg/ml. Diclofenac sodium, a standard anti-inflammatory drug showed the maximum inhibition of 90% at the concentration of 500 µg/ml, as shown in **Table 4**.

The erythrocyte membrane may be considered a

model of the lysosomal membrane which plays an important role in inflammation.<sup>24</sup> During inflammation, lyses of lysosomes release their component enzymes which lead to a variety of disorders.<sup>25</sup> Prevention of the lysis of membrane by compounds caused by the release of hydrolytic enzymes contained within the lysosomes may relieve some symptoms of inflammation.<sup>26</sup> It has been reported that certain saponins and flavonoids exerted profound stabilizing effect on lysosomal membrane both *in vivo* and *in vitro*.<sup>27</sup> The anti-inflammatory activity of chloroform extract of *Combretum punctatum* var. *squamosum* was investigated by HRBC membrane stabilization method. The percentage protection of erythrocyte membrane by the extract and standard was given in **Table 5** and **Figure 7**. The extract shows maximum inhibition of 70.51% at 500 µg/ml compared with the standard which shows maximum inhibition of 91.66% at 500 µg/ml.

## Conclusion

The results in the present study showed good *in*

**Table 4** | Effect of chloroform extract of *Combretum punctatum* var. *squamosum* on albumin denaturation.

Sl. No	Concentration (µg/ml)	% Inhibition of standard (Diclofenac sodium)	% Inhibition
1	100	66.66	13.33
2	200	80	20
3	300	83	26.66
4	400	86.66	43.33
5	500	90	56.66

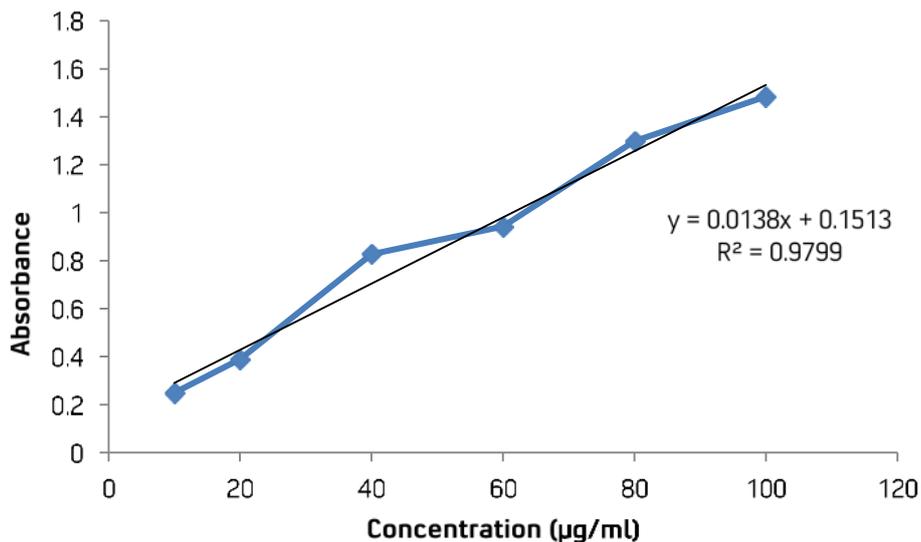


Figure 3 | Calibration curve for total phenolic content.

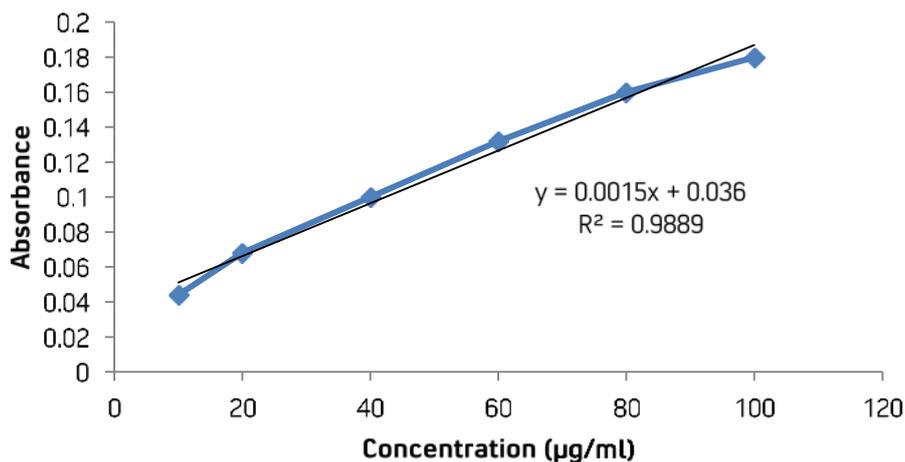


Figure 4 | Calibration curve for total flavonoid content.

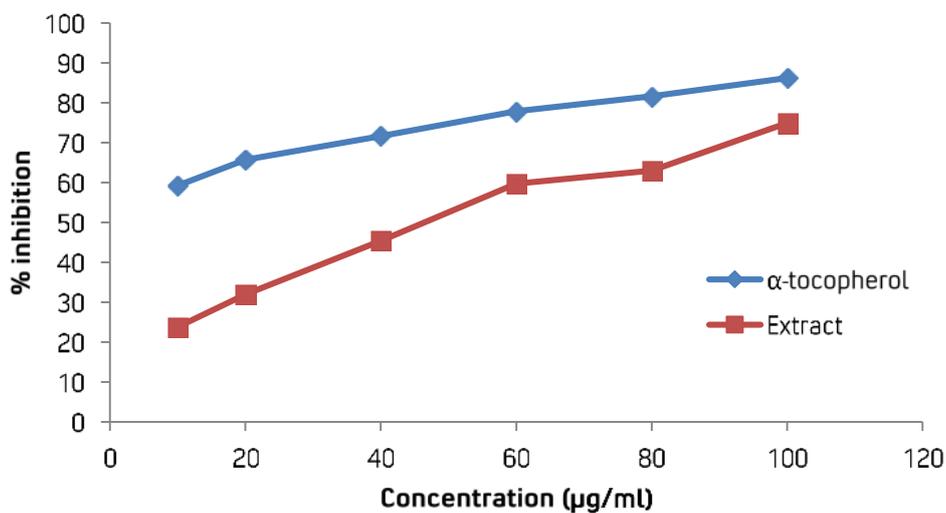
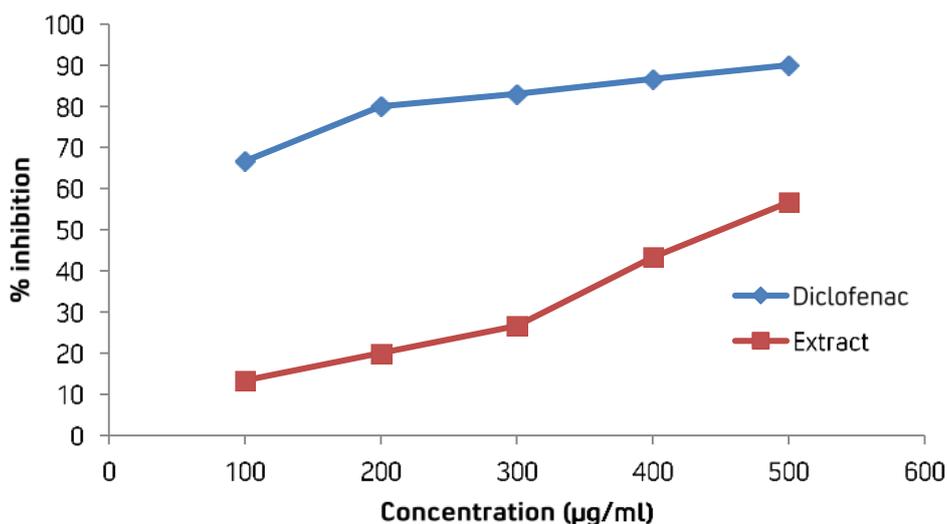
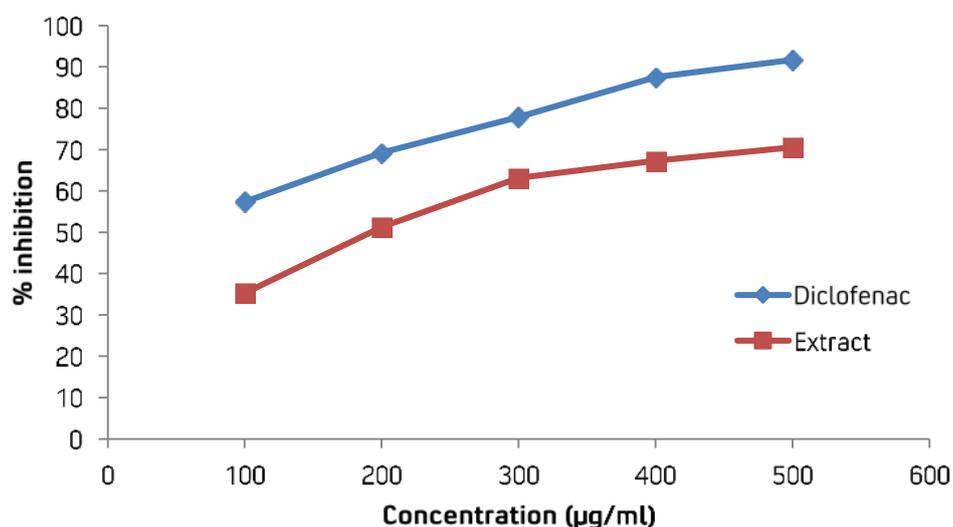


Figure 5 | Graph representing Inhibition of lipid peroxidation of standard ( $\alpha$ -tocopherol) and chloroform extract of *Combretum punctatum* var. *squamosum*.



**Figure 6** | Graph representing % inhibition of albumin denaturation of standard (Diclofenac) and chloroform extract of *Combretum punctatum* var. *squamosum*.



**Figure 7** | Graph representing % inhibition of HRBC membrane stabilisation of standard (Diclofenac sodium) and chloroform extract of *Combretum punctatum* var. *squamosum*.

**Table 5** | Effect of chloroform extract of *Combretum punctatum* var. *squamosum* on HRBC membrane stabilisation.

SL. No	Concentration (µg/ml)	% Inhibition of standard (Diclofenac sodium)	% Inhibition
1	100	57.37	35.25
2	200	69.23	51.28
3	300	77.88	63.14
4	400	87.5	67.3
5	500	91.66	70.51

*in vitro* anti-inflammatory and antioxidant activity of chloroform extract of *Combretum punctatum* var. *squamosum* even though lesser as compared to standard drugs. But it should be kept in mind that the crude chloroform extract is a mixture of several compounds while standard drugs are pure compounds; and if the compound responsible for such activities can be isolated from the crude extract, there is a possibility that the activities may be even better than the standard drugs. Consequently, to conclude flavonoids and related phenolics present in studied plant materials may possibly be responsible for the important anti-inflammatory and antioxidant activities. However, further investigations are required for identifying the unknown bioactive compounds and to establish their pharmacological

properties involved in the activities.

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## Conflict of interest

None declared.

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