Rationale Behind the Use of *Temnocalyx Obovatus* Roots as Antivenins Against Snakebites in Primary Health Care

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**ABSTRACT**

Worldwide snakebites are a health hazard. In recent years there has been an increased interest in alternative therapeutics especially those derived from plants. The aim of the present study was to provide a rationale behind the use of *Temnocalyx obovatus* roots in the treatment of snakebites in folk medicines. Thin Liquid Chromatography (TLC) analysis of *Temnocalyx obovatus* methanolic extract revealed five spots ($R_f$ 0.65, $R_f$ 0.71, $R_f$ 0.72, $R_f$ 0.77 and $R_f$ 0.78). On spraying with standard revealing reagents it was noted that the extract consisted of appreciable amounts of flavonoids, saponins and moderate amounts of tannins. Alkaloids were detected in trace amounts. Cardiac glycosides anthraquinones, steroids and terpenoids were not detected. Results of caseinolytic activity of metalloproteinase show that flavonoids inhibited significantly the digestion of casein by the enzyme ANOVA analysis $p > 0.05$. Tannins and saponins also revealed a greater anti-proteolytic activity while anti-proteolytic activity of alkaloids was poor. On anti-phospholipase $A_2$ activity analysis, flavonoids and saponins revealed superior inhibitory effect with a 100% effective dose of 10 and 15µg/ml respectively. Anti-phospholipase $A_2$ activities of alkaloids were negligible. Tannins also exhibited good inhibitory activity however 100% protection was not reached. Metalloproteinase and phospholipase $A_2$ are the major enzymes in snake venom and inhibition of these enzymes prevent local tissue damage and entrance of other venom toxins. Inhibitory activity of *Temnocalyx obovatus* against metalloproteinase and phospholipase $A_2$ authenticate its use as an antivenin against snake bites in primary health care.

**Keywords:** Antivenins, Snakebites, *Temnocalyx obovatus*, Primary health care.
INTRODUCTION

Researches in alternative antivenins against snakebites and envenomations continue to rise over the years\(^1\text{-}^6\). This is due to the realization of the limitations inherent in the use of horse serum based antivenins\(^7\). Horse serum based antivenins consist of antibodies from the immunized animals which may invoke adverse reactions due to activation of the immune system. Side effects may range from less severe symptoms like chills, nausea and fever, to serious problems such as bronchospasms and anaphylactic shock\(^8\). In addition, production of such antivenins is very expensive, pushing up the prices of acquiring them from drug shops.

Venoms are complex mixtures of components which have a diverse array of actions. These components appear differently in snakes even of the same species. Each population of snakes’ requirements depends on the type of prey it seeks, temperature and place of origin. Chippaux et al.\(^9\) reports a seasonal variation in the European viper (Vipera aspis) venom, with spring venom being more effective than autumn venom and bites inflicted in spring or summer being more severe. This makes it very difficult to develop universal antivenins calling for researches in local and cheap snakebites treatment alternatives. Antioxidants from plants have been shown to interact with enzymes from snake venoms and act as an antidote\(^10\text{-}^14\). Antioxidants principles can form hydrogen bonds with the three histidine residues in the zinc or magnesium binding motifs and chelate the metal atoms of venom enzymes; antimeatalloproteinase, phosphodiesterases and phospholipase A\(_2\). This can result in total inhibition of the venom enzymatic activities and thereby inhibit tissue necrosis. In Zimbabwe Temnocalyx obovatus roots are acclaimed to be an antidote for snakebites in folk medicine\(^15\). Therefore this research sought to explore antiproteinase and lipase activity of Temnocalyx obovatus root extracts so as to provide the rationale behind its use in primary health care needs.

MATERIALS AND METHODS

Plant Material

Plant roots were collected with the assistance of a local traditional healer, from St Albert’s areas in Mashonaland central, Zimbabwe and validated by a taxonomist at Harare botanical garden. A voucher sample No. 2012/12 was then kept in the chemistry department of Bindura University of Science Education for future reference. One kilogram of the fresh plant was dried at room temperature for two weeks and then pulverized and stored in plastic bags.

Extraction of Plant Material

A mass, (20g) was extracted with 100 ml absolute methanol on a shaker at room temperature for 2 hours. Methanol was selected because Mahamadi et al.\(^15\) reported that methanol was the best solvent for extracting bioactive from Temnocalyx obovatus. The extraction was repeated three times. The resultant extracts were filtered and evaporated on a rotary evaporator and freeze dried to give the crude dried extract. A yield of 4.71g g\(^{-1}\) dry weight was obtained. The dried extract was redissolved in 100ml of methanol and stored in a freezer (0-5\(^\circ\)C) until used.

Thin Layer Chromatography (TLC) analysis of phytocompounds

Analysis of different bioactive components in Temnocalyx obovatus root extract was performed using TLC. A small volume of the methanolic extract was spotted on silica gel G plate which was developed using acetone: hexane 3:7 (v/v). The separated bands were identified using different revealing reagents, Table 1.
Fractions of different $R_f$ values were scratched into tubes and dissolved in methanol. The contents were then filtered through Whatman No. 1 filter paper and then tested for protease and phospholipase enzyme inhibition activity.

**Anti-proteolytic activity**

Caseinolytic activity of metalloproteinase enzyme was determined by modifying the methods of Kunitz\(^\text{16}\) and Tan et al.,\(^\text{17}\). Two millilitres of 1% casein was placed in 0.25 M sodium phosphate buffer at pH 7.75 and 0.1 ml of metalloproteinase enzyme (0–1000 µg/ml) were incubated for 1 h at 37 °C. The undigested casein was precipitated and the reaction terminated by adding 2 ml of 5% trichloroacetic acid. The contents were then centrifugation at 10,000×g for 10 min and the absorbance of the supernatant was measured at 280 nm. The initial proteolytic dose of the enzyme was obtained from the plot between proteolytic activity and the enzyme doses. Solutions of 0–20 mg/ml of each extracts were evaluated for their anti-proteolytic potentials against the enzyme. This was done by taking each 0.05 ml of the test solution and pre-incubating it for 1 h at 37 °C with an equal amount of the enzyme solution (200 µg/ml) before the mixture was subjected to proteolytic activity evaluation. The experiments were performed five times.

**Anti-phospholipase A\(_2\) activity**

Anti-phospholipase activity was carried out by a slight modification of the methods reported by Araujo and Radvanyi\(^\text{18}\). A 1ml substrate solution (3.5mM Phosphotidyl choline in a solution containing 7mM Triton X100, 100mM NaCl, 10mM CaCl\(_2\) and pH adjusted to 6.8 with 0.1M NaOH) was mixed with 10 µl of phospholipase A\(_2\) (100 µg/ml) and incubated at 37°C for 10 minutes. The reaction was stopped by adding one ml of acetone-ethanol solution (1:1v/v). The mixture was centrifuged at 2000 rpm for 5 minutes and supernatant collected. The action of the phospholipase enzyme produces fatty acid and the amount of fatty acid in the reaction mixture was determined by titration with 0.01M NaOH solution. Phenolphthalein was used as an indicator. The amount of NaOH that reacted was noted and taken as phospholipase enzyme activity. To determine the inhibitory action of *Temnocalyx obovatus* root extracts 1ml of phosphatidyl choline solution and different concentration of extract solution Fig 3 at pH 6.8 were combined and used as the substrate inhibitor mixture for the determination of phospholipase activity of 10 µl of the phospholipase enzyme. The amount of 0.01M NaOH used for substrate solution without inhibitor was taken as 100% enzyme activity and percentage of enzyme activity with *Temnocalyx obovatus* root extracts relative to control (phospholipase only) was calculated as follows:

\[
\% \text{ activity} = \left( \frac{\text{Volume of NaOH used in the presence of phospholipase and extract solution}}{\text{Volume of NaOH used in the presence of phospholipase only}} \right) \times 100 \]

**Statistical analysis**

The results are expressed as mean ± standard error for at least five determinations. Statistical analysis to determine the extract with the greatest inhibition activity was carried out using SPSS 17.0. Significant differences ($p = 0.05$) between means were assessed by one-way ANOVA, followed by Tukey’s significant difference test.

**RESULTS**

Results of TLC analysis are shown in Table 1. Extracted compounds resolved in acetone: hexane 3:7 (v/v) formed five spots ($R_f$ 0.65, $R_f$ 0.71, $R_f$ 0.72, $R_f$ 0.77 and $R_f$ 0.78). After spraying with revealing agents it was
noticed that (Table 2) *Temnocalyx obovatus* root extract consist of appreciable amounts of flavonoids and saponins and moderate amounts of tannins. Alkaloids were detected in trace amounts. Cardiac glycosides, anthraquinones, steroids and terpenoids were not detected. Occurrence of flavonoids and saponins on different spots may reveal the presents of compounds with different structures.

Results of caseinolytic activity of a metalloproteinase are shown in Fig 1. The absorbance of casein decreased with an increase in concentration of enzyme. After an enzyme concentration of 200µg/ml the rate of decrease leveled off. The optimum substrate enzyme dose was found to be 200µg/ml. Fig 2 depicts anti-proteolytic activity of compounds extracted from *Temnocalyx obovatus* roots. Flavonoids inhibited significantly the digestion of casein by the enzyme ANOVA analysis p > 0.05. Tannins and saponins also revealed a greater anti-proteolytic activity while anti-proteolytic activity of alkaloids was poor. Anti-phospholipase activities of compounds isolated from the extract are shown in Fig 3. Once more flavonoids and saponins revealed superior anti-lipase activity with a 100% effective dose of 10 and 15µg/ml respectively. Anti-phospholipase activities of alkaloids were negligible. Tannins also exhibited inhibitory activity however 100% protection was not reached. The present results compare with the results obtained by (Pithayanukul et al.\textsuperscript{19} and Mors et al.\textsuperscript{19}). Polyphenols from tea and flavonoids from plants extract consisted of inhibitory action against venom enzymes such as metalloproteinase and phospholipase A\textsubscript{2}. While hundred percent was not achieved in Pithayanukul et al.\textsuperscript{19} and Mors et al.\textsuperscript{20} studies, in the present study flavonoids and saponins extracted from *Temnocalyx obovatus* exhibited 100% protection against both proteolytic enzyme and phospholipase enzyme. One hundred percent inhibitory activity was reached at very low inhibitor concentration of 10 and 15µg/ml. In similar studies performed by Ushanandini et al.\textsuperscript{21}, Meenatchisundaram et al.\textsuperscript{22} extracts from *Anacardium occidentale* and *Andrographis paniculata* exhibited anti-proteolytic and anti-phospholipase activity. In the present study alkaloids exhibited weak neutralization activity however in 1987 Vishwanath and Gowda\textsuperscript{23} reported that phospholipase A\textsubscript{2} enzymes from *Vipera russelli* activity was neutralized by an alkaloid aristolochic acid.

DISCUSSION

It is beyond doubt that bioactive compounds from medicinal plants may act as a new source of antivenins against snakebites. Many researches are now focusing on traditional medicinal plants not only for the discovery for new therapeutics but possibly for discovering compounds with novel mechanisms of action that can lead to discovery of new fields of research. High cost involved in production of conventional antivenins and the significant percentage of patients who develop side effects to them provides a rationale for systematic investigation of plant-based antivenins against snake bites. In the current study neutralizing power of *Temnocalyx obovatus* roots extracts were tested against two enzymes found in most snake venom metalloproteinase and phospholipase A\textsubscript{2}. Flavonoids and saponins separated by TLC and identified through standard revealing agents exhibited significant inhibitory activity. The most probable mechanism for the inhibition displayed by flavonoids and saponins lies in the structure of these compounds. Flavonoids and saponins consist of polyphenolic structures that can complex metal ions of both enzymes through hydroxyl groups thereby causing inhibition\textsuperscript{24}. Soares et al.\textsuperscript{25} reported that plant extracts showing inhibition activity of enzymes consist of compounds that bind to
divalent metal ions such as magnesium, zinc and copper, which act as cofactors of enzymatic activities. Phospholipase A2 hydrolyzes lecithins to lysolecithins that lyse erythrocyte membranes and cause internal homolysis. Therefore inhibition of such enzymes result in prevention of erythrocyte ruptures and decrease hemorrhage in local tissues. Proteases in snake venom cause envenomation by acting as digestive agents. This gives allowance of other venom constituents’ access to local tissues. It has been shown that snake proteases are able to hydrolyze various components of extracellular matrices that is fibronectin, collagen type I, III, IV and V, gelatins, laminin and proteoglycans. Proteolytic damage of these compounds lead to structural integrity disturbances and therefore inhibition of proteases enzymes result in prevention of extracellular damage and decrease the diffusion of toxins through the tissues.

CONCLUSION

Temnocalyx obovatus roots consist of appreciable levels of bioactive compounds that can inhibit metalloproteinase and phospholipase A2. Inhibition of these enzymes in snake venom prevents local tissue hemorrhage and damage and prevents entrance of other venom toxins therefore acting as an antidote against snake bites.

ACKNOWLEDGEMENTS

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REFERENCES

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Table 1. TLC analysis of *Temnocalyx obovatus* methanolic roots extract

<table>
<thead>
<tr>
<th>Plant Sample</th>
<th>Spot No.</th>
<th>R&lt;sub&gt;f&lt;/sub&gt; value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. obovatus</em> roots</td>
<td>1</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Table 2. Phytochemical analysis of *T. obovatus* methanolic roots extract

<table>
<thead>
<tr>
<th>R&lt;sub&gt;f&lt;/sub&gt;</th>
<th>Tests</th>
<th>Observation</th>
<th>Phytocompounds present</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.65</td>
<td>Ferric chloride test and Gelatin test</td>
<td>++</td>
<td>Tannins</td>
</tr>
<tr>
<td>0.71</td>
<td>Dragendorffs test and Wagner`s reagent</td>
<td>+</td>
<td>Alkaloids</td>
</tr>
<tr>
<td>0.72</td>
<td>Sodium hydroxide test</td>
<td>++</td>
<td>Flavonoids</td>
</tr>
<tr>
<td>0.77</td>
<td>Frothing test</td>
<td>+++</td>
<td>Saponins</td>
</tr>
<tr>
<td></td>
<td>Sodium hydroxide test</td>
<td>+</td>
<td>Flavonoids</td>
</tr>
<tr>
<td>0.78</td>
<td>Frothing test</td>
<td>+</td>
<td>Saponins</td>
</tr>
<tr>
<td></td>
<td>Sodium hydroxide test</td>
<td>+++</td>
<td>Flavonoids</td>
</tr>
</tbody>
</table>

+ = trace levels, ++ = Moderate amounts, +++ = Significant amounts
Figure 1. Caseinolytic activity of a metalloproteinase enzyme.

Figure 2. Anti-proteolytic activity of *Temnocalyx obovatus* root extracts.
Figure 3. Anti-lipase activity of Temnocalyx obovatus root extracts