Inhibition of *Naja nigricolis* (Reinhardt) venom protease activity by *Luffa egyptiaca* (Mill) and *Nicotiana rustica* (Linn) extracts

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*Luffa egyptiaca* and *Nicotiana rustica* are used in traditional medicine to treat snakebites and were evaluated for inhibitory activities on *Naja nigricolis* venom protease. The aqueous and ethanolic extracts of *L. egyptiaca* significantly reduced the maximum velocity (*V*_max*) and the computed index of physiological efficiency (*K*_p*) of the enzyme in a dose dependent fashion. The protease activity was non-competitively inhibited by the aqueous extract of *N. rustica* with the *V*_max* significantly decreased and the *K*_p* remained unchanged. However, the *N. rustica* ethanol extract completely inhibited the protease activity. Ethyl acetate fractions partitioned from ethanol extracts of both plants were also found to completely inhibit the *N. nigricolis* venom protease activity at 0.1 and 0.05%. The use of these plants could be important in the treatment of snakebites.

**Keywords:** *Luffa egyptiaca*, *Naja nigricolis*, *Nicotiana rustica*, Protease, Venom

Snakebite envenomations constitute a medical hazard in many regions of the world1. One of the commonest causes of envenomation in Northern Nigeria is *Naja nigricolis* (black necked spitting cobra). Snake venoms are composed of complex mixture of active substances, mainly peptide and proteins which are able to interfere with biological processes including thrombosis by affecting platelet aggregation and blood coagulation2. Some of these proteins include enzymes like phospholipase A2 and metalloproteases3.

Antivenom immunotherapy is the only contemporary treatment against snake envenomations with various side effects such as anaphylactic shock, pyrogen reaction and serum sickness. Most of these symptoms may be due to the action of high concentrations of non-immunoglobulin proteins present in commercially available antivenom4,5. Plant preparations have been used in folk medicine to treat snakebites and the efficacy of some of them have been scientifically validated6,7 and attempts are now being made to develop snake venom antagonists from plants sources. *Luffa egyptiaca* Mill (Cucurbitaceae) and *Nicotiana rustica* Linn (Solanaceae) are two plants mainly used, separately or in combination, for the treatment of snakebite by traditional herbalists in Katsina state of Nigeria. Therefore an attempt has been made to validate this claim by investigating *in vitro*, the inhibitory effects of the plants extracts on *N. nigricolis* venom protease activity.

**Snake venom**—Freeze dried *N. nigricolis* venom was obtained from the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria.

**Plant material**—Fresh leaves of *Luffa egyptiaca* and *Nicotiana rustica* were collected in September, 2009 from Yantumaki town, Dan Musa village, Katsina state of Nigeria and were identified by U. S. Gallah of the herbarium unit of Biological Sciences Department, Ahmadu Bello University, Zaria. Voucher specimen numbers 336 and 2542 for *L. egyptiaca* and *N. rustica* respectively were deposited. The leaves were thoroughly washed and shade-dried for two weeks to a constant weight. The dried leaves were pounded to fine powder with mortar and pestle, and stored in dry containers until needed.

**Extract preparation**—Fine powdered dried leaves (100 g) were soaked in 300 ml of distilled water or ethanol and sequentially extracted by shaking for 6 h on wrist action shaker. The preparations were then kept for 24 h at room temperature. The extracts were filtered through Whatmann’s filter paper and samples were concentrated by vaporization in a water bath at 40°C and refrigerated at about 4°C until required. Further, the crude ethanol extracts were suspended in distilled water and partitioned with equal amounts of ethyl acetate. The ethyl acetate fraction was concentrated by drying with a rotar vapor at 35°C.

**Protease assay**—The protease activity was assayed as described by Fahmy *et al*7. Briefly, 50 µl of the crude venom solution (10 mg/ml) was incubated with 500 µl of 100 mM sodium acetate buffer pH 4.5, and 100 µl of 3% casein at 37°C. The mixture was then made up to 1 ml with distilled water. Assays were carried out after 1 hour, the reaction was stopped by

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the addition of 200 µl of 20% trichloroacetic acid. The precipitated proteins were removed by centrifugation at 10,000 g. The absorbance of the supernatant was measured at 366 nm. The activity of protease was defined as the amount of enzyme that hydrolyzes 1 µmol of amino acids (in terms of tyrosine) from casein per minute under the standard assay conditions.

**Effects of L. egyptiaca and N. rustica extracts on crude N. nigricolis protease**—This was carried out using the same procedure for protease activity assay only that varying concentrations of the substrate (0.375-3%) casein was used and additional 100 µl of 0, 5, 10 and 15% (w/v) of the aqueous or ethanol extracts of either L. egyptiaca or N. rustica were added to the reaction mixture. The initial velocity data obtained were used to plot Lineweaver-Burke’s plot to determine $K_M$, $V_{max}$ and hence the turnover number ($K_{cat}$) of the N. nigricolis protease in the absence and presence of each concentration of the extracts. The kinetic parameters of the N. nigricolis venom protease were also determined in presence of 0.025, 0.05 and 0.1% of the ethyl acetate fractions of both plants.

**Statistical analysis**—The results were presented as mean ± SD and Students’ t-test was used to compare paired means and a difference was considered statistically significant when $P < 0.05$.

The Michaelis constant ($K_M$) and maximum velocity ($V_{max}$) of the N. nigricolis venom protease were found to be 1.88 ± 0.04 mg/ml and 86.96 ± 3.26 µmol/min respectively and thus the computed physiological index of efficiency ($K_{cat}$) was 46.34 ± 1.60 min⁻¹.

The results of the effects of aqueous and ethanolic extracts of *L. egyptiaca* and *N. rustica* on *N. nigricolis* venom protease activity are presented in Table 1.

**Naja nigricolis** venom protease activity was not observed in the presence of ethyl acetate fractions obtained from the ethanolic extracts of both plants at 0.1 and 0.05% extract concentrations (Table 2) but non-competitive inhibition was observed in the presence of *L. egyptiaca* ethyl acetate fraction at 0.025% extract concentration.

*Luffa egyptiaca* extracts produced dose dependent decrease in the computed physiological index of efficiency of *N. nigricolis* protease. This indicates reduction in the number of casein molecules hydrolysed to products at saturation of the enzyme. This may further suggests that, the extracts reduce the amount of protein degradation by the *N. nigricolis* venom protease and consequently decrease other pathological effects caused by the venom proteases.

The mechanism of inhibition of *N. rustica* aqueous extract reveals a non-competitive inhibition pattern which indicates that the extract binds the venom protease at other sites rather than the active site. It further suggests that the extract contain some

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### Table 1—Effect of different concentrations of *L. egyptiaca* (A) and *N. rustica* (B) extracts on *N. nigricolis* venom protease activity [Values are mean ± SD of 3 replicates]

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Aqueous extract of <em>L. egyptiaca</em> and <em>N. rustica</em></th>
<th>Ethanol extract of <em>L. egyptiaca</em> and <em>N. rustica</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_M$ (mg/ml) A</td>
<td>1.88±0.04&lt;sup&gt;a&lt;/sup&gt; 1.92±0.12&lt;sup&gt;a&lt;/sup&gt; 2.29±0.04&lt;sup&gt;b&lt;/sup&gt; 3.38±0.41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.88±0.04&lt;sup&gt;a&lt;/sup&gt; 1.98±0.09&lt;sup&gt;a&lt;/sup&gt; 2.41±0.03&lt;sup&gt;d&lt;/sup&gt; 2.83±0.06&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>B</td>
<td>1.88±0.04&lt;sup&gt;a&lt;/sup&gt; 1.88±0.04&lt;sup&gt;e&lt;/sup&gt; 1.88±0.04&lt;sup&gt;a&lt;/sup&gt; 1.88±0.04&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.88±0.04&lt;sup&gt;a&lt;/sup&gt; CI CI CI</td>
</tr>
<tr>
<td>$V_{MAX}$ (µmol/min) A</td>
<td>86.96±3.26&lt;sup&gt;a&lt;/sup&gt; 45.45±6.20&lt;sup&gt;a&lt;/sup&gt; 25.00±2.92&lt;sup&gt;c&lt;/sup&gt; 13.70±0.95&lt;sup&gt;d&lt;/sup&gt;</td>
<td>86.96±3.26&lt;sup&gt;a&lt;/sup&gt; 76.80±1.33&lt;sup&gt;c&lt;/sup&gt; 58.82±1.19&lt;sup&gt;f&lt;/sup&gt; 47.50±1.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>B</td>
<td>86.96±3.26&lt;sup&gt;d&lt;/sup&gt; 52.63±2.71&lt;sup&gt;b&lt;/sup&gt; 47.61±0.59&lt;sup&gt;d&lt;/sup&gt; 32.26±1.78&lt;sup&gt;d&lt;/sup&gt;</td>
<td>86.96±3.26&lt;sup&gt;a&lt;/sup&gt; CI CI CI</td>
</tr>
<tr>
<td>$K_{cat}$ (min⁻¹) A</td>
<td>46.34±1.60&lt;sup&gt;a&lt;/sup&gt; 23.60±1.71&lt;sup&gt;b&lt;/sup&gt; 10.90±1.07&lt;sup&gt;c&lt;/sup&gt; 4.08±0.48&lt;sup&gt;d&lt;/sup&gt;</td>
<td>46.34±1.60&lt;sup&gt;a&lt;/sup&gt; 37.51±2.55&lt;sup&gt;d&lt;/sup&gt; 24.40±0.43&lt;sup&gt;b&lt;/sup&gt; 16.79±0.76&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>B</td>
<td>46.34±1.60&lt;sup&gt;a&lt;/sup&gt; 28.06±1.79&lt;sup&gt;b&lt;/sup&gt; 25.37±0.37&lt;sup&gt;c&lt;/sup&gt; 17.21±1.39&lt;sup&gt;d&lt;/sup&gt;</td>
<td>46.34±1.60&lt;sup&gt;a&lt;/sup&gt; CI CI CI</td>
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</tbody>
</table>

Values with different superscript (a, b, c, d, e, f) within a row are significantly different from each other ($P>0.05$)

CI=complete inhibition of enzyme activity

### Table 2—Effect of different concentrations of ethyl acetate fractions of *L. egyptiaca* and *N. rustica* ethanol extracts on *N. nigricolis* venom protease activity [Values are mean ± SD of 3 replicates]

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>Ethyl acetate fraction of <em>L. egyptiaca</em></th>
<th>Ethyl acetate fraction of <em>N. rustica</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_M$ (mg/ml)</td>
<td>1.88±0.04&lt;sup&gt;a&lt;/sup&gt; CI CI CI</td>
<td>1.88±0.04&lt;sup&gt;a&lt;/sup&gt; CI CI CI</td>
</tr>
<tr>
<td>$V_{MAX}$ (µmol/min)</td>
<td>86.96±3.26&lt;sup&gt;a&lt;/sup&gt; CI CI CI</td>
<td>86.96±3.26&lt;sup&gt;a&lt;/sup&gt; CI CI CI</td>
</tr>
<tr>
<td>$K_{cat}$ (min⁻¹)</td>
<td>46.34±1.60&lt;sup&gt;a&lt;/sup&gt; CI CI CI</td>
<td>46.34±1.60&lt;sup&gt;a&lt;/sup&gt; CI CI CI</td>
</tr>
</tbody>
</table>

Values with different superscript (a, b) within a row are significantly different from each other ($P>0.05$)

CI=complete inhibition of the enzymatic activity
phytochemicals capable of interacting with the *N. nigricolis* protease-casein complex. The dose dependent reduction in the $V_{\text{max}}$ and $K_{\text{cat}}$ of the venom protease in the presence of *N. rustica* aqueous extract implies that the amount of the active component in this extract could have an effect on the action of this enzyme and that higher amount of the extract would lead to lowered protease activity and consequently ameliorate the clinical symptoms related to the physiological activity of the enzyme. The complete inhibition of the *N. nigricolis* protease by the ethanol extract of *N. rustica* implies that the plant contains some ethanol extractable phytochemicals that could completely inhibit venom protease.

In order to predict the role of phytochemicals for the observed inhibitions, the ethanol extracts of both plants were subjected to further fractionation with ethyl acetate—a less polar solvent. Ethyl acetate is known to extract most flavonoids present in plant extracts. The complete inhibition of the enzymatic activity by the ethyl acetate fraction of both extracts at lower concentrations implicates flavonoids as important components responsible for the observed inhibitions of the venom protease activity. Since the enzyme activities were detected in the presence of 0.025% of ethyl acetate fraction of *L. egyptiaca*, it may indicate that the amount of the flavonoids, especially from this plant, has to reach some threshold before completely inhibiting the protease activity.

It can therefore be concluded that the use of *L. egyptiaca* and *N. rustica* leaf extracts inhibit the proteolytic activity of *N. nigricolis* venom. Further studies to isolate the phytochemical(s) and flavonoids responsible for such action are in progress.

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References


