Anti-Inflammatory Effects of Rothmannia longiflora Fruit Extract

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ABSTRACT:
The anti-inflammatory activity of the fruit extract of Rothmannia longiflora was carried out by evaluating its inhibitory effect on Phospholipase A2 and Prostaglandin Synthetase. As R. longiflora was found to have febrifugal and analgesic properties by reducing oedema formation; the ability of the extract to inhibit the synthesis of prostaglandin which is one of the mediators of inflammation, was utilized as the model for anti-inflammation. R. longiflora was found to significantly (p<0.05) inhibit the activity of both Phospholipase A2 and Prostaglandin Synthetase activities. The results of this study shows the anti-inflammatory activity of R. longiflora extract and suggest further investigations into the effects of specific constituent(s) of the plant on inflammation.

KEYWORDS: Rothmannia longiflora; Inflammatory; Prostaglandin Synthetase; Phospholipase A2.

INTRODUCTION:
The use of plants as a source of relief for illness is as old as mankind, with recorded practices dating back at least 4000 years (Christophersons et al., 1991). Rothmannia longiflora (Family Rubiaceae) commonly referred to as 'Uru' (Igbo Eastern Nigeria) is a shrub or small tree and occasionally herbs or climbers which grow in old farms of secondary forest and thickets (Joffe, 2005). The fruit of the plant is widely distributed in tropical and sub-tropical regions of the globe and sometimes they are found in cold regions. They abound in Ghana through Nigeria to Congo. R. longiflora is also found from east of Camibia to Sudan and Kenya, and South of Tanzania and Angola (Coates, 2002). The fruits are ingredients of common anti-inflammatory remedies used locally (Abubakar et al., 2007).

Inflammatory reactions in humans are usually characterized by pains, swelling and fever and a major inflammatory disease (rheumatoid arthritis) is one of the most distressing and disabling syndromes encountered in medical practice and although it is one of the oldest disease, there is no drug leading to a permanent cure. The side effects of existing steroidal and non-steroidal anti-inflammatory drugs have led to increasing efforts in search of novel compounds especially from plants which would possess long acting anti-inflammatory activity with minimum side effects.
In the present study, we have examined the anti-inflammatory potential of *R. longiflora* using its ability to inhibit the synthesis of inflammatory mediators like prostaglandins. Aspirin and indomethacin inhibited the synthesis of prostaglandins (example PGF and PGE series) from arachidonic acid (Vane, 1971); therefore any agent that acts in a similar manner should be considered as an anti-inflammatory agent.

**MATERIALS AND METHODS:**

**Plant Material:**
Mature fruits of *R. longiflora* were harvested from Nkpologo in Uzo-uwani Local Government Area of Enugu State and authenticated by Mr. M. C. Eze of the Department of Botany, University of Nigeria, Nsukka, Enugu State, Nigeria. Voucher specimen of the fruits are retained as reference in the herbarium unit of the department of Botany, University of Nigeria, Nsukka.

**Animal Material:**
Ox-testes were purchased from the local slaughter house in Nsukka market and processed as the source of prostaglandin synthetase.

**Chemicals:**
All chemicals used in this study were of analytical grade and they were used as such. They were products of May and Baker, England and Merck, Darmstadt, Germany. All laboratory reagents were freshly prepared.

**Preparation of plant extract:**
Fresh fruits of *R. longiflora* were extracted using chloroform and methanol in the ratio of 2:1 (v/v) (Folch et al., 1957). The fruits were pulverized and soaked for 24 hours with a mixture of 1260ml of chloroform and 630ml of methanol. The extract was filtered first through calico, and then through a (Whatman No.4) filter paper. Next, 360ml of water was added to 1800ml of the filtrate and shaken to give upper and lower layers; methanol-water filtrate and chloroform filtrate respectively. The upper aqueous methanol layer was drawn out and lyophilised. The concentrated portion gave 5.95% of methanol fraction. Bambara oil was also extracted using the Folch method.

**Preparation of Phospholipase A2:**
Specimen (enterobacter) in agar slant in bijou bottles was incubated overnight at 37°C and then inoculated on nutrient agar slant already prepared. The inoculated slants were incubated for 24 hour at 37°C. The nutrient broth used was prepared by dissolving 13g of nutrient broth in 1000ml of distilled water, homogenized in a water bath for 10 min and 5ml dispensed into two clean bijou bottles. The broth was autoclaved at 121°C for 15 min. The broth was allowed to cool to normal room temperature, and then the organisms in the slant above were aseptically inoculated into the broth and incubated for 24hr at 37°C. Bacterial cells were harvested by centrifuging at 3000rpm for 15min at 4°C, then washed in 0.02M Tris-HCl buffer, pH 7.4 and sonicated. The sonicate was then centrifuged at 3000 rpm for 30min at 4°C. The supernatant was decanted and stored at 15°C and was used as an enzyme preparation (Nwodo and Eze, 2001).

**Preparation of Prostaglandin Synthetase (PGS):**
The method of Nugeyen et al. (1966) was used in the isolation of the enzyme from beef seminal vesicle (the source of prostaglandin synthetase). The frozen beef seminal vesicle (50g) obtained was thawed and freed of fat and connective tissues. Then the seminal vesicle was sliced and homogenized in 40ml of 0.02M Tris-HCl buffer, pH 7.6 for 2min at 4°C with a blender. The homogenate was centrifuged at 6000 rpm for 10min at 4°C. The supernatant was decanted and centrifuged twice and the resulting supernatant was then used as the crude enzyme preparation.

**Anti-inflammatory test:**
**Effect of *Koelreuteria longiflora* fruit extract on Phospholipase A2 activity:**
The method of Vane (1971) was used. Aliquots (0.1ml) of re-suspended fresh human red blood cell (HRBC), 0.2ml of CaCl2 (2mM) and 0.2ml of enzyme preparation from enterobacter and varying concentrations of the extract were incubated for one hour. The incubates were centrifuged at 3000rpm for 10min. Samples of the supernatant were diluted with 10ml normal saline and the absorbance of the solutions read at 418nm using distilled water as blank.

The formula below was used to calculate the maximum enzyme activity:

\[
\% \text{Maximum enzyme activity} = \frac{\text{O.D. of test sample} - \text{O.D. of control}}{100}
\]

Percentage inhibition = 100 - % Maximum enzyme activity.

**Effect of the extract on Prostaglandin Synthetase activity:**
The resultant supernatant of the crude enzyme preparation above was used as the source of the enzyme. The method of Yoshimoto et al. (1970) was used for the determination of the effect of the extract on prostaglandin synthetase activity. Thus, increase in absorbance was monitored at 278nm due to formation of PGB from PGE2 and the difference in absorbance reading determines the degree of the activity of the extract.

The cofactor solution was prepared by mixing 33mM hydroquinone, 2mM glutathione and 40μM haemoglobin in the ratio of 1:1:8. Thus, 8mg solution of prostaglandin synthetase was weighed into each test tube and 1.5ml of cofactor solution was also added, then the mixtures were allowed to pre-incubate for 2 min at 37°C. The reaction was started by adding 0.2cm3 of the substrate (bambara oil) and allowed to proceed for 2 min at 37°C. Varying concentrations of the extract and buffer were added to make the volume up to 2.5cm3. The mixtures were incubated for 2 min and the reaction was terminated by the addition of 0.5ml of citric acid.
Table 1: Effects of Extract on Egg albumin-Induced Oedema and Average Inhibition of Inflammation of the Rat Paw

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Δ Paw volume (oedema) ml and average oedema</th>
<th>30 mins (Mins)</th>
<th>1hr</th>
<th>2hrs</th>
<th>3hrs</th>
<th>4hrs</th>
<th>5hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>200</td>
<td>0.96±0.006 (0.24)</td>
<td>0.84±0.021 (0.24)</td>
<td>1.06±0.000 (0.24)</td>
<td>0.95±0.021 (0.24)</td>
<td>0.67±0.000 (0.24)</td>
<td>0.50±0.021* (0.24)</td>
<td></td>
</tr>
<tr>
<td>Extract</td>
<td>400</td>
<td>0.44±0.000 (0.23)</td>
<td>0.67±0.000 (0.23)</td>
<td>0.81±0.000 (0.23)</td>
<td>1.05±0.021 (0.23)</td>
<td>0.77±0.021 (0.23)</td>
<td>0.51±0.021* (0.23)</td>
<td>0.47±0.021* (0.23)</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>25</td>
<td>0.80±0.021 (0.25)</td>
<td>1.05±0.021 (0.25)</td>
<td>1.28±0.000 (0.25)</td>
<td>1.14±0.021 (0.25)</td>
<td>1.00±0.000 (0.25)</td>
<td>0.90±0.020 (0.25)</td>
<td>0.86±0.021* (0.25)</td>
</tr>
<tr>
<td>Normal Saline</td>
<td>–</td>
<td>0.25±0.021 (0.25)</td>
<td>0.51±0.000 (0.25)</td>
<td>0.77±0.021 (0.25)</td>
<td>0.99±0.000 (0.25)</td>
<td>1.06±0.000 (0.25)</td>
<td>1.01±0.020 (0.25)</td>
<td>1.02±0.020 (0.25)</td>
</tr>
</tbody>
</table>

* Reduction in oedema is significant at p<0.05 compared to the control. Values of oedema shown are mean ± SD (n=5).
Values in parenthesis are Average oedema calculated relative to the paw volume at time zero.

Table 2: Effect of the methanolic fruit extract of R. longiflora on rat erythrocyte haemolysis

<table>
<thead>
<tr>
<th>Test Tube</th>
<th>RBC (ml)</th>
<th>Normal Saline (ml)</th>
<th>Extract (mg/ml)</th>
<th>Indomethacin (mg/ml)</th>
<th>Distilled H2O (ml)</th>
<th>OD (418nm)</th>
<th>% Inhibition of Haemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1</td>
<td>2.4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.030±0.001</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>1.9</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.099±0.003</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>1.8</td>
<td>0.1</td>
<td>–</td>
<td>–</td>
<td>0.075±0.003</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>0.1</td>
<td>1.7</td>
<td>0.2</td>
<td>–</td>
<td>–</td>
<td>0.070±0.001</td>
<td>33</td>
</tr>
<tr>
<td>5</td>
<td>0.1</td>
<td>1.5</td>
<td>0.4</td>
<td>–</td>
<td>–</td>
<td>0.060±0.001</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>0.1</td>
<td>1.3</td>
<td>0.6</td>
<td>–</td>
<td>–</td>
<td>0.058±0.001</td>
<td>53</td>
</tr>
<tr>
<td>7</td>
<td>0.1</td>
<td>1.7</td>
<td>–</td>
<td>0.2</td>
<td>–</td>
<td>0.059±0.003</td>
<td>52</td>
</tr>
<tr>
<td>8</td>
<td>0.1</td>
<td>1.5</td>
<td>–</td>
<td>0.4</td>
<td>–</td>
<td>0.045±0.001</td>
<td>60</td>
</tr>
<tr>
<td>9</td>
<td>0.1</td>
<td>1.3</td>
<td>–</td>
<td>0.6</td>
<td>–</td>
<td>0.040±0.001</td>
<td>83</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD of six experiments.

The reaction mixtures were then extracted twice with 5ml ethyl acetate and centrifuged at 2,500 x g for 10 min. For each extraction, 4ml of the top organic layer was pipetted out into a clean test tube. The combined ethyl acetate extract (8ml) was evaporated to dryness using sand bath. The dried residue was then dissolved in 2ml methanol and 0.5ml of 3M KOH solution was also added and was allowed to stand for 15 min. The absorbance of test solution was read at 278nm. The experiment was compared with the results of the blank (boiled enzyme) and that of the positive control (indomethacin).

To calculate the enzyme activity = \( \frac{\Delta \text{Absorbance} 278\text{nm} \times 10 \times 2.5 \times 100}{25.6 \times 8 \text{ (mg of enzyme best)}} \)

Data analysis
Data obtained from this study were analyzed statistically and expressed as mean ± SD. The results were compared using one way ANOVA and regarded as significant at p<0.05.

RESULTS:
Acute toxicity studies:
From the acute toxicity and mortality (LD₅₀) tests for the extract, no death was recorded in the two stages of the test using intraperitoneal (i.p.) route. Therefore the LD₅₀ was greater than 5g/kg.

Effect of extract on egg albumin-induced oedema in rats:
Data from Table 1 shows that the paw volume taken immediately after fasting the animals, and soon after the injection of the egg albumin was assumed to be at time zero (t = 0), while the mean paw volumes for the various groups were taken at different time intervals. The oedema swellings induced by egg albumin in rats were significantly (p<0.05) inhibited by the extract especially after the third hour. However, the oedema reduction for the R. longiflora treated groups was more than that observed for the standard anti-inflammatory drug, indomethacin.

Effect of extract on erythrocyte membrane-stability:
The extract at different concentrations significantly (p<0.05) protected the rat erythrocyte membrane against lysis induced by hypotonic solution. However, at a concentration of 0.2mg/ml, indomethacin (standard drug) produced 52.0% inhibition of RBC haemolysis as compared with 33.0% produced by the extract at the same concentration (Table 2).

DISCUSSION:
The results of this study showed that Rothmannia longiflora fruit extract possesses anti-inflammatory property as it significantly (p<0.05) inhibited oedema induced by egg albumin in rats. Although the reduction of the oedema took effect after the third hour. This report agrees with earlier reports of Ely et al. (2006) which showed that suppression of inflammation at one hour post injection of phlogistic...
agent tends to show that the agent could be antihistamine, whereas reduction of inflammation at three hours and beyond, shows that the agent could have caused inhibition of arachidonic acid pathway.

In inflammatory reactions, there is increased vascular permeability which leads to exudation of fluid rich in plasma proteins including immunoglobulins, coagulation factors and cells into the injured tissue with subsequent oedema at the site (http://www.Afr.Biotech. 2006). Oedema results from the action of inflammatory mediators such as histamine, serotonin, prostaglandin and bradykinin at the site of a local inflammatory insult (Harriot et al., 2004). So the ability of the extract to reduce the size of oedema produced by egg albumin in the third hour suggests that it was active at the later phase of the oedema and not at the early phase.

Furthermore, the extract did show membrane stabilizing effect, as it offered significant (p<0.05) protection of the erythrocyte against lyses induced by hypotonic solution but the mechanism of action was not known. The erythrocyte membrane resembles lysosomal membrane and such the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane (Oyedapo et al., 2004). Also according to Nwodo and Eze (2001), when lytic enzyme activity and membrane-stabilization were investigated with Abrus precatorius, it was found that the extract stabilizes membrane. This may suggest that the anti-inflammatory properties of this extract may be due to the inhibition of enzymes or processes, which may enhance the release of lytic enzymes.

Therefore, as the mechanism of action of Rothmannia longiflora in stabilizing membrane was not known, further studies will be undertaken to determine whether R. longiflora fruit extract will be able to inhibit these enzyme that may enhance the release of lytic enzyme.

REFERENCES:
