Anti-Inflammatory, Fibrinolytic and Anti-Oxidant Activities of the N-Hexane Extract of *Ficus sur* Forssk (Moraceae) Leaves

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**Abstract**

This study investigates the anti-inflammatory, fibrinolytic and anti-oxidant activities of the n-hexane extract and fractions of *Ficus sur* Forssk leaves. The extract was separated on column chromatography using a gradient elution of hexane, dichloromethane and ethanol. Pooled column fractions were assayed for membrane stabilizing for anti-inflammatory, Streptokinase inhibition for fibrinolytic and 2, 2-dichloromethane and ethanol. Pooled column fractions were assayed for membrane stabilizing for anti-inflammatory and fibrinolytic ability. The leaf extract of *F. sur* showed negligible anti-inflammatory activity. Except for F1 all the fractions and the crude extract showed very high dose-dependent anti-inflammatory property even significantly higher (p <0.05) than the reference drug – diclofenac. Crude extract, F2 and F3 exhibited fibrinolytic ability. F1 showed the lowest assayed biological activity consistently. The study shows that the column fractions of n-hexane extract of *F. sur* leaves have fibrinolytic and anti-inflammatory activity and can be used in the management of thrombosis and inflammation.

**Keywords:** *Ficus sur*, Moraceae, Anti-inflammatory, Fibrinolysis, DPPH reduction, Membrane stabilizing, Diabetes, n-hexane extract.

**INTRODUCTION**

A metabolic syndrome is a group of symptoms that occur at the same time and raise the risk of heart disease, stroke, and type-2 diabetes. Insulin resistance, visceral adiposity, atherogenic dyslipidaemia, endothelial dysfunction, genetic susceptibility, elevated blood pressure, hypo-fibrinolysis, hypercoagulable state, and chronic stress are the several factors that constitute the syndrome. Chronic inflammation is known to be associated with visceral obesity and insulin resistance which is characterized by the production of abnormal adipocytokines such as tumour necrosis factor α, interleukin-1 (IL-1), IL-6, leptin, and adiponectin (Kaur, 2014). The creation of inflammatory mediators is stimulated by oxidative stress, and inflammation in turn, increases the formation of reactive oxygen species (Giacco & Brownlee, 2010; Mu et al., 2018; Ogundele et al., 2017). Complex haemostasis abnormalities, such as increased coagulation, platelet dysfunction, and hypo-fibrinolysis, are associated with type 2 diabetes (DM2) which could lead to deep vein thrombosis and eventually to death. Type 2 diabetics are predisposed to increased morbidity and mortality from thrombotic complications as a result of these clinical diseases (Bryk-Wiązania & Undas, 2021; Kearney et al., 2017; Trost et al., 2006). Hypercoagulability, usually strongly influenced by the resistance of clot formed to physiological lysis (hypo-fibrinolysis) goes hand in hand with inflammation (Kell & Pretorius, 2015). The relationship between C-reactive protein (an inflammatory mediator) and hypo-fibrinolysis is such that C- reactive protein may impede fibrinolysis by causing the release of plasminogen activator inhibitor-1 (PAI-1) from human aortic endothelial cells (Zouaoui Boudjeltia et al., 2004).
Plant extracts contain useful phytochemicals that can be used as alternatives to conventional pharmaceuticals in the treatment of a variety of human ailments. Given the relationship and link between inflammation and oxidative stress, as well as the common occurrence of hypo-fibrinolysis in a disease state like diabetes, it is advantageous to have a medication that has different medicinal actions to the events that can occur in a disease state like diabetes. *Ficus sur* Forssk is a round-crowned tree that grows up to 25 meters tall and may be found in a variety of environments, including grassland, woodland, riverine forest, semi-deciduous forest, and rocky outcrops. Its young shoot sand leaves are cooked and eaten as vegetables (Achigan-Dako et al., 2010; Facciola, 2020). It can be used in the treatment of burns (Hyde et al., 2020). In vitro antibacterial and anti-inflammatory action has been demonstrated in bark, leaf, and root extracts (Facciola, 2020). In vivo antimalarial activity has been reported using methanol extracts of the leaves, stem bark, and root bark (Facciola, 2020). In vivo anti-ulcer activity and in vitro spasmytotic effects have been shown in aqueous and methanol extracts of the dried leaves (Ayiinde & Owolabi, 2009; Fern et al., 2014). Wounds, burns, toothaches, eye issues, general bodily discomfort, lung and throat problems, gonorrhoea, and emetic are all treated with its latex (Hyde et al., 2020). The stem and leaf have been shown to have anti-microbial activity (Solomon-Wisdom et al., 2011) and antidiabetic activity (Shi et al., 2018). However, having reported the antidiabetic effect of *Ficus sur* hexane extract and its fractions in Suleiman and Kio (2018), this study aimed at investigating the anti-inflammatory, anti-oxidant and fibrinolytic activities of *Ficus sur* n-hexane leaf extract.

**MATERIALS AND METHODS**

**Plant Collection and Identification**

*Ficus sur* Forssk (Moraceae) leaves were collected from Ahoada Local Government Area of Rivers State respectively. The leaves were identified and authenticated by Dr A.T. Oladele and Herbarium Specimens with voucher numbers UPF300 was deposited in the Herbarium Section of the Department of Forestry, University of Port Harcourt and in the Herbarium of Department of Pharmacognosy and Phytotherapy, University of Port Harcourt. The leaves were air-dried at room temperature, pulverized and preserved for further use.

**Extraction and Column Fractionation**

A total of 2 kg powdered *F. sur* leaves were exhaustively macerated in n-hexane for three days. The extract was filtered and concentrated en vacuo with a rotary evaporator (England lab science). The oily dark greenish extract was kept in airtight glassware and stored in a desiccator until it was needed. The n-hexane extract was fractionated using column chromatography packed with silica gel G (60-120 mesh) and eluted with mixtures of n-hexane, dichloromethane, and ethanol in gradients, as previously reported (Suleiman & Kio, 2018). Fractions were developed in dichloromethane on a pre-coated analytical thin-layer chromatography (TLC) GF254, monitored, and bulked into five fractions (F1 through F5). The fractions were tested separately.

**In vitro anti-inflammation assay**

The red blood membrane stabilization assay was used to test for the anti-inflammatory activity of the n-hexane extract and fractions of the leaves of *F. sur* (Mikailu et al., 2019). To avoid clotting, 5 mL of fresh human blood was transferred to an ethylene diamine tetraacetate (EDTA) centrifuge tube. To remove the supernatant, it was centrifuged at 2000 rpm for 10 minutes. The remaining erythrocytes were centrifuged after being rinsed three times with an equivalent volume of normal saline. The erythrocytes were suspended in isotonic phosphate buffer (pH 7.4) to make a 40 % v/v suspension. A suspension of 20, 40, 60 and 80 µg of each of the fractions F1 – F5 was made using dichloromethane in four (4) sets each and allowed to evaporate. A five (5) mL of phosphate buffer solution (pH 7.4) and 1-ml Tween 80 were introduced to each tube. Control tubes contained 5 mL of phosphate buffer solution (pH 7.4) without extracts (negative control) and 5 mL of diclofenac 100 µg/mL (positive control). A 0.5 µg/mL erythrocyte suspension was added into each tube and gently mixed. In a controlled water bath, a pair of tubes from each set were incubated at 54 ºC for 20 minutes. The second pair was kept in ice for 20 minutes at a temperature of 0-4 ºC. The reaction mixture was centrifuged at 1000 rpm for 3 minutes in each of the four tubes, and the absorbance of the supernatant was measured at 540 nm. The percentage of haemolysis inhibition was estimated as follows:

\[
\text{Percent haemolysis inhibition} = 100 \times \left(1 - \frac{OD_1 - OD_3}{OD_2 - OD_3}\right)
\]

OD1 = Unheated test sample, OD2 = Heated test sample and OD3 = Heated control sample

The concentration of extracts exhibiting 50% inhibition of enzyme activity (IC50) was determined graphically.

**Fibrinolytic activity**

The Prasad technique for determining fibrinolytic activity, which was previously reported (Ebenezer et al., 2014), was employed. Five (5) mL of sterile injection water were carefully combined with commercially available streptokinase (Ickinase ®) - 1,500,000 IU. From this stock, 100 µL (30,000 IU) of streptokinase was obtained. Healthy participants provided venous blood (5 mL), which was put into 500 µL Eppendorf tubes that had already been weighed. These were incubated at 37 °C for 45 minutes. After the clotting, a Pasteur pipette was used to extract serum entirely from each tube. The leftover clots as well as the tube were weighed. The plant fractions were dissolved in dichloromethane to achieve concentrations.
of 10 µg/mL, 50 µg/mL, and 100 µg/mL, respectively, afterwards each was added to the various Eppendorf tubes containing clots in triplicates. A positive control of 100 µL of streptokinase was utilized, whereas negative control was dichloromethane and water respectively without streptokinase or plant fraction. All tubes were incubated for 90 minutes at 37°C. The fluids recovered were fully aspirated after incubation, and the tube including clot for each sample was reweighed to check for clot weight differences. The following formula was used to calculate clot weight in all cases:

\[ \text{Clot weight} = \text{Weight of clot + tube} - \text{weight of empty tube} \]

Using the equation below, the difference in clot weight before and after the addition of the plant fractions and streptokinase was represented as a percentage of clot lysis:

\[ \text{Percentage weight of blood clot lysed} = \frac{\text{Average difference in clot weight}}{\text{Average original weight of clot}} \times 100\% \]

**Antioxidant assay**

The modified 2,2-diphenyl-1-picrylhydrazyl (DPPH) model of the free radical scavenging activity technique was utilized. 5mg of DPPH were weighed and put to a 100mL conical flask covered with foil paper. In aliquots, 100mL of methanol was added to the reagent, with intermittent shaking at intervals. The solution was stored at room temperature in the dark. The fractions 1 - 5 obtained from column chromatography separation were prepared using dichloromethane at 1mg/ml. After that, 2.0 mL of 50 µg/mL DPPH in methanol was added to the varied concentrations of the test samples and incubated for 30 minutes in the dark. A 2ml methanol:2ml DPPH (1:1 v/v) was used as a negative control. The blank – methanol – was used to calibrate the UV spectrophotometer. The spectrophotometer was used to measure the absorbance of the individual test samples and the standard at 517nm. Positive control was ascorbic acid, which was generated at a concentration of 80 µg/mL. The following approach was used to compute the % inhibition of the various concentrations:

\[ \text{Percentage inhibition} = \frac{\text{Absorption of control} - \text{Absorption of test}}{\text{Absorption of control}} \times 100\% \]

Anti-oxidant activity was recorded as the percentage reduction in the absorbance of DPPH by the test samples or standard.

**Gas Chromatography-Mass Spectrometer (GC-MS)**

The fraction F3 that is consistently exhibiting activity in the assays was analysed on GC-MS to determine the various compounds present in it with their appropriate masses. It was dissolved in n-hexane using an Agilent gas chromatograph Model 6890, coupled to a Mass spectrometer equipped with a DB-1MS capillary column (30m long × 320 µm nominal diameter), programmed and ran to obtain the mass spectra data. The compounds from fraction F3 were identified by comparing their retention time and mass with those of reference compounds in the NIST library. Acceptance was based on a quality factor greater than 80.

**Statistical Analysis**

The data was presented as mean ± standard error of the mean (SEM). Graph pad Prism version 6 was used to conduct one-way and two-way ANOVA testing. Significance was defined as a p-value of less than 0.05.

**RESULTS**

**Percentage Yield**

Table 1 shows the percentage yields of the n-hexane extract and the five (5) fractions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight (g)</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Hexane extract</td>
<td>39.05</td>
<td>1.95</td>
</tr>
<tr>
<td>Fraction 1</td>
<td>1.99</td>
<td>15.31</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>1.44</td>
<td>11.1</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>1.96</td>
<td>15.1</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>1.72</td>
<td>13.2</td>
</tr>
<tr>
<td>Fraction 5</td>
<td>3.86</td>
<td>29.7</td>
</tr>
</tbody>
</table>

**Anti-inflammatory / Membrane stabilizing effect of the fractions from n-hexane extract**

Figure 1 illustrates the effect of the fractions and crude extract on membrane stabilisation while Figure 2 displays their IC\(_{50}\). The activity trend was almost dosage dependent. As the concentration of each fraction and extract changed, there was a significant variation in the membrane stabilising effect (p < 0.05). F3 and F4 had a reduced membrane stabilising impact as concentration increased despite having a considerable membrane stabilising effect at all doses. Only F1 did not exhibit a significant membrane stabilizing effect. When compared to diclofenac, the anti-inflammatory activity of the fractions and n-hexane was sometimes superior. The activity of the fractions and crude extract differed significantly according to a two-way ANOVA analysis (p < 0.05).
Figure 1: Membrane stabilizing effect of the fractions, n-hexane crude extract and diclofenac. Inhibition above 50% was considered significant

Figure 2: IC_{50} showing membrane stabilizing effect of the fractions and the crude extract

Fibrinolytic effect of the fractions from n-hexane extract

The fibrinolytic activity of the crude extract and fractions is shown in Figure 3. A clot lysis of less than 30% was deemed negligible. Figure 3 demonstrates a significant dosage dependent rise in fibrinolytic activity of F2, F3, and the crude hexane extract (p < 0.05), with 100 µg/ml concentration exhibiting the only significant fibrinolytic activity among fractions and extract. Streptokinase had higher fibrinolytic activity when compared to fractions. F3 showed the strongest fibrinolytic activity in all concentrations, followed by n-hexane and then F2 (p < 0.05).

Figure 3: Fibrinolytic effect of the samples. Clot lysis above 30% was considered significant
Anti-oxidant / DPPH reduction effect of the fractions from n-hexane extract

Figure 4 reveals the DPPH reduction models low anti-oxidant effect on the fractions and crude extract of F. sur. As F2 was not soluble in methanol, its DPPH reduction activity could not be determined. Even at a low concentration of 80 µg/mL, ascorbic acid had extremely potent anti-oxidant activity. Since the extract and fractions of F. sur demonstrated less than 50% anti-oxidant activity, a dose-dependent experiment was not performed.

Figure 4: Anti-oxidant / DPPH reduction effect of the fractions from n-hexane extract. All test samples were used at concentration of 1mg / ml. Ascorbic was used at 80 µg/ml. Percent inhibition greater than 50% was considered significant

Gas Chromatography-Mass Spectrometer (GC-MS)

Two major compounds; 9,12- octadecadienoic acid and Phytol were identified from the compounds eluted from the gas chromatograph (Figure 5) based on their fragmentation and match with the library as presented in Table 2.

Table 2: Gas Chromatography-Mass Spectrometer (GC-MS) of Fraction F3

<table>
<thead>
<tr>
<th>S/ no</th>
<th>Peak area</th>
<th>Retention time</th>
<th>Compound</th>
<th>Percentage</th>
<th>Reported Biological activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23.9</td>
<td>12.095</td>
<td>9,12- octadecadienoic acid</td>
<td>6.96</td>
<td>Antiarthritic, anti – histaminic (Rajeswari et al., 2012), anti-diabetic (Moloney et al., 2007)</td>
</tr>
<tr>
<td>2</td>
<td>6.96</td>
<td>9.000</td>
<td>9,12- octadecadienoic acid</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>13.7</td>
<td>16.577</td>
<td>Phytol</td>
<td>13.7</td>
<td>Antimicrobial (Islam et al., 2018; Taj et al., 2021), anti-inflammatory (Islam et al., 2020; Taj et al., 2021)</td>
</tr>
</tbody>
</table>
DISCUSSION

Several works have shown the link between diabetes and oxidative stress (Crook, 2004; Giacco & Brownlee, 2010; Ma et al., 2018; Oguntibeju, 2019; Vikram et al., 2014), inflammation (Crook, 2004; Festa et al., 2000; Rehman & Akash, 2017) and hypo fibrinolysis (Kearney et al., 2017; Staško et al., 2011; Trost et al., 2006). Since the fractions of the n-hexane extract demonstrated anti-hyperglycaemia (Suleiman & Kio, 2018), a good drug candidate will be one that does not only bring about anti-hyperglycaemia but also have potential in ameliorating interaction of endocrine system with other disease conditions. Some drugs have the complementary effect of preventing blood clot, fibrinolysis and reducing free radicals. These were investigated on the n-hexane leaf extract of Ficus sur.

From the result of this study, it can be seen that the extract has anti-inflammatory and fibrinolytic activities but very low anti-oxidant potential. The fibrinolytic activity shown by some concentrations of F2, F3 and the n-hexane extract tallies with the work carried out by Nnah (2015) where there was an increased bleeding time and clotting time observed when the methanol extract of F sur was administered to diabetic rats. The enhanced fibrinolytic activity means that when treating diabetes with F sur, thrombosis could also be prevented.

The idea behind using erythrocyte membrane stabilizing assay to test for anti-inflammatory activity is based on the fact that erythrocyte membrane are similar to the lysosomal membrane (Gunathilake et al., 2018). Therefore, any extract that stabilizes the erythrocyte membrane will be able to stabilize the lysosomal membrane and therefore prevent the release of lysosomal components which initiate inflammatory processes (Saleem et al., 2011; Yesmin et al., 2020). All the fractions and the crude extract exhibited in vitro anti-inflammatory activity even higher than the drug of reference diclofenac. Anti-inflammatory activity has also been demonstrated in the ethanolic leaf extract of F. sur Forsk (Omodamiro et al., 2021). The GC-MS analysis revealed the compound, 9,12-octadecadienoic acid. This compound happens to be a poly unsaturated fatty acid and has been reported to have anti-inflammatory, anti-diabetic and fibrinolytic activities (Moloney et al., 2007; Rajeswari et al., 2012). These support the anti-inflammatory and fibrinolytic activities exhibited by F. sur.

CONCLUSION

Fractions obtained from n-hexane extract of Ficus sur demonstrated anti-inflammatory and fibrinolytic activities in the in vitro studies conducted. It is also evident that the anti-inflammatory activities exhibited is due to the presence of 9,12-octadecadienoic acid in the fraction. It could also be suggested that the fractions of this plant will not only serve as an anti-diabetic but also in ameliorating the secondary complications that accompanies diabetes.

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