IN-VITRO LIPOXYGENASE INHIBITORY ACTIVITY AND TOTAL FLAVONOID OF STRYCHNOS SPINOSA LEAF EXTRACTS AND FRACTIONS

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ABSTRACT

Strychnos spinosa Lam. is a deciduous tree used in traditional medicine to treat inflammation and infectious diseases. This study was undertaken with the objectives of establishing the inhibitory effect of extracts and fractions of Strychnos spinosa leaves on the mediator of inflammation lipoxygenase (LOX), and to evaluate the total flavonoid content of the crude extracts and fractions of Strychnos spinosa leaves. The water fraction, chloroform fraction and the n-butanol fraction were the most active with median inhibitory concentration (IC₅₀) values of 70.75 ± 0.03, 85.27 ± 0.06 and 127.85 ± 0.06 µg/mL, respectively. The methanol, acetone, dichloromethane/methanol and the alkaloid extracts had IC₅₀ values of 143.21 ± 0.00, 149.14 ± 0.05, 154.66 ± 0.01 and 164.03 ± 0.00 µg/mL, respectively. The least active is the hexane and ethyl acetate fractions with IC₅₀ values of 204.02 ± 0.01 and 231.49 ± 0.00 µg/mL, respectively. Apart from the water and chloroform fractions, the anti-inflammatory activity of all the extracts and fractions were lower than the positive controls. The nButF contains the highest level of flavonoid (128.87 ± 2.96 mg QE/g plant material), while the least amount is found in the WatF (0.20 ± 0.00 mg QE/g plant material). In the acute toxicity studies, the median lethal dose (LD₅₀) in all the crude extracts were found to be above 5000mg/kg. The results obtained support the use of S. spinosa leaves in traditional medicine for the treatment of inflammatory related conditions.

Key words: Strychnos spinosa; Inflammation; Lipoxygenase; Flavonoid

INTRODUCTION

Plant species of the genus Strychnos have been used in folk medicine and in arrow and dart poisons in many parts of the world (Thongphasuk et al., 2003). Its common names include: spiny monkey-orange (English), Kokiya (Hausa), Atako (Yoruba) (Neuwinger, 1996). The plant is used in traditional medicine from Senegal through tropical Africa to South Africa as
an analgesic and in the treatment of venereal diseases, stomach disorders, and snake bites (Hedberg et al., 1983). A decoction of the leaf or root is used as an analgesic in Central Africa. In Gambia, leaf decoction with barks powder are used for the treatment of wounds, while in Cameroon dried and powdered leaves are taken in food for liver damage (Neuwinger, 1996). The Zulus of South Africa use the green fruits as an antidote to snakebite (Hedberg et al., 1983; Mors et al., 2000). The genus is a member of the Loganiaceae family, comprising about 200 species. The fruit is generally considered by botanical collectors to be edible, but there are poison-makers, who express a contrary opinion and consider the unripe fruit poisonous (Neuwinger, 1996). Pharmacological properties of S. spinosa include antiplasmodial (Bero et al., 2009), antitrypanosomal (Hoet et al., 2007) and anthelmintic (Waterman et al., 2010) activities. Antimicrobial activity has also been reported from the stem bark extract of S. spinosa (Kubmarawa et al., 2007; Nwozo et al., 2010; Ugo and Beijide, 2013), and the antioxidant activity of the fruit extract of the plant has been documented (Nhukarume et al., 2010). Secondary metabolites as triterpenoids, sterols and essential oils (Hoet et al., 2007), secoiridoids (Msonthi et al., 1985; Itoh et al., 2005), alkaloids (Ohiri et al., 1984), and monoterpenes (Adesogan et al., 1981) have been reported from S. spinosa. Flavonoids are naturally occurring polyphenolic compounds ubiquitously found in plants. They have long been recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anti-carcinogenic activities (Middleton et al., 2000).

MATERIALS AND METHODS
Collection and preparation of plant materials

Fresh leaf of Strychnos spinosa leaves were collected in January, 2013 in Sakara village, located along new Jos road, Zaria. The plant was identified and authenticated by a taxonomist, (Mal Musa Muhammad) in the Herbarium section, Department of Biological Sciences, Ahmadu Bello University, Zaria as compared by a voucher specimen No. 900161. The leaves were sorted from the stem, packed in a well perforated bag and air dried under shade at room temperature for 2 weeks. The dried leaves were ground, powdered and kept in an air tight polyethylene bag until needed for the extraction process.

Preparation of extracts and fractions

Acetone extraction

Two kilograms of the dried powdered leaves was macerated three times in six litres of acetone to give acetone extract (AcetE) after filtration and removal of the solvent in vacuum.

Methanol extraction

The residues left after extraction with acetone were further macerated three times in six litres of methanol using the same procedure as described above for acetone to give the methanol extract (MetE).

Dichloromethane/methanol extraction

One kilogram of the dried powdered leaves was macerated three times in three litres of mixture (50/50, v/v) of dichloromethane / methanol to give the dichloromethane / methanol extract (DcmMetE) after filtration and removal of the solvent in vacuum.

Fractionation of the acetone extract

The acetone extract (70 g) was dissolved in 700 mL of chloroform and partitioned with 700 mL of water to obtain the water and chloroform components. The water component was further partitioned with 600 mL of n-butanol to afford n-butanol and water (Wat1) fractions while the chloroform component was concentrated to dryness and dissolved in 100 mL (10%)
water in methanol and partitioned with 2.5 L of n-hexane to obtain the hexane fraction and the residue of 10% water in methanol. The hexane fraction was concentrated to dryness and the residue of 10% water in methanol was further diluted using distilled water (162.5 mL) to give 35% water in methanol. The 35% water in methanol was partitioned with 2 L of chloroform which finally gave the chloroform and 35% water (wat2) fractions. From the comparative TLC, water (Wat1) and 35% water in methanol (Wat2) fractions were combined into one fraction.

**Alkaloids extraction**

The leaves of *S. spinosa* (1 kg) was macerated with the mixture (96:3:1, v/v) of EtOAc-EtOH-NH₄OH (600 mL) and then percolated with EtOAc to give the extract after removal of the solvent using rotary evaporator under reduced pressure. The extract was further dissolved in EtOAc and extracted with 4% acetic acid to afford EtOAc fraction (EtAcF) and the acidic solution (pH 3-4) was basified to pH (8-9) with Na₂CO₃ and extracted three time with DCM to give crude alkaloids extract (AlkE) after removal of the solvent in vacuum.

**Instruments and reagents**

Beckman LS6000LL scintillation counter, indomethacin (Sigma), quercetin, catechin, Lipoxidase (soybean) Lot 050M1910V Sigma, United States, boric acid, linoleic acid (Merck, Germany), Sodium Hydroxide, micro plate reader (Labotec).

**Determination of total flavonoids**

The total flavonoids content of the extracts was determined by aluminum chloride method as described by Abdel-Hamed *et al* (2008) with some modification. Briefly 100 µL of the extract was mixed with 100 µL of 20 % AlCl₃ and two drops of glacial acetic acid. The mixture was diluted with methanol to 3000 µL and the absorbance was read at 415 nm after 40 mins. Blank samples were prepared using the extract only. Standard curve was prepared using quercetin (3.9-500 µg/ml) in methanol. The amount of flavonoids was expressed as mg quercetin equivalent/g of dry plant material.

**Lipoxygenase inhibition assay**

Lipoxygenase activity was determined spectrophotometrically according to (Kumaraswamy and Satish, 2008) which is based on the enzymatic oxidation of linoleic acid to the corresponding hydroperoxide. Ninety microlitres of 5-LOX (bought from Sigma as 5-LOX, and made to a working solution of 200 units/mL, kept on ice) enzyme was added to test extracts dissolved in DMSO (10 µL). The solution was, incubated at room temperature for 5 min after which 100 µL substrate solutions (10 µL linoleic acid dissolved in 30 µL ethanol, made up to 120 mL with 2 M borate buffer at pH 9.0) was added to the solution containing extracts in different concentrations ranging from 15.6-250 µg/mL. After 5 min of incubation at room temperature, the absorbance was read at 234 nm with a microplate reader (SpectraMax 190, Molecular devices). Indomethacin, quercetin and catechin were used as positive controls, while DMSO was used as negative control. The percentage enzyme inhibition of each extract compared with negative control as 100 % enzyme activity was calculated using the equation:

\[
\%\text{ inhibition by extract} = \left( \frac{OD\text{ extract} - OD\text{ blank}}{OD\text{ negative control} - OD\text{ blank}} \right) \times 100\%
\]

**Acute Toxicity studies**

The LD₅₀ determination was conducted using the method of Lorke (1983). The study was conducted into two stages.
In the first phase, the mice were divided into three. Each group comprises three mice. The animals were treated with the aqueous extract of *Strychnos spinosa* plant as follows: 10, 100, and 1000mg/kg orally and observed for 24 hours after the administration. In the second phase, the mice were grouped into three groups of one mouse each and treated with dose-extract of 1,600, 2,900 and 5,000 mg/kg and observed for 24 hours after the administration.

**Statistical Analysis**

The data obtained were analyzed using one-way ANOVA. This was followed by Dunnett's or Tukey's post hoc tests. All data were expressed as the mean ± SEM. The tests were performed using Statistical Package for the Social Sciences/version 20 Stat. Differences were considered significant, when p values were < 0.5.

**RESULTS AND DISCUSSION**

The median lethal dose was above 5000mg/kg. The yield of the crude extracts of *S. spinosa* leaves of various polarities using acetone, methanol, mixture (v/v) of dichloromethane / methanol (50/50) and alkaloids extraction are presented in Table 1. The methanol extract were an extremely efficient solvent with about 11.9% followed by dichloromethane / methanol (50/50) while alkaloid extract yielded the least (0.28%). Table 1: Extraction Yields from leaf of *S. spinosa* using different solvents.

The lipoxygenase inhibitory activity of the extracts and fractions expressed as median inhibitory concentration 50 (IC\(_{50}\)) is presented on Table 1. MetE is the most active, while the alkaloid extract produced the lowest activity with IC\(_{50}\) of 164.03 ± 0.00 µg/mL. Among the fractions tested, the WatF is the most active (IC\(_{50}\); 70.75 ± 0.03µg/mL), while the EtAcF had the lowest IC\(_{50}\) (231.49 ± 0.00 µg/mL).

Lipoxygenases (LOX) are the members of a class of non-haeme iron containing dioxygenases that catalyze the addition of molecular oxygen to fatty acids containing a cis-1, 4-pentadiene system to give unsaturated fatty acid hydroperoxides. In mammals, LOX catalyze the first step in the arachidonic acid cascade (Mohan et al., 2013). The 5-LOX and 15-LOX’s together lead to formation of biologically active lipoxins whereas 5-LOX’s lead to formation of 5, 6-epoxy-leukotrienes, which are involved in the variety of inflammatory responses, including neutrophil chemotaxis, vascular permeability and smooth muscle contraction. There exists a good correlation between the inhibitory activity of mammalian 5-LOX and soya bean LOX (Mohan et al., 2013). In this study, the leaf extracts and fractions of *S. spinosa* were evaluated for anti-inflammatory activity *in vitro* using LOX derived from soya bean.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Amount (g)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcetE</td>
<td>75</td>
<td>3.7</td>
</tr>
<tr>
<td>MetE</td>
<td>119.9</td>
<td>11.9</td>
</tr>
<tr>
<td>DcmMetE</td>
<td>114g</td>
<td>11</td>
</tr>
<tr>
<td>AlkE</td>
<td>2.8</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Key: AcetE: Acetone Extract, MetE: Methanol extract, DcmMetE: Dichloromethane/methanol extract, AlkE: Alkaloid extract
Table II: Lipoxygenase inhibitory activity of S. spinosa leaves Extract

<table>
<thead>
<tr>
<th>Samples</th>
<th>IC₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Indomethacin</td>
<td>49.79 ± 0.01</td>
</tr>
<tr>
<td>Quercetin</td>
<td>50.58 ± 0.04</td>
</tr>
<tr>
<td>Catechin</td>
<td>91.76 ± 0.00</td>
</tr>
<tr>
<td>Extracts</td>
<td></td>
</tr>
<tr>
<td>AcetE</td>
<td>149.14 ± 0.05</td>
</tr>
<tr>
<td>MetE</td>
<td>143.21 ± 0.00</td>
</tr>
<tr>
<td>DCM/MetE</td>
<td>154.66 ± 0.01</td>
</tr>
<tr>
<td>AlkE</td>
<td>164.03 ± 0.00</td>
</tr>
<tr>
<td>Fractions</td>
<td></td>
</tr>
<tr>
<td>NButF</td>
<td>127.85 ± 0.06</td>
</tr>
<tr>
<td>WatF</td>
<td>70.75 ± 0.03</td>
</tr>
<tr>
<td>HexF</td>
<td>204.02 ± 0.01</td>
</tr>
<tr>
<td>ChlF</td>
<td>85.27 ± 0.06</td>
</tr>
<tr>
<td>EtAcF</td>
<td>231.49 ± 0.00</td>
</tr>
</tbody>
</table>

Lipoxygenases (LOX) are the members of a class of non-haeme iron containing dioxygenases that catalyze the addition of molecular oxygen to fatty acids containing a cis-1, 4-pentadiene system to give unsaturated fatty acid hydroperoxides. In mammals, LOX catalyze the first step in the arachidonic acid cascade (Mohan et al., 2013). The 5-LOX and 15-LOX’s together lead to formation of biologically active lipoxins whereas 5-LOX’s lead to formation of 5, 6-epoxy-leukotrienes, which are involved in the variety of inflammatory responses, including neutrophil chemotaxis, vascular permeability and smooth muscle contraction. There exists a good correlation between the inhibitory activity of mammalian 5-LOX and soya bean LOX (Mohan et al., 2013). In this study, the leaf extracts and fractions of S. spinosa were evaluated for anti-inflammatory activity in vitro using LOX derived from soya bean.

Results in this study showed the WatF and ChlF have the highest lipoxygenase inhibitory activity and were found to be dose-dependent, while the positive controls (indomethacin, quercetin and catecin) had significant and dose-dependent inhibitory effect against LOX activity and found to be more active than all the extracts and fractions.
The inhibitory activities of the extracts and fractions of *S. spinosa* could be attributed to phenolics such as flavonoids or triterpenoids and sterols contents of the extracts and fractions of the plant. Previous studies have shown the role of flavonoids in the inhibition of inflammatory enzymes (Middleton *et al.*, 2000; Arts and Hollman, 2005). Studies have implicated oxygen free radicals in the process of inflammation and phenolic compounds may block the cascade process of arachidonic acid metabolism by inhibiting lipoxygenase activity, and may serve as a scavenger of reactive free radicals which are produced during arachidonic acid metabolism (Trouillas *et al.*, 2003). Furthermore, lipoxygenases inhibition correlate to antioxidants because lipid hydroperoxide formations are usually inhibited as a result of the scavenging of lipid-oxy- or lipid-peroxyradicals formed in the course of enzymatic peroxidation. Consequently, limiting the availability of lipid hydroperoxide substrates required for the catalytic cycle of lipoxygenase oxidative process. The antitrypanosomal activity of the dichloromethane fraction from the leaves of *Strychnos spinosa* was previously reported, (Hoet *et al.*, 2007) and the activity was attributed to triterpenoids and sterols.

The total flavonoid levels in the extracts and fractions of *S. spinosa* is shown on Figure 1 and ranges from 0.20 ± 0.00 to 128.87 ± 2.96 mg QE/g plant material. The nButF contains the highest level of flavonoid (128.87 ± 2.96 mg QE/g plant material), while the least amount is found in the WatF (0.20 ± 0.00 mg QE/g plant material).

**Figure 1: Total flavonoid content of *S. spinosa* leaves and fractions.**

Acetone Extract (AcetE), Methanol extract (MetE), Dichloromethane/methanol extract (DcmMetE), n-butanol fraction (n-butF), water fraction (Wat F), Hexane fraction (HexF), Chloroform fraction (ChlF), Ethyl acetate fraction (EtAcF). Means with different superscript letters are significantly (p<0.05) different.
Flavonoids are C6-C3-C6 polyphenolic compounds present in food, beverage and medicinal plants. They have been reported to have useful pharmacological properties. Flavonoids are known to inhibit inflammatory responses via many routes and block inflammatory molecules such as COX, inducible nitric oxide synthase iNOS, cytokines, nuclear factor-cB and matrix metalloproteinases. In addition, flavonoids possess good antioxidant, free radical scavengers that donate hydrogen, inhibit lipid peroxidation (Havsteen, 2002) and metal ion chelators. In this study all the extracts and fractions of *Strychnos spinosa* inhibited LOX could be attributed to flavonoids contents of the plant.

**CONCLUSION**

The results obtained support the use of *S. spinosa* leaves in traditional medicine for the treatment of inflammatory related conditions.

**REFERENCES**


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