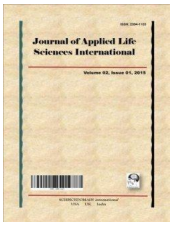

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In-vitro Anti-inflammatory Activities of Extract of the Leaves of *Sphenocentrum jollyanum* Pierre

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Authors' contributions

This work was carried out in collaboration between all authors. Author OOS designed the study and wrote the protocol. Author FOS managed the analyses of the study and wrote the first draft of the manuscript. Author OO performed the statistical analysis and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To evaluate the anti-inflammatory potential of *Sphenocentrum jollyanum* Pierre leaf.
Study Design: Red blood cell Membrane stabilization, anti-lipoxygenase and proteinase inhibitory activities of the extracts were assayed *in-vitro* as a measure of anti-inflammatory potential of *Sphenocentrum jollyanum* leaf.
Place and Duration of Study: All the work was carried out in the Department of Biochemistry, Faculty of Basic Medical Science, Ladoke Akintola University of Technology, Ogbomoso, Nigeria between April 2015-February, 2016.
Methodology: Aqueous, ethanol extracts and the secondary metabolites were extracted using standard techniques. Inhibitory effect of the extracts on erythrocytes membrane stabilization, trypsin and lipoxygenase (*in vitro*) were used to assess anti-inflammatory properties of the leaf. The reactions were performed in triplicates and changes in optical density of test samples and control were measured using a 96-well micro plate reader Spectra Max 384 plus (Molecular Devices, USA) and inhibition were calculated.

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Results: The result of extraction showed that the aqueous and tannin rich extract has the highest yield of 27.50 and 12.00 grams of the crude and secondary metabolites rich extracts respectively. The aqueous extract and saponin rich faction demonstrated the highest dose dependent erythrocyte membrane stabilizing potential among the crude and secondary metabolites rich extracts. It was also observed, that the aqueous extract exhibited a significant (P>0.05) dose dependent lipoxygenase inhibitory activities with IC_{50} of (637 µg/ml) when compared with other extracts. It was observed in the proteinase inhibitory assay, that the ethanol and tannins rich fraction exhibited the maximum inhibitory potential with IC_{50} (840 and 1810 µg/m) among the crude and factions respectively. However, the standard drugs demonstrated the strongest anti-inflammatory activities in all the assays.

Keywords: Anti-inflammatory; Sphenocentrum jollyanum Pierre; leaf extracts; membrane stabilization; lipoxygenase; proteinase.

1. INTRODUCTION

Sphenocentrum jollyanum Pierre (menispermaceae) is a shrub commonly found in West – African countries of Nigeria, Ghana and Cameroun [1]. It is commonly known locally as *Akerejupon* (Yoruba), *Krakoo* (Asante), *Oban abe* (Republic of Benin). Various organs of *S. jollyanum* are employed in folkloric medicine in the management of several ailments such as malaria, fever, rheumatism, and infertility [2,3]. Several scientific studies have reported *S. jollyanum* to demonstrate wide arrays of biological and pharmacological activities such as anti-malaria, anti-allergy [4,5], hypoglycaemic, hypolipidemic [6,7], anti-diabetic activity and haematinic activities [8,9]. Phytochemical analysis of various part of the plant revealed that it contains bioactive compounds such as flavonoids, saponin, tannins, alkaloids and terpenes and these might be responsible for the reported pharmacological and biological activities [1].

Inflammation can be defined as the natural defence system of mammals against invading physical agents, pathogens and microbes [10]. Although, it is a defence mechanism, it produces a variety of undesirable disorders that is characterized by pain, increase in vascular permeability and edema [11]. According to Perianayagam et al. [12] a number of enzymes such as cyclooxygenase, peroxidases, lipoxygenase and arachidonic acid metabolites acid are implicated in the aetiology of inflammatory disorders, while extracellular release of histamine and serine proteases leads to alteration of cellular membranes and denaturation of proteins [10,13].

Globally, steroidal and non-steroidal anti-inflammatory drugs (NSAIDS) are widely employed in the management of inflammatory diseases. Although, these drugs are potent beyond reasonable doubt, however, their prolonged usage results in undesirable and unpalatable side effects such as gastrointestinal disturbances, peptic ulceration, osteoporosis, intestinal bleeding and toxicity [14,15]. Therefore, it is necessary to search for natural, less toxic, more effective therapeutic alternative for the management of inflammatory related disorders. Thus, this study was aimed at evaluating the anti-inflammatory potential of crude and secondary metabolites rich fractions of *S. jollyanum* leaf using standard *in-vitro* approach.

Fig. 1. *Sphenocentrum jollyanum* Pierre

2. MATERIALS AND METHODS

2.1 Plant Materials

The Fresh leaves of *Sphenocentrum jollyanum* were collected from the Botanical garden of University of Ibadan, Nigeria and were identified by Professor A. J. Ogunkunle of the Department of Pure and Applied Biology, Ladoke Akintola University of Technology. A voucher (LHO 241) sample of the plant was also deposited at the University herbarium. The leaves were rinsed with clean water to remove sandy particles and air dried for two weeks after which they were

blended into fine powder with electrical blender (Tower brand, 220-240v 50/60HZ 500W)

2.2 Preparation of Ethanol Leaf Extract of *Sphenocentrum jollyanum*

This was carried out according to method of Mbaka et al. [8]. Finely blended leaf of *S. jollyanum* (300 g) was loaded in a soxhlet extractor in batches for 5 hours each and subjected to extraction with ethanol. After extraction, the solvent was evaporated at 45°C using a rotary evaporator and the extract were kept in a refrigerator (4°C) for further analysis.

2.3 Preparation of Aqueous Leaf Extract of *Sphenocentrum jollyanum*

The aqueous extraction was carried out according to the modified method of Mbaka et al. [8]. Briefly, 300 g of blended plant material was loaded in batches into soxhlet extractor. This was extracted with water in four cycles for about 96 hours. The filtrate obtained was dried in an electric oven between 30-36°C and in kept in a refrigerator (4°C) until it was needed.

2.4 Extraction of Secondary Metabolites Rich Fractions of *Sphenocentrum jollyanum* Leaf

2.4.1 Extraction of Saponin rich fraction

Extraction of Saponin rich fraction of *S.jollyanum* leaf was carried out according to the described method of Obdoni and Ochuko [16] with slight modification. In brief, 300 g of blended plant material was immersed in 250 ml of distilled water. This was extracted thrice with 150 ml diethyl ether. The diethyl ether layer was discarded and the retained aqueous layer was extracted further with 150 ml butanol (four times). The n-butan-1-ol extracts was pulled together and washed four times using 40 ml of 5% sodium chloride (NaCl). The washed extract was concentrated at < 60°C in an electric oven. The extract was air dried at room temperature and stored in the refrigerator (4°C) till it was needed for anti-inflammatory evaluation.

2.4.2 Extraction of tannins rich fraction

Extraction of tannins rich fractions was extracted by method described by Mohamad et al. [17] with slight modifications. Briefly, powdered materials (300 g) were macerated in 1500 ml acetone for 72 hours. The supernatant was then separated from the residue by filtration using Whatman No.1 filter paper. The fraction was concentrated using a rotary evaporator at 45°C and the residue obtained was stored in a (4°C) before further analysis. Qualitative test for presence of tannins was carried out by method described by Harbone [18].

2.4.3 Extraction of alkaloids rich fraction

The extraction of the alkaloids rich fraction was carried according to method of Delima [19] with slight modifications using the continuous extraction method and soxhlet apparatus.

2.4.4 Extraction of flavonoids rich fraction

Flavonoids rich extract was prepared according to method described by Harbone [18]. Exactly 300 g of blended material was immersed in 400 ml ethanol (90% v/v) for 24 hours at room temperature using magnetic stirrer. The mixture was then filtered using Whatman No. 1 filter paper. The process was repeated using the remaining residue with 300 ml ethanol to ensure the proper extraction of the plant material. Filtrates were treated with 150 ml lead acetate (1%) for 4 hours for precipitation. The mixture was filtered and a mixture of 300 ml acetone and 50 ml concentrated hydrochloric acid was added to the precipitate and filtered. The extract was again dissolved in ethanol and extraction process was repeated for 1 hour, filtered to produce red filtrate. The powder was placed in a clean and dry Petri dish away from light at room temperature until deep red brown powder was obtained. This was later stored in the refrigerator (4°C) till it was needed.

2.5 Laboratory Animals and Ethical Protocol

A Total of 10 adult male albino rats with average weight of 160 g were used for the *in-vitro* anti-inflammatory assay. The animals were obtained from the Department of Anatomy Animal House, LADOKE Akintola University of Technology (LAUTECH). They were fed standard food pellets throughout the period of investigation and were allowed access to clean fresh water *ad libitum* in bottles. The experiment was carried out after its approval by the ethics committee of the Ladoke Akintola University of Technology in accordance with the recommendations of the proper care and use of laboratory animals.

2.6 *In-vitro* Anti-inflammatory Assays

Three *in-vitro* procedures were carried out to evaluate anti-inflammatory activities of the

extracts. Human red blood cell (hrbc) membrane stabilization assay was carried out according to the modified method of Sadique et al. [20] and Sakat et al. [21], while anti-lipoxygenase and proteinase inhibition were assayed according to the modified method of Tappel [22] and Oyedepo [23], Sakat et al. [21] respectively. Various concentrations (100, 150, 200, 250 and 300 µg/ml) of extracts were prepared. Diclofenac sodium and Indomethacin were used as reference standard drug.

3. RESULTS AND DISCUSSION

3.1 Extraction Yield

Extraction is the crucial first step in the analysis of medicinal plants, because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization. Proper actions must be taken to assure that potential active constituents are not lost, distorted or destroyed during the preparation of the extract from plant sample [24]. The result of extraction of *S. jollyanum* leaf revealed that the aqueous and tannin rich extract has the highest extraction yield of the crude and secondary metabolites rich extracts with 27.50 and 12.00 grams respectively. This result is similar to the previous observation of [5]. The disparity in yield observed in the various extracts (Table 1) can be attributed to many factors. According to earlier reports, extraction yield can be affected by extraction solvent [25,26], drying procedure, duration of extraction [27] and exposure to light and heat [28].

Table 1. Approximate Yield (grams and grams) of various extracts from 300 grams of *Sphenocentrum jollyanum* leaf

Extract	Yield (g)	Yield (%)
Aqueous	27.50	9.16
Ethanol	24.00	8.00
Saponin rich extract	7.20	2.40
Tannin rich extract	12.00	4.00
Flavonoids rich extract	6.00	2.00
Alkaloid rich extract	9.00	3.00

3.2 *In-vitro* Anti-inflammatory Assay

3.2.1 Membrane stabilization activity of *S. jollyanum* leaf extracts

Natural products, such as plants extracts in form of pure compounds or standardized extracts, provide unlimited opportunities for new drug discoveries because of the un-paralleled availability of chemical diversity [29]. Evaluation of the ability of plant extracts or test compounds to prevent or minimize red blood cell haemolysis has always been taken as a measure of their anti-inflammatory potential. As shown in Table 2, the aqueous extract of the leaf exhibited a stronger dose dependent erythrocyte membrane stabilization activity than the ethanol extract with IC_{50} of 637± 1.20 µg/ml. The table also showed that the saponin rich extracts exhibited the highest stabilization activity between the secondary metabolites rich fractions. While no activity was demonstrated by the alkaloids and flavonoids rich extracts. Although at present, the likely mechanism of action remains elusive, probably the saponins and tannins in the extract might be responsible for the considerable membrane stabilization activity. Saponin and tannin have been reported to elicit diverse biological and pharmacological activities to prevent inflammation and reduce generation of reactive oxygen species [30]. Phenolic compounds, tannins and flavonoids have been reported to have multiple biological effects, including ability to scavenge reactive oxygen species (ROS) thus preventing anti-inflammatory disorders [31]. Earlier studies by Garai et al. [32, 33-38] revealed that saponins have a wide range of biological activities which include anti-inflammatory anti-fungal, cytotoxic and anti-tumor activities. The result of this study concurred with submissions of Prakash et al. [39,40] that reported membrane stabilization activity of different plant extracts.

3.2.2 Lipoxygenase inhibitory activity S. J leaf extracts

Lipoxygenases (LOX) are a group of non-heme iron-containing enzymes that mediate the addition of molecular oxygen to into poly-unsaturated fatty acids (PUFAs) such as arachidonic acid and linoleic acid. This results in the formation of arachidonic acid metabolites, which have been implicated in the development inflammatory-related diseases such as arthritis, allergic asthma, psoriasis, inflammatory bowel disease, immune responses and other physiological processes [41-46]. Hence, lipoxygenase inhibitors may open new gateway for design of biological and pharmacological therapeutic strategies against active metabolites involved in the development of metabolic disorders. Fig. 2 revealed that aqueous extract demonstrated an insignificantly ($p<0.05$) higher

lipoxygenase inhibitory activity with IC_{50} of 637±6.89 µg/ml when compared to the ethanol extract with IC_{50} of 656±4.13. Among the secondary metabolites rich extract, alkaloids exhibited the LOX inhibitory activity, while saponins exhibited the lowest activity with IC_{50} value of 2420±6.72 µg/ml and 10120±3.50 µg/ml respectively. However the standard drug indomethacin demonstrated the highest LOX inhibitory activity IC_{50} of 172±5.95 µg/ml. The observed high LOX activity of the crude extracts when compared to the individual secondary metabolites might be due to the synergistic activity of the different constituent phytochemicals. According to submissions of Basole and Rodolfo [47,48], beneficial health effects observed from plants are as a result of the synergistic activities of phytochemicals and other nutrients. Furthermore, Xi and Liu [49], reported that phytochemicals isolated from plant sources taken alone as a supplement do not have consistent health benefits when compared to crude extracts. However, the result of this study is in agreement with previous submissions of [50-54] on anti-lipoxygenase activities of various medicinal plant extracts.

3.2.3 Proteinase inhibitory activity *S. jollyanum* leaf extracts

With the aim of discovering new plants as a source of management of inflammatory related disorders, *Sphenocentrum jollyanum* leave extracts were evaluated for anti-proteinase activity at varying concentrations. Proteinases are enzymes that mediate the hydrolysis of the peptide bonds, thereby changing the primary configuration of proteins [55]. Overall, they assist in processing of the cellular information and as molecular mediators in immune and inflammatory responses [56,57]. Results of the current study (Table 3), indicates that the various extracts *of Sphenocentrum jollyanum* leaf demonstrated considerable protein inhibitory activities. The maximum inhibition was observed from the ethanol extract with IC_{50} of (840 µg/ml), in decreasing order was aqueous (910 µg/ml), tannins (1810 µg/ml), saponin (2900 µg/ml) and flavonoids (2466 µg/ml), while the standard drug demonstrated the highest activity with 246 µg/ml inhibiting 50% of the enzyme. The observed proteinase inhibition may be attributed to the activities of phytochemicals present in the extracts. According to Li et al. [58], Kanakis et al. [59] and Jia et al. [60] the inhibition of proteases by phytochemicals may be related to the structure-function relationship. Li et al. [58], also suggested that the trypsin inhibitory potential of polyphenols increases with increased number hydroxyl group.

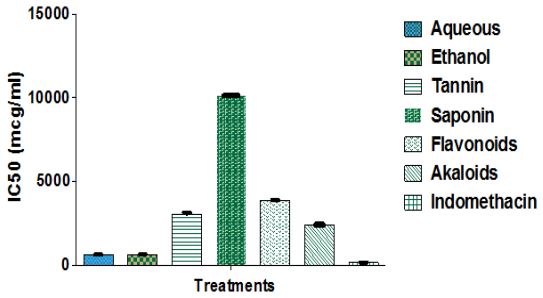

Fig. 2. IC_{50} Values of Anti-lipoxygenase Activity of *Sphenocentrum jollyanum* leaf extracts and Standard Drug (Indomethacin). Results are expressed as mean±SEM of triplicate determinations

Table 2. IC_{50} (µg/ml) values and percentage (%) membrane stabilization activity of leaf extracts of *S. jollyanum*

Extract/Drug	% Stabilization					
	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml	300 µg/ml	IC_{50} (µg/ml)
Ethanol	21.48±0.07	23.26±0.07	26.17±0.06	28.92±0.07	31.68±0.07	656±4.13*
Aqueous	42.97±0.03	43.00±0.04	43.25±0.04	44.60±0.03	45.70±0.07	637± 1.20*
Tannin	22.70±0.04	24.00±0.06	24.24±0.02	25.12±0.01	28.00±0.04	1299±1.98*
Saponin	8.60±0.06	16.90±0.08	23.00±0.09	26.63±0.07	29.10±0.01	489±8.24*
Alkaloids	N/A	N/A	N/A	N/A	N/A	N/A
Flavonoids	N/A	N/A	N/A	N/A	N/A	N/A
Diclofenac	54.60±0.03	55.00±0.06	62.50±0.05	65.67±0.09	68.00±0.04	52±6.12

*Values represent mean±SEM (n=3) *p<0.05 considered as IC_{50} values significant when compared to the Standard Drug (Diclofenac). N/A- No activity was observed*

Table 3. IC_{50} (µg/ml) values and Percentage (%) Protein inhibitory activity of leaf extracts of *S. jollyanum*

Extract/Drug	% Inhibition					
	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml	300 µg/ml	IC_{50} (µg/ml)
Ethanol	9.20±0.056	12.20±0.050	13.00±0.46	16.84±0.78	20.80±0.87	840±4.49*
Aqueous	8.88±0.011	13.20±0.031	16.93±0.76	17.00±0.42	19.23±0.23	910±4.07*
Tannin	15.96±0.13	17.05±0.85	18.14±0.76	18.14±0.95	19.26±0.65	1810±7.05*
Saponin	13.10±0.47	14.40±0.13	15.50±0.84	15.58±0.65	15.79±0.48	2900±1.11*
Alkaloids	N/A	N/A	N/A	N/A	N/A	N/A
Flavonoids	5.04±0.35	5.72±0.18	7.23±0.48	8.18±0.24	8.59±0.25	2466±1.53*
Indomethacin	40.00±0.05	43.20±0.06	47.00±0.05	51.00±0.06	54.00±0.03	246±5.66

*Values represent mean±SEM (n=3) *p<0.05 considered as IC50 values significant when compared to the Standard Drug (Indomethacin). N/A- No activity was observed*

4. CONCLUSION

These findings provide scientific evidence to support traditional medicinal uses and indicate a promising potential for development of anti-inflammatory agent from *Sphenocentrum jollyanum* plant.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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