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The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
TPI 2024; 13(3): 189-192

www.thepharmajournal.com
Received: 20-01-2024
Accepted: 24-02-2024

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Screening of antiaflatoxin, anti-inflammatory performance and biosafety of *Spondias mombi* Linn. (Hog Plum) leaf extract

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Abstract
Spondias mombi belonging to the *Anacardiaceae* family has demonstrated efficacy in treating inflammatory conditions, wounds, and infections. Found commonly in Africa, Americas and Asia, its leaf extract was tested for antiaflatoxin, anti-inflammatory and biosafety properties. A thin layer chromatographic (TLC) technique was employed to assess aflatoxin levels in *Aspergillus flavus*, *Aspergillus versicolor* and *Fusarium oxysporum*. The inhibition of aflatoxin increased with higher concentrations of *Spondias mombi* leaf extract, with aflatoxin B1 being completely inhibited at 300mg/ml concentration. Additionally, the plant extract showed significant protection against hemolysis, particularly at higher concentrations and no hemolysis was observed in the absence of aflatoxin. This study highlights the potent antiaflatoxin, anti-inflammatory and biosafety of properties of *Spondias mombi* leaf extract, suggesting potential for further exploration of plant-based remedies for various medical conditions.

Keywords: *Spondias mombi*, aflatoxin, antiaflatoxin, anti-inflammatory, biosafety, infections

Introduction
Despite remarkable advancements in modern science, technology, and the allopathic healthcare system, providing health care to all remains a challenge. Factors such as pollution, unhealthy lifestyle, and environmental toxins contribute to an increased risk of diseases. Additionally, the overuse and misuse of allopathic drugs have raised concerns about their side effects. Oxidative stress and inflammation are recognized as major contributions to various diseases, including cancer, atherosclerosis, and brain dysfunction, garnering significant research attention in recent decades. Oxidative stress arises from an imbalance between the production of reactive oxygen species (ROS) and antioxidant defense mechanisms within cells. Reactive oxygen species formation also plays a role in inflammatory responses. Inflammation, a localized protective immune response of vascular tissues against harmful stimuli, is crucial in skin pathologies and can delay wound healings in conditions like leprosy and sexually transmitted infections (Tan and Lim, 2015) [1]. Antioxidants, compounds that inhibit or delay substrate oxidation, even in small amounts compared to the oxidized substrate, play a vital role in combating oxidative stress. Natural antioxidants found in medicinal herbs have the potential to protect the body from free radicals and slow the progression of many chronic diseases (Patel *et al.*, 2011) [2].
Research has linked traditional medicinal plant use with phytochemical and pharmacological investigations, aiming to discover new drugs and herbal remedies (Medeiros *et al.*, 2013) [3]. *Spondias mombi* Linn, belonging to the *Anocardiceae* family, is renowned for its versatile folklore application. Known as “hog plum” in English and “linkara” by the Ibos of Southeastern Nigeria (Aromolaran and Badejo, 2014; Sampaio *et al.*, 2018) [4, 5], this medium to large-sized tree is widespread in tropical regions of America, Asia, and Africa. Its traditional use spans treating inflammatory conditions, wounds, and infections, attributed to its rich bioactive compounds such as tannins, saponins, flavonoids, phenolics and anthraquinone glycosides (Sabiu *et al.*, 2015; Ishola *et al.*, 2018) [6, 7]. Leaf decoctions are commonly employed for ailments like laryngitis, sore throat, tooth decay and traditional birthing practices. Additionally, a tree brewed from its flowers and leaves is used to alleviate stomach aches, biliousness, urethritis, cystitis, and soothe eye and throat inflammation. The barks astringent decoction has been historically used as an emetic, diarrhea and dysenteryremedy, and for treating hemorrhoids and gonorrhea (Ayoka *et al.*, 2008) [8].

Furthermore, the leaves contain antiviral ellagitannins and caffeoyl esters, antibacterial and mulluscicidal phenolic acids, and exhibit anti-helminthic and abortifacient properties (Nworu *et al.*, 2007) [9].

Materials and Methods

Plant material

Collection of plant material

Fresh leaves of *Spondias mombi* were collected from botanical garden Federal Polytechnic Bida, Niger State Nigeria and authenticated at International Institute of Tropical Agriculture (IITA) Ibadan following the criteria stipulated by International Committee for Botanical Nomenclature (ICBN). The samples were packaged in polythene bags, appropriately labelled, and stored for subsequent use.

Extraction of plant material

The plant leaves were air-dried in the shade at room temperature, and the extraction of plant material was carried out following the procedure of adopted by Banso *et al.* (2023a) [10] with a slight modification. Ten grams of plant powder was extracted with 200ml of methanol in conical flask covered with aluminium foil and plugged with sterile cotton wool, placed in a rotary shaker for 24 hrs for complete homogenization at 35°C. After homogenization, the extract was filtered using Whatman no 1 filter paper and subsequently stored in the refrigerator until needed.

Test organism

Fungal strains including *Aspergillus terreus*, *Aspergillus nidulans and Fusarium oxysporum* were employed in this study and maintained on Potato Dextrose Agar slants. The organisms were obtained from National Institute of Pharmaceutical Research, Abuja.

Antiaflatoxin assay

The requisite amount of extract of *Spondiamombi* Linn was dissolved to achieve concentrations from 50 to 300 mg/ml. The medium was inoculated with 10^6 spores/ml of seven days old culture of the test organism. The control contained no extract.

Thin layer chromatography (TLC) technique was used for detection of aflatoxins. Both the filtrates and the standards were spotted on the prepared TLC plates at 1.5cm from the lower edge and left for 1 minute to dry. The solvents that were used were chloroform, methanol (97:3 and 50:50 v/v) to detect the mycotoxins produced by the test organisms. The TLC plates were removed when the solvent reached a mark 1cm from the upper edge of the plates. The plates were left to dry up and the solvent front marked across the plate. The colour spot was marked with pencil and visualized under UV lamp. The retention factor (RF) of the compounds/toxins in the filtrates were calculated as; Rf = distance travelled by compound /distance travelled by solvent (Banso *et al.*, 2023b; Banso and Banso, 2023) [11, 12]. The intensities of fluorescence of the separated mycotoxin spots were then measured with a fluorodensitometer (model MPF-2A. Hitachi Ltd., Tokyo).

Anti-Inflammatory assay

Human red blood cell (HRBC) suspension preparation and HRBC membrane stabilization

Alsever solution was prepared by dissolving 2g dextrose, 0.8gsodium citrate, 0.05g citric acid and 0,42g sodium chloride in 100ml distilled water. Hyposaline and isosaline solutions were prepared by dissolving 0.36 and 0.85g NaCl in 100ml distilled water, respectively. All three solutions prepared were autoclaved before use. Human blood was collected from volunteers. 2ml of the blood sample was added to 2ml of Alsever solution and the contents were then centrifuged at 3000rpm for 5minutes, and the supernatant was discarded. To the pellet 2ml of isosaline was added slowly along the side of the centrifuge tube and again centrifuged at 3000rmp for 5minutes and this process was repeated 2-3 times till we got a clear supernatant. The clear supernatant which was obtained after repeated centrifugation was discarded and iso0saline was to make 10% HRBC suspension and then stored at a low temperature inside the refrigerator for further use.

Different concentrations (50,100, 250,500, 1000 and 2000µg/ml in isosaline) of the ethanolic extract of leaf were prepared, 0.5ml of each concentration was taken and 1ml of phosphate buffer pH 7.4, 0.5ml of HRBC suspension and 2ml of hyposaline solution were added and incubated at 37°C for 30 minutes. Diclofenac was used as the standard drug and processed in the same manner as the plant extract. A control was also prepared by adding 2.5ml distilled water, 0.5ml HRBC suspension and 1ml phosphate buffer pH 7.4. The blank was made by adding 1ml phosphate buffer pH 7.4, 2ml hyposaline solution, and 1ml distilled water. After incubation, centrifugation was done at 3500 rpm for 5minutes. The supernatant was taken, and the absorbance was recorded using UV-Vis spectrophotometer at 360nm. After taking the absorbance percentage of hemolysis the percentage of protection was determined using the following formulae.

$$\text{Percentage of hemolysis} = \frac{\text{Absorbance of test sample}}{\text{Absorbance of control}} \text{ x } 100$$

$$\text{Percentage protection } = 100 - \frac{\text{Absorbance of test sample}}{\text{Absorbance of control}} \text{X}100$$

Biosafety assay

Aflatoxin was isolated as described earlier. Intravenous random blood samples were collected in EDTA tubes from healthy humans. The blood samples were washed several times with normal saline and centrifugation at 1000xg for 10minutes. The supernatant was discarded each time. The final RBC suspension was prepared in normal saline to have $2x10^4$cells/ml. To examine the hemolysis due to AFB1 on RBC and its amelioration by the ethanolic extract, four sets of tubes were prepared.

Control tubes: 2.0ml of RBC suspension.
Control tubes: 0.25, 0.5, 1, 1.5 and 2mg/ml.
Plant extract added to 2.0ml of RBC suspension.
Treated tubes: 2µg/ml of AFB_1 added to
2.0ml of RBC suspension (100% hemolysis)
Treated tubes: 0.25, 0.5, 1, 1.5 and 2mg/ml plant extract added to RBC suspension treated with 2µgml of AFB1.

AFB1 solution and plant extract were prepared in normal saline. The total volume of each tube was made up to 4.0ml by normal saline. All tubes were incubated at 37°C for 4 hours then centrifuged at 1000xg for 10 minutes.

The colour density of the supernatant absorbance was measured spetrophotometrically at 540nm and the hemolysis percentage calculated by the formula as:

Hemolysis % = Absorbance of individual tube/absorbance with 100% hemolysis x100

Data analysis

The results were subjected to analysis of variance and means comparisons were performed by Turkey's multiple range tests using SPSS version 20.0 (package for social sciences, Inc. Chicago IL, United States). Differences between means were considered significant at $p<0.05$.

Results

This study showed that A. flavus produced aflatoxin B_1 while *A. versicolor* produced Zearalenone. Ochratoxin was produced by *F. oxysporum.* The retention factors recorded against *Aspergillus flavus, Aspergillus versicolor* and *Fusariun oxysporum* were 0.46, 0.42 and 0.56 respectively (Table 1).

Table 1: Mycotoxin identified in test fungi.

Fungi	Retention factor (R_f)	Aflatoxin
Aspergillus flavus	0.46	Aflatoxin B_1
Aspergillus versicolor	0.42	Zearalenone
Fusariun oxysporum	0.56	Ochratoxin

The results showed that inhibition of aflatoxin increased with increase in the concentration of leaf extract of *Spondias mombi* (Table 2). Aflatoxin B_1 was inhibited by 100% when 300mg/ml of the leaf extract was assayed against the aflatoxin. The percentage inhibition decreased to 55% as the concentration of the extract decreased to 150mg/ml. The percentage inhibition recorded against Zearalenone was 40% when 100mg/ml of the extract was assayed against the aflatoxin. Ochratoxin was inhibited by 80% when 250mg/ml of the extract was tested against the aflatoxin (Table 2).

Table 2: Percentage inhibition of aflatoxin with different concentrations of leaf extract of *Spondias mombi*

	Extract (mg/ml)	Aflatoxin B_1	Zearalenone	Ochratoxin
Control	0	0	0	0
	300	100	99	100
	250	80	78	80
	200	70	64	50
	150	55	45	40
	100	30	40	35
	50	10	30	20

The anti-inflammatory activity of the leaf extract was compared with diclofenac. Analysis of inhibition of hemolysis and protection from hemolysis of the extract and standard drug diclofenac is described in Table 3. The anti-inflammatory activity of the extract was concentration dependent. The highest inhibition of hemolysis by the extract was reported at 300mg/ml and at this concentration only 3.5±1.5% of hemolysis was recorded by the extract. The percentage hemolysis was reduced from 90.40±2.0% to 29.90±3.0% when the concentration of diclofenac was increased from 50mg/ml to 300mg/ml. Hemolysis protection was also observed at higher concentrations (300mg/ml) (Table 4).

Table 3: Percentage of hemolysis at different concentrations of diclofenac and leaf extract of *Spondias mombi*

Concentration (mg/ml)	Diclofenac	Leaf extract
50	90.40 ±2.0	80.60 ±2.5
100	82.45± 2.5	40.50± 2.0
150	56.70± 3.0	35.62± 3.0
200	45.35 ±2.5	24.50 ±2.5
250	36.64 ±3.0	43.20 ±2.0
300	29.90± 3.0	3.50 ±1.5

Table 4: Percentage protection at different concentrations of diclofenac and leaf extract of *Spondias mombi*

Concentration (mg/ml)	Diclofenac	Leaf extract
50	9.60±2.0	19.40±2.5
100	17.55±2.5	59.50±2.0
150	43.30±3.0	64.38±3.0
200	54.65±2.5	75.50±2.5
250	63.36±3.0	86.80±2.0
300	70.10±3.0	96.50±1.5

The results showed that no hemolysis was recorded in the absence of aflatoxin (Table 5). 44.8% was recorded against 2µg/ml of aflatoxin in the absence of leaf extract of *Spondias mombi*. Retardation of hemolysis was recorded with increase in the concentration of the leaf extract. Hemolysis retarded from 44.8 to 6.0% as the concentration of the leaf extract increased from 5mg/ml to 25mg/ml (Table 5).

Table 5: Aflatoxin induced hemolysis retardation by leaf extract of *Spondias mombi*

Aflatoxin B_1(µg/ml)	Leaf extract(mg/ml)	Hemolysis (%)
0	0	0
0	5	0
0	10	0
0	15	0
0	20	0
0	25	0
2	0	44.8
2	5	12.5
2	10	10.3
2	15	8.4
2	20	8.1
2	25	6.0

Discussion

A. flaavus, A. versicolor and *F. oxysporum* produced aflatoxin B1, Zeralenone and ochratoxin respectively. Xiang *et al.* (2020) [13] suggested that aflatoxigenic fungi produce aflatoxin via complex biochemical system called aflatoxin biosynthesis. Aflatoxin B1 causes membrane permeability change and then cell lysis.

Leaf extract of *Spondias mombi* inhibited aflatoxin B1. This agrees with the report of (Alpsoy, 2010; El-soud *et al.*, 2015) [14, 15]. The inhibition of aflatoxin B1 production by leaf extract of *Spondiamombi* is related to the downregulation of aflatoxin biosynthesis genes (Xiang *et al.*, 2020) [13]. Antiaflatoxigenic actions may be related to the ternary steps of aflatoxins biosynthesis involving lextract of lipid peroxidation and oxygenation (Alpsoy, 2010; El-soud *et al.*, 2015) [14, 15]. The results indicate that the leaf extract of *Spondias mombi*s a promising alternative for inhibiting aflatoxin production. Velazhahan*et al.* (2010) [16] reported that the aqueous seed extract of *G. cowa* showed antiaflatoxin

B_1 was retarded by activity (61%). Ethanol extract of *T. daenensis* inhibited aflatoxin B_1production (51-87%). The current study also revealed that anti-inflammatory activity (determined by percentage inhibition of hemolysis) was concentration dependent and as the concentration of the ethanolic leaf extract increased, the anti-inflammatory activity was also increased. The protection potential of the leaf extract was much higher than the standard drug diclofenac. Phytochemicals in plant extracts are responsible for their potential anti-inflammatory properties (Oyedepo*et al.*, 2010; Anosike *et al.*, 2017) [17, 18]. The results showed that aflatoxin induced hemolysis was reduced by leaf extract of *Spondias mombi*. The reason for production inhibition or depression was explained by few reports that the plant extract might change the chemical structure of toxins present in the. In addition, plant extracts might interact with aflatoxins production genes (metabolic pathway) affecting the gene expression level. Since leaf extract of *Spondias mombi* does not produce hemolysis at concentrations ranging from 5mg/ml to 25mg/ml, the extracts could be regarded as biocompatible with human cells hence the biosafety.

Conclusion

The current research highlights the strong antiaflatoxin, anti-inflammatory, and biosafety properties of *Spondias mombi* leaf extract. Through *in vitro* and clinical investigations, it is necessary to validate their efficacy in treating inflammatory conditions. If successful outcomes are obtained, *Spondias mombi* leaf extract could emerge as a natural solution for managing infections. Moreover, the demonstrated potency of the leaf extract suggests its potential application as a natural inhibitor derived from herbs, safeguarding stored crops against aflatoxin contamination.

Declaration

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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