



# Antioxidant, anti-inflammatory, analgesic and in vitro-in vivo cytotoxicity effects of *Spondias Venulosa* (Engl.) Engl. leaf extracts on MCF-7/S0.5 and OV7 cancer cell lines

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## ABSTRACT

**Ethnopharmacological relevance:** *Spondias Venulosa* is a plant whose ethnobotanical uses were neither scientifically investigated nor documented despite the overwhelming use of the leaf extract as antioxidant, pain relief, anti-inflammatory, and anticancer agent in traditional medicine in Nigeria.

**Aim of the study:** To evaluate the antioxidant, anti-inflammatory, analgesic and cytotoxic activity of *S. venulosa* leaf extracts on MCF-7/S0.5 and OV7 cancer cell lines and isolate the phyto-constituent responsible for its possible bioactivity.

**Materials and methods:** The antioxidant activity was determined by DPPH and H<sub>2</sub>O<sub>2</sub> radical scavenging activities, anti-inflammatory was evaluated by carrageenan induced paw edema in mice, analgesic activity was carried out by acetic acid induced writhing in mice while cytotoxicity activity of the extract was investigated *in vitro* and *in vivo* by MTT assay and tumor induction model by trypan blue dye exclusion assay respectively. Identification and characterization of the bioactive compound present in *S. Venulosa* were done using the GC-MS, FTIR, 1D and 2D NMR spectroscopy.

**Abbreviations:** OV7, Human epithelia ovarian cancer cell line; MCF-7/S0.5, Tamoxifen sensitive human breast cancer cell line; DMEM, Modified eagle medium; PBS, Phosphate buffer saline; DPPH, 2,2'-diphenyl-1-picrylhydrazyl radical; PC, Pure compound; NNMDA, Nigerian natural medicine development agency; Na<sub>2</sub>CO<sub>3</sub>, Sodium carbonate; GAE, Gallic acid equivalents; AAE, Ascorbic acid equivalents; EF, Ethyl acetate fraction; HF, n-hexane fraction; BF, n-butanol fraction; AA, Aqueous fraction; D/H<sub>2</sub>O, Distilled water; H<sub>2</sub>O<sub>2</sub>, Hydrogen peroxide; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; DMSO, Dimethyl sulphoxide; IC<sub>50</sub>, Inhibitory concentration; CC<sub>50</sub>, Lethality concentration; SI, Selective index; ANOVA, Analysis of variance; SVM, *Spondias venulosa* methanol extract; MST, Mean survival time; %ILS, Percentage increase in lifespan; VCC, Viable cell count; NVCC, Non-viable cell count; WBC, White blood cell; RBC, Red blood cells; PCV, Packed cell volume; AST, Aspartate amino transaminase; ALP, Alanine phosphatase; ALT, Alanine amino transaminase; NSAID, Non-steroidal anti-inflammatory drugs; NMR, Nuclear magnetic resonance; GC-MSD, Gas chromatography-mass spectrometry detector; HSQC, Heteronuclear single quantum coherence; HMBC, Heteronuclear multiple bond correlation; DEPT, Distortionless enhancement by polarization transfer; COSY, Correlated spectroscopy; HPLC, High performance liquid chromatography; TLC, Thin layer chromatography; FTIR, Fourier transformed infrared spectroscopy.

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**Results:** The  $^1\text{H-NMR}$  showed six protons signals while  $^{13}\text{C-NMR}$  showed twenty-six carbons consisting of methyl, methylene and quaternary groups characteristic of lactones. Ethyl acetate (EF) fraction showed highest biological activities in terms of antioxidant, anti-inflammatory, analgesic and cytotoxic activities on MCF-7/S0.5 and OV7 cancer cells because of higher amount of phenolic and flavonoid contents. The EF showed an  $\text{IC}_{50}$  values of  $12.18 \pm 0.20$  ( $\text{SI} = 1.97$ ) and  $4.12 \pm 0.001$  ( $\text{SI} = 77.67$ ) on MCF-7/S0.5 and OV7 cell lines respectively. Most of the biological activities of the extracts were dose-dependent. The isolated compound showed the highest selective index on cancer cells especially against OV7 with  $\text{IC}_{50}$  value of  $2.01 \pm 0.01$ ;  $\text{SI} > 100$  when compared with the control drug doxorubicin with  $\text{IC}_{50}$  value of  $2.20.80 \pm 0.01$ ;  $\text{SI} = 25.57$ . Extracts of *S. venulosa* showed strong MST and %ILS on tumor-treated mice in dose-dependent fashion. The extract showed significant reductions in TCV and VCC at higher concentration, and low levels of liver biomarker (AST, ALP and ALT) which is an indication of the tolerance of *S. Venulosa* in the animals. In addition, a lactone flavonoid compound 6-hydroxy-4,4,7a-tetrahydrobenzofuran-2(4H)-one (i.e. 6-OH-THBF) was identified as one of the bioactive compounds largely responsible for some of the observed pharmacological activities.

**Conclusion:** The 6-OH-THBF isolated from EF fraction was the most potent on the two cancer cell lines, and it showed the highest selective against cancerous and normal Vero E6 cell line. Our study showed that *S. Venulosa* extract possessed the acclaimed pharmacological action in traditional medicine.

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## Introduction

From ancient times till date, plant parts or whole plant has been used in the treatment of various diseases such as ulcer, hypertension, haemorrhoids, infertility, convulsion, fevers (like malaria, yellow fever, and typhoid), weak erection, coughs, cataract, fibroid, microbial infections, among others. Plants were able to achieve several pharmacological potentials on these diseases due to the presence of biologically active constituents such as alkaloids, vitamins, terpenes, flavonoids, proteins, sterols, other phenolic, and polyphenols, among others [1–16]. For instance, flavonoids have been reported to possess strong antioxidant, anticancer, antiulcer, anti-inflammatory and analgesic activities, while alkaloids possessed anticancer and anti-malarial activities ([28]; Sofowora, 2006; [22]).

In most cases, some phyto-constituents have been isolated in their purest form, and have been used as therapeutic alternatives for diseases like cancers, tumors, diarrhoea, and viral infections (Sofowora, 2006; [15–18]). The ethno-pharmacology of the various plants are unique to the customs and traditions of the people within the locality. Use of these plants are done in a careful manner in order to prevent their poison effects. Currently, it has been reported that about 50% of conventional drugs in use are derived from plant parts such as leaves, stems, barks, roots, flowers, seeds, among others [16]. Despite the successes recorded with the use of plants, there are still numerous medicinal plants whose ethno-pharmacological activity are either scientifically un-documented or unknown, one of such plant is *Spondias venulosa* commonly called coarse mombin (plum), and a member the Family Anacardiaceae [16]. It is a rare mombin species, that is scarcely seen in the wild and in cultivation. Its tree is semi-deciduous which grows up to 50ft [3]. The plant is well distributed in Taraba and Adamawa States, north-east Nigeria, where it is locally called “*Barmagaadaa*” in Hausa language [1].

In this present study, we evaluated the major ethno-botanical uses of *Spondias venulosa* (coarse mombin) leaf extract which includes: antioxidant, anti-inflammatory, analgesic, and cytotoxicity. The leaves of *S. venulosa* have been used in the Nigerian traditional medicine for treating pains, diarrhoea, malaria fever, inflammation, diabetes and cancers, just like most plums [3,27]. Despite its popular uses in the Nigerian traditional medicine, there were little or no scientific documentation on its biological activities. However, the leaves have been reported to contain these flavonoid compounds: rutin (quercetin 3-O- $\beta$ -D-rutinoside), rhamnetin 3-O- $\beta$ -D-rutinoside and quercetin 3-O-[ $\alpha$ -rhamnopyranosyl-(1 $\rightarrow$ 2)]- $\alpha$ -rhamnopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -glucopyranoside [20–24]. Aside this, no other works have been reported scientifically on the biological activities of *S. venulosa* may be due to its limited geographical distribution [3]. Most of the works on the genus were performed on species such as *S. mobin*, *S. pinnata*, *S. purpurea*, *S. dulcis* and *S. tuberosa* [32,37].

The genus *Spondias* L. belongs to Family Anacardiaceae which comprises 70 genera and 600 species. It is endogenous mostly in the tropics and subtropics worldwide, as well as extends into the temperate zone. Members of this family are used in traditional medicine for the treatment of many diseases [2,13]. They are extensively used in traditional medicine for the treatment of many diseases in Nigeria, and in some African countries [16]. Previous studies on the genus in ethno-pharmacology, has shown that numerous *Spondias* species possess antioxidant, hepa-toprotective, antidiarrheal, cytotoxic, ulcer protective, hepa-toprotective, photo-protective, anti-inflammatory, antiviral, antipyretic, analgesic, thrombolytic, hypoglycaemic, anti-fertility, antihypertensive, antimicrobial, and anthelmintic activities ([37]; Engels et al., 2012). This is due to

the presence of arsenals of phyto-constituents that abound in the genus such as tannins, flavonoids, sterols, triterpenes, saponins, essential oils, amino acids, and polysaccharides ([13]; Iwu, 2004; [5]).

The purpose of this present study was to evaluate and determine the pharmacological activities of *S. Venulosa*, which is popularly used in Nigerian folkloric medicine in treating many diseases.

This current study was carried out to evaluate the biological activities such as antioxidant, anti-inflammatory, analgesic and cytotoxicity of the *S. Venulosa* leaf extract preparations originating from Nigeria.

## Materials and methods

### Materials and reagents

Methanol, n-hexane, ethyl acetate, acetic acid, n-butanol and DMSO was purchased from JoeChem, Nig. Ltd (for Sigma Co. St Louis, USA), DMEM was purchased from Gibco (NY, USA), gallic acid; 97.7% v/v (Sigma-Aldrich), trypan blue solution; 0.4%; 0.4µm filtered (Sigma Co. St Louis, USA), disposable hemacytometer (Nexcelom Bioscience, USA), tamoxifen sensitive human breast cancer; MCF-7/S0.5, human epithelial ovarian cancer cell lines; OV7 and normal cell; Vero E6 from an African green monkey were purchased from ATCC (USA), MTT dye (Sigma, US), Folin-Ciocalteu reagent, DPPH and carrageenan were purchased from Sigma Co. St Louis, USA, silica gel 60 × 120 mesh (Merck, Germany), UV-vis spectrophotometer (Jenway™ model 7315, Fisher Scientific, UK), GC-MSD (Agilent technologies, alpha II FTIR and NMR apparatus (Ascend 600 MHz, Bruker, USA), rotary evaporator (BUCHI, Germany). All the other chemicals and reagents were of analytical grades.

### Collection of plant material and extraction

The leaves of *Spondias venulosa* were collected fresh in the early hours of the morning (5am and 6am) on April, 2020, from Bali, Taraba State (Nigeria). A voucher number of MPLB 002012 was deposited for the plant at the herbarium unit of Plant Research Laboratory, Bali. The leaves were air-dried for two weeks and pulverized into fine powders, weighed, and then extracted with methanol (80% v/v) by cold maceration technique. Sub-fractions of the MeOH extract was obtained by liquid-liquid phase (LLP) extraction using n-hexane, ethyl acetate, and n-butanol and aqueous in increasing order of their polarities. Ethyl acetate fraction (EF) produced the highest yield of 48.12%, while the aqueous fraction (AF) yielded 42.01% followed by n-butanol (BF) and n-hexane (HF) with 33.54% and 18.34% respectively.

### In vitro bio-assays

#### Evaluation of antioxidant activity

**DPPH radical scavenging activity.** *Spondias venulosa* MeOH extracts (SVME) was partitioned with n-hexane (HF), ethyl acetate (EF), n-butanol (BF), and aqueous (AF). Free radical scavenging activity of each fraction and isolated compound(PC) from EF was evaluated by DPPH assay. In this method, 0.1 mM DPPH solution was prepared in MeOH. Briefly, to various concentrations of each fraction (0, 5,10,20, 40, 80, and 100 µg/mL), 1 mL of DPPH solution was separately added. The absorbance was measured at 517 nm with reference to that of ascorbic acid [8,21–25]. The radical scavenging activity (RSA) of each fraction as well as that of the isolated compound (PC) was calculated from the formula below:

$$\text{RSA (\%)} = \left( \text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test sample}} \right) / \text{Absorbance}_{\text{control}} \times 100 \quad (\text{i})$$

**Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) scavenging activity.** H<sub>2</sub>O<sub>2</sub> scavenging activity was determined using the procedure previously described by Ruch *et al.* (1989). Briefly, various concentrations of each fraction and isolated compound (PC) (0, 5,10,20, 40, 80, and 100 µg/mL), was separately mixed with 0.8 mL of 4 mM H<sub>2</sub>O<sub>2</sub> solution in 0.1 M PBS solution with pH 7.4, and incubated at 45 °C for 10 min. The solution was measured at 230 nm absorbance. Ascorbic acid (Vitamin C) was used as positive control. The % of inhibition was then evaluated by comparing the absorbance of the control to that of the test sample from the equation below. Data obtained for anti-oxidation were compared with the reference ascorbic acid.

$$\text{H}_2\text{O}_2\text{RSA (\%)} = \left( \text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test sample}} \right) / \text{Absorbance}_{\text{control}} \times 100 \quad (\text{ii})$$

Where, RSA = radical scavenging activity.

**Determination of total phenolic content.** The total phenolic content in the MeOH extract was evaluated using the Folin-Ciocalteu reagent previously described by Spanos and Wrolstad [40] with slight modification. Briefly, 0.1 mL of suspension containing 1 mg MeOH extract dissolved in 40 mL distilled water and made up to 46 mL was transferred into a 100 mL flask. Then, 1 mL of Folin-Ciocalteu reagent was added to the mixture followed by addition of 3 mL Na<sub>2</sub>CO<sub>3</sub>, and incubated at 45 °C for 10 min. The mixture was measured with UV-vis spectrophotometer at 765 nm at room temperature. Total phenolic content was determined from the linear calibration curve using gallic acid as a reference where, R<sup>2</sup> = 0.9907.

**Determination of total flavonoid content.** In determining the flavonoid content, aluminium chloride ( $\text{AlCl}_3$ ) colorimetric assay was used. Briefly, 0.5 mL SVME suspension was added to 1.5 mL MeOH, 0.1 mL of 10 %  $\text{AlCl}_3$ , 0.1 mL of 1 M potassium acetate ( $\text{C}_2\text{H}_3\text{KO}_2$ ) and 2.5 mL distilled water at 45°C for 30 min. The absorbance of the mixture was recorded at 415 nm [11]. Gallic acid solution prepared calibration curve was at 0–100  $\mu\text{g}/\text{mL}$  concentrations. Flavonoid content was then calculated and expressed in  $\mu\text{g}/\text{mL}$  from the formula shown below:

$$\text{Absorbance} = 0.001 \times \text{gallic acid concentration } (\mu\text{g}) + 0.00179 \quad (\text{iii})$$

#### Cytotoxicity assay of extract

MTT bioassay was used to determine the effects of the various fractions on cancer cells [25]. Briefly, MCF-7/S0.5 breast cancer and OV7 ovarian cancer cell lines of human as well as a normal cell line Vero E6 from an African green monkey, were each cultured in a 96 well cell culture plates (Costar, USA) at  $1 \times 10^5$  cells/per well containing 100  $\mu\text{L}$  of RPMI1640, and incubated at 37°C for 24 h in 5 %  $\text{CO}_2$ . A fresh medium containing various concentrations of the various fractions from the SVME and isolated compound (PC) (0.1, 2.5, 5, 10, 20, 40, 80, 100  $\mu\text{g}/\text{mL}$ ), was replaced with old media and further incubated at normal temperature for 48 h. Then, 20  $\mu\text{L}$  MTT dye solution containing 5  $\mu\text{g}/\text{mL}$  PBS were each added to each well, and further incubated for 2 h. The medium was then removed, and 200  $\mu\text{L}$  DMSO solution was added to each well in order to dissolve the MTT formazan crystals. The culturing plate was then shaken at 200 rpm for 5 min, and absorbance was measured at 570 nm. 0.5  $\mu\text{g}/\text{mL}$  doxorubicin (Adriamycin, USP) a first-line anticancer drug was the standard drug used. This procedure was repeated in triplicate for each fraction. The percentage cytotoxicity ( $\text{IC}_{50}$ ) and selective index (SI) were calculated from the formulae below:

$$\text{IC}_{50} = (\text{Absorbance}_{\text{cells without treatment}} - \text{Absorbance}_{\text{cells with treatment}} / \text{Absorbance}_{\text{cells without treatment}}) 100 \quad (\text{iv})$$

$$\text{SI} = \text{CC}_{50} \text{ value for Vero E6} / \text{IC}_{50} \text{ for cancer cells} \quad (\text{v})$$

#### Characterization of bioactive compound

##### Isolation and purification of the most active fraction

The most active fraction obtained from the bio-guided experiment (ethyl acetate fraction; EF) was purified in a silica gel column chromatography, and eluted in step-wise with n-hexane: ethyl acetate (30:70). The EF sub-fractions were also bio-guided by analgesic, anti-inflammatory, antioxidant and anticancer activities. The most active sub-fraction for each of these activities was then checked for purity by obtaining a single spot on TLC plates, and uniform peak area as well as retention time from the HPLC [26–31,43].

**HPLC analysis.** High performance liquid chromatography (HPLC) analysis of sub-fraction was carried out in an Agilent Technologies 1260 infinity II LC system HPLC apparatus consisting of a multi-sampler and a UV detector. The separation was carried out on a SGE protecol PC18GP120 (250 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ ) column at room temperature using methanol: water (70:30) as mobile phase. Elution was isocratic, flow rate was 1 mL/min. All HPLC data were processed using autochro-3000 software [20].

##### FTIR analysis

The ethyl acetate most active sub-fraction was scanned at 4000–600  $\text{cm}^{-1}$  wavelength using alpha II FTIR spectrometer with dimensions 208  $\times$  330  $\times$  260 mm (Bruker optics, USA).

##### NMR spectra analysis

NMR analysis of bioactive compound was recorded on a Bruker NMR 600MHZ with deuterated chloroform ( $\text{CDCl}_3$ ) as standard solvent for  $^1\text{H}$ -NMR, COSY-NMR,  $^{13}\text{C}$ -NMR, DEPT-135, HSQC and HMBC data.

##### GC-MSD analysis

GC-MSD analysis of compound was carried out on an Agilent Technologies 7890A GC coupled to a MS detector with DB23 column model number J&W 1222362, and internal diameter of 60 m  $\times$  250  $\mu\text{m}$   $\times$  0.25  $\mu\text{m}$  (250°C Max).

#### In vivo experiments

##### Experimental animals

Swiss albino mice of both sexes numbering seventy-five weighing 20–30 g purchased from the Department of Pharmacology, University of Jos, Nigeria, were used for this study. The mice were kept in well-ventilated cages at 37  $\pm$  2  $^\circ\text{C}$  with access to food and water *ad libitum*. Ethical approval for the use of these animals was obtained from the Ethical Committee of the University of Jos, Nigeria (Reference number: UJ/FPS/F17-00379). The use of these animals was strictly in compliance with ethical guidelines of the University of Jos and international laws for the use of animals in research [17].

### Anti-inflammatory study of *S. Venulosa* extract

Mice were divided into five groups of five mice per group as follows:

Group 1: Control group (5 % tween 80).

Group 2: Aspirin (300 mg/kg).

Group 3: 300 mg/kg b.w. SVME.

Group 4: 600 mg/kg b.w. SVME.

Group 5: 1,200 mg/kg b.w. SVME.

Anti-inflammatory activity of the MeOH extract fractions and the isolated compound (PC) was determined by carrageenan-induced paw edema in mice [44]. In this procedure, the mice were fasted overnight with free access to water. The right hind paw basal volume of each mouse was noted before the administration of the various fractions orally by using a plethysmometer (Model 7140, Italy) [31]. This was followed by the induction of edema by sub-plantar injection of 0.05 mL 1 % freshly prepared carrageenan solution which was previously dissolved in 5 mL normal saline to the left hind paws of each mouse of all treated groups. Variations in paw volumes of each mouse was then recorded in 1, 2, 3, and 4 h of administration of carrageenan (intradermal). Increase in paw volume following the administration of carrageenan at these hours was regarded as the sign of inflammation. Acetylsalicylic acid 200 mg/kg (aspirin, BP) was used as a positive control. The mean of data obtained from swelling feet in SVME treated groups and of the aspirin was compared with negative control [1,4,6]. % inhibition of edema was calculated from the following formula below:

$$\% \text{ inhibition of edema} = [(C_0 - C_t)/C_0] \times 100 \quad (\text{vi})$$

where,  $C_0$  is the mean value of inflammation (in hind paw edema) of the control group at a given hour, and  $C_t$  is the mean value of inflammation of the plant extract.

### Analgesic study of *S. Venulosa* extract

Acetic acid-induced writhing model in mice was used with little modification. Briefly, twenty-five Swiss albino mice were randomly grouped into five groups of five mice per group. Group 1 was the negative control group which received 10 mL/kg distilled water, group 2 was the positive control which received 200 mg/kg ibuprofen BP (Motrin; Bristol, UK), while group 3, 4 and 5 were the treatment groups which received 250, 500, and 1000 mg/kg b.w. SVME fractions and isolated compound respectively following oral administration (o.p.). After one hour of treatment, 0.5 mL of 1 % acetic acid (v/v) in 0.9% saline solution was administered to the animals intra-peritoneal (i.p.) in order to induce abdominal writhing in the mice. Writhing was observed and counted in each mouse 5 min later, for 30 min [18,21,30]. The inhibition of abdominal writhing in mice by the extract was determined using from the following formula:

% inhibition

$$= (\text{Average number of writhing}_{\text{control}} - \text{Average number of writhing}_{\text{treated}} / \text{Average number of writhing}_{\text{control}}) 100 \quad (\text{vii})$$

### In vivo cytotoxicity study of extract

In order to determine the effect of *S. venulosa* on tumor cells, sarcoma-180 ascites model was used [7,24], with slight modifications. Tumor was induced in male Swiss albino mice weighing 25–28 g by allowing the animal free access to 10 % formalin in 100 mL distilled water for 2 weeks. Tumor induction was confirmed by ectopic appearance of tumors in mouth, skin, and rectum of mice. Tumor infected mice then grouped into 6 groups of 5 mice per group. Group 1 served as the negative control group which received normal saline 0.2 mL/mice, group 2 was the positive control which received 25 mg/kg 5-fluorouracil (Sigma Co., USA) standard drug, whereas group 3, 4, 5 and 6 were treated groups administered with 100, 200, 400 mg/kg b.w. SVME (non-partitioned), and 2.5 µg/mL isolated pure compound from *S. venulosa* respectively (i.p.) from day one to fourteen. All the mice were sacrificed after 2 weeks under chloroform anaesthesia. Ascites fluid was collected immediately from the tumor site for evaluation of tumor parameters after treatment. Trypan blue dye exclusion assay was then used to determine cell viability of tumor cells by staining dead cells blue [36,39,41]. At the end of the dye exclusion assay, the amount of tumor inhibition by the samples was evaluated as follows:

$$\% \text{ tumor inhibition} = (\text{Average number of cells}_{\text{control mice}} - \text{Average number of cells}_{\text{treated mice}}) \quad (\text{viii})$$

$$\text{Mean survival time (MST) in days} = \text{Day of first death} + \text{Day of last death} / 2 \quad (\text{ix})$$

$$\% \text{ increase in life span (\% ILS)} = (\text{MST}_{\text{treated}} / \text{MST}_{\text{normalcontrol}}) 100 \quad (\text{x})$$

Other biochemical parameters were determined using a mindray BC-3600 auto hematology analyzer (large colour touch screen resolution 800 × 600, 45% humidity, 30 °C temperature, USA).

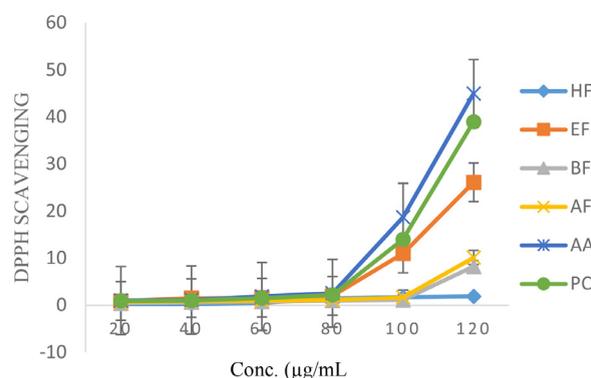
### Statistical analysis

Results from the studies were as Mean ± SE. Significance difference between treated and control groups was analyzed by analysis of variance (one-way ANOVA) followed by Dunnett's multiple comparison test analyzed by SPSS version 22.  $p < 0.05$  were statistically considered significant.

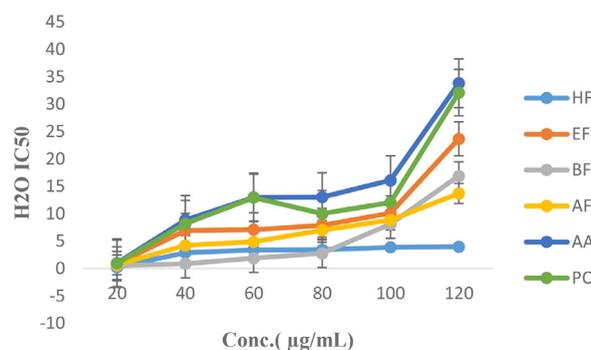
**Table 1**  
Scavenging effects of various fractions of *S. venulosa* extracts on DPPH and H<sub>2</sub>O<sub>2</sub>.

Sample	Inhibition of scavenging radicals' activity (µg/mL)	
	DPPH	H <sub>2</sub> O <sub>2</sub>
n-Hexane	72.55 ± 0.62	68.19 ± 0.42
Ethyl acetate	16.13 ± 0.02	13.11 ± 0.02
n-Butanol	46.12 ± 0.42	33.02 ± 0.40
Aqueous	33.16 ± 0.20	28.08 ± 0.04
Ascorbic acid	0.08 ± 0.01	0.06 ± 0.01
Pure compound	0.16 ± 0.01	0.10 ± 0.02

Concentration; 10–125 µg, abs; 517 and 230 nm respectively for DPPH and H<sub>2</sub>O<sub>2</sub>.



**Fig. 1.** % inhibition of DPPH radicals by ascorbic acid and *S. venulosa* fractions. HF; n-hexane fraction; EF; ethyl acetate fraction; BF; n-butanol; AF; aqueous fraction; AA; ascorbic acid, PC; isolated pure compound from EF sub-fraction,  $p < 0.05$  (one-way ANOVA).



**Fig. 2.** % inhibition of H<sub>2</sub>O<sub>2</sub> radicals by ascorbic acid and *S. venulosa* fractions. HF; n-hexane fraction; EF; ethyl acetate fraction; BF; n-butanol; AF; aqueous fraction; AA; ascorbic acid, PC; isolated pure compound from EF sub-fraction, \*values are Means ± SEM,  $p < 0.05$  (one-way ANOVA).

## Results

### Antioxidant activity, total phenolic and flavonoid contents

The result obtained indicated that, *S. venulosa* leaf extract showed slightly lower antioxidant effect on DPPH scavenging radicals. The ethyl acetate fraction (EF) has an IC<sub>50</sub> value of 16.13 ± 0.02 µg/mL, while the isolated compound had 0.16 ± 0.01 µg/mL. The H<sub>2</sub>O<sub>2</sub> reduction activity of the isolated pure compound was the highest with an IC<sub>50</sub> value of 0.10 ± 0.02 µg/mL followed by that of the EF with IC<sub>50</sub> value of 13.11 ± 0.02. These values are significantly different when compared to that of the ascorbic acid with an IC<sub>50</sub> value of 0.08 ± 0.01 µg/mL and 0.06 ± 0.01 µg/mL for DPPH and H<sub>2</sub>O<sub>2</sub> respectively (Table 1; Fig. 1 and 2). Total phenolic content was 199 µg/mg of gallic and ascorbic acids equivalent while flavonoid content was 74.1 µg/mg (Figure SM 1), which resulted in significant antioxidant effect on the scavenging radicals.

**Table 2**  
Cytotoxic effects of different fractions of *S. Venulosa* extract of MCF-7/S0.5 and OV7 cancer cell lines.

Sample	Cancer cell lines IC <sub>50</sub> and normal cell CC <sub>50</sub> (μg/mL)					
	MCF-7/S0.5	Vero E6	SI <sup>a</sup>	OV7	Vero E6	SI <sup>b</sup>
HF	98.28 ± 0.72	150 ± 0.8	1.53	200.1 ± 2.14	240.2 ± 0.67	1.20
EF	12.18 ± 0.20*	24 ± 0.4	1.97	4.12 ± 0.01*	320 ± 0.47	77.67
BF	32.01 ± 0.42	63.1 ± 0.2	1.97	68.14 ± 0.20	100.2 ± 0.32	1.47
AF	48.2 ± 0.41	88.12 ± 0.4	1.83	55.03 ± 0.13	102.4 ± 0.51	1.86
PC	2.12 ± 0.01*	260.11 ± 0.6	>100	2.01 ± 0.01*	286.8 ± 0.62	>100
Dox.	0.8 ± 0.01*	52.0 ± 0.3	65	2.20 ± 0.01*	56.26 ± 0.21	25.57

HF; n-hexane fraction; EF; ethyl acetate fraction; BF; n-butanol; AF; aqueous fraction; AA; ascorbic acid, PC; isolated pure compound from EF sub-fraction, Dox.; doxorubicin USP, SI<sup>a</sup> and SI<sup>b</sup> are SI (selective index) for breast and ovarian cancer cell lines respectively, \*values are Means ± SEM,  $p < 0.05$  (one-way ANOVA).

#### *In vitro* cytotoxicity activity of *S. Venulosa* fractions and isolated compound

The results in Table 2 revealed that most of the fractions do not show significant inhibition of growth of the cancer cell lines except EF which showed remarkable cytotoxic effect on MCF-7/S0.5 and OV7 with IC<sub>50</sub> value of 12.18 ± 0.20 (SI = 1.97) and 4.12 ± 0.01 (SI = 77.67) respectively. The isolated pure compound showed the strongest cytotoxicity activity for MCF-7/S0.5 and OV7 cancer cell lines with IC<sub>50</sub> values of 2.12 ± 0.01 (SI > 100) and 2.01 ± 0.01 (SI > 100). These values were statistically different from the control drug doxorubicin USP with IC<sub>50</sub> values of 0.80 ± 0.01 (SI = 65) and 2.20 ± 0.01 (SI = 25.57) against MCF-7/S0.5 and OV7 cell lines respectively ( $p < 0.05$ ; one-way ANOVA).

#### Phytochemical analysis of *S. Venulosa* leaf extracts

Analysis of various fractions of *S. Venulosa* leaf methanol extract revealed the presence of saponins, terpenoid, cardiac glycosides, flavonoids, anthraquinones and reducing sugars in the ethyl acetate fraction (EF) with flavonoids occurring in largest amount than any other secondary metabolites. The n-hexane fraction (HF) revealed the presence of trace amount of saponins and flavonoids (Table SM 1).

#### HPLC, GC-MSD, FTIR and NMR analysis of isolated compound

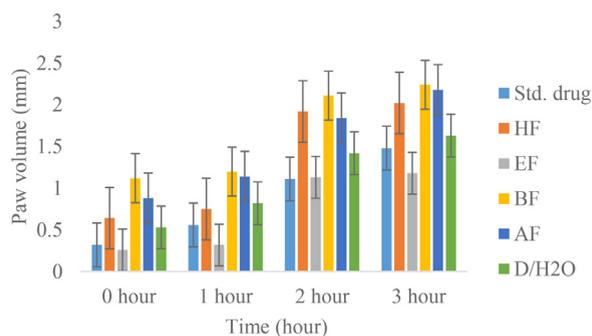
The purity of isolated was confirmed using a sharp peak area and retention time with HPLC standard. The MS data showed a compound with molecular weight of 196 mol/g, and a base peak at 111 with several fragmentation patterns and retention time of 14.08 min, which corresponds with the lactone group. FTIR data also showed aromatic, furan rings and hydroxyl groups in the region 4000 to 500 wavenumber cm<sup>-1</sup> which is typical of lactones. <sup>1</sup>H-NMR (600 MHz, CDCl<sub>3</sub>) showed eight signals with the following data: 1H δ (ppm) 0.78 (d, J = 2.0 Hz), 2H δ 0.98 (m, J = 11.0 Hz), 3H δ 1.08 (s, J = 1.0 Hz), 4H δ 1.13 (s, J = 1 Hz), 5H δ 1.33 (q, J = 16.0 Hz), 6H δ 1.47 (q, J = 10.0 Hz), 7H δ 1.73 (q, J = 10 Hz), 8H δ 1.78 (s, J = 10.0 Hz). The <sup>13</sup>C-NMR showed 30 carbon atoms with δ values from 22.94 to 33.79 ppm which are characteristic positions of methine, methyl and methylene carbons. There are correlations between protons (<sup>1</sup>H-NMR) and carbons atoms (<sup>13</sup>C-NMR) as shown by DEPT-135, COSY, HSQC and HMBC spectra, which confirmed the proposed compound as 6-hydroxy-4,4a,7a-tetrahydrobenzofuran-2(4H)-one (i.e. 6-OH-4,4a,7a-THBF) (Figure SM 2-10).

#### Anti-inflammatory and analgesic activities

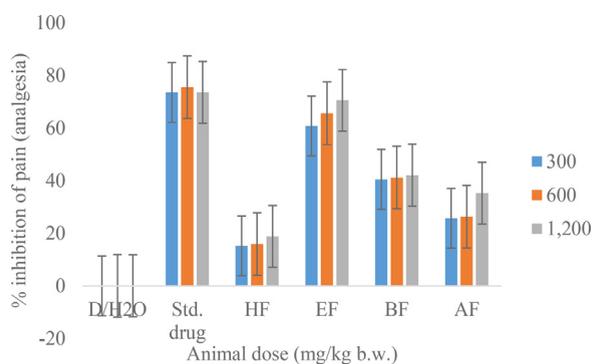
The results from anti-inflammatory study showed that the *S. venulosa* crude leaf methanol extract in carrageenan-induced edema in mice possessed significant reduction in paw edema in dose-dependent fashion (Fig. 3). The highest reduction in paw volume (anti-inflammatory) was witnessed in the ethyl acetate fraction (EF) after 3 h with reduction from 0.25 ± 0.01 to 1.10 ± 0.02 mm at highest dose of extract. There is significant difference between this and aspirin (acetylsalicylic acid) with values of 0.26 ± 0.01 to 2.1 ± 0.01 mm ( $p < 0.05$ ; one-way ANOVA). Similarly, there is significant reduction in pain in the mice in dose dependent fashion. Pronounced reduction in the number of writhing (analgesia) in mice was witnessed in the EF (64.21 % writhing inhibition) when compared to other treatment groups (Fig. 4). These results were statistically significant when compared to the negative control group that received only distilled water ( $p < 0.05$ ).

#### *In vivo* anticancer activity of *S. Venulosa* extract

From the results obtained, Mean Survival Time (MST) increased with increase in life span (% ILS) in dose-dependent fashion in all the tumor-treated groups. However, the MST and % ILS of the isolated pure compound was the highest with 0.76 ± 0.02 (MST) and 76.02 (% ILS). These data were statistically different from the control at  $p < 0.05$  (Table SM 2). Growth



**Fig. 3.** Anti-inflammatory activities of various fractions of *S. venulosa* extract at various doses. HF (n-hexane fraction), EF (ethyl acetate fraction), BF (n-butanol fraction), AF (aqueous fraction), Std. drug (standard drug), D/H<sub>2</sub>O (distilled water). Results are Mean±SE;  $p < 0.05$  (one-way ANOVA),  $n = 5$ .



**Fig. 4.** Analgesic effects of fractions of *S. venulosa* leaf extract. HF (n-hexane fraction), EF (ethyl acetate fraction), BF (n-butanol fraction), AF (aqueous fraction), Std. drug (standard drug), D/H<sub>2</sub>O (distilled water). Results are Mean±SE;  $p < 0.05$  (one-way ANOVA),  $n = 5$ .

of tumor cells were inhibited with increased concentration of plant extract from 100 to 400 mg/kg b.w. SVME (Table SM 3). Again, the isolated compound showed the highest % inhibition of tumor cell growth with 73.80 %. These values were significantly different when compared with the negative control ( $p < 0.05$ ; one-way ANOVA). Most of the serum biochemical parameters were elevated with the except of bilirubin. The extract does not cause significant reductions in the values of AST, ALP, ALT and WBC in mice after treatment (Figure SM 11).

## Discussion

Nowadays, the use of plant parts as ethno-pharmacological remedy in traditional medicine has gain global recognition due to the numerous research carried out on plants with regards to their uses in treatment of diseases. Many plants contain antioxidants which are plant-derived compounds that prevent human body against the hazardous effects from damage free radicals. Antioxidants help in preventing oxidation, boost immune systems, reduce the potentials for disease infection such as heart and cancer diseases. They are capable of interacting with free radicals thereby neutralizing their damage to the biological tissues. About 35 % medicinal plants contain antioxidants which are derived from various morphological parts of plants like leaves, fruits, flowers, stembarks, and seeds [9,16,35]. Though, the body cells produce some free radicals, and numerous oxygen reactive species, their overproduction have been reported to result in certain diseases [14,23,45]. Many secondary metabolites from plants have shown promising antioxidant potentials as well as remedies for several illnesses such as inflammation, analgesia, and diabetes.

In this study, antioxidant evaluation of *S. venulosa* leaf extracts showed that ethyl acetate fraction (EF) had the highest reductive effect on DPPH scavenging radicals with  $IC_{50}$   $16.13 \pm 0.02$   $\mu\text{g}/\text{mg}$  and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) scavenging radicals with  $IC_{50}$   $11.02 \pm 0.01$   $\mu\text{g}/\text{mg}$ , when compared to that of the isolated compound (6-OH-4.4a.7-THBF) with an  $IC_{50}$  value of  $0.16 \pm 0.01$   $\mu\text{g}/\text{mg}$  and  $0.01 \pm 0.02$   $\mu\text{g}/\text{mg}$  respectively. These reductions in DPPH and  $\text{H}_2\text{O}_2$  scavenging radicals were concentration-dependent (Table 1; Fig. 1 and 2). The data from  $\text{H}_2\text{O}_2$  further affirmed that *S. venulosa* leaf extract had high reducing power on DPPH radical cations, and displayed good antioxidant ability.

From the study, the determination of the total phenolic and flavonoid contents for the leaf extracts revealed that it contains  $199 \pm 0.2$  mg AAE / GAE/g and  $74.1 \pm 0.4$  mg AAE/ GAE/g respectively (Figure SM 1). The high levels of these polyphenols obtained from the plant might be due to the contributions of carbohydrate, and the solvents used for the extraction [32–42]. These data were not compared with those of the previous studies as there has not been any work of such scientifically documented on the plant as at the time of this present study. The antioxidant activity of the leaf extract at

various concentrations was due to the presence of high amount these polyphenols in the EF, and other fractions with trace amount of polyphenols. Previous studies on some members of the genus *Spondias* showed that most species in the genus contain high levels of flavonoids, and had shown high levels of reduction in DPPH scavenging radicals. The presence of high amount of phenolic and flavonoid could account for its cytotoxic effects on the cancer cell lines. In this present study, high cytotoxic effect was obtained with the isolated pure compound (PC) showing an  $IC_{50}$  value of  $2.12 \pm 0.01 \mu\text{g}/\text{mg}$ ;  $SI > 100$  against MCF-7/S0.5 cell line, and  $2.01 \pm 0.01 \mu\text{g}/\text{mg}$ ;  $SI > 100$  against OV7 cancer cell line in *in vitro* experiment, while the EF had an  $IC_{50}$  value of  $12.18 \pm 0.20 \mu\text{g}/\text{mg}$ ;  $SI = 1.97$  against the breast cancer cell line MCF-7/S0.5, and  $4.12 \pm 0.01 \mu\text{g}/\text{mg}$ ;  $SI = 77.67$  against the ovarian cancer cell line OV7. These data obtained were significantly different from those of the first-line anticancer drug doxorubicin USP (Adriamycin) with an  $IC_{50}$  value of  $0.80 \pm 0.01 \mu\text{g}/\text{mg}$ ;  $SI = 65$  against MCF-7/S0.5 cell line, and  $2.20 \pm 0.01 \mu\text{g}/\text{mg}$ ;  $SI = 25.57$  against OV7 cell line ( $p < 0.05$ ; one-way ANOVA). The ability of these extracts (PC and EF) to show high cytotoxicity activity and selective indices conferred on *S. Venulosa* leaf extract a potential anticancer agent due to its ability to differentiate cancerous cells from normal cell (Vero E6) as seen from the study (Table 2). The poor selective index obtained for doxorubicin USP against the cancer cell lines in this study was due to inability of most cancer chemotherapeutic drugs to target cancerous cells successfully and induce cellular apoptosis [15]. The present study showed that the *S. Venulosa* extract successfully targeted cancer cells thereby differentiating them from the mitochondria of other healthy tissues or cells.

The antioxidant and anticancer activities of the genus *Spondias* has also been reported in some members of the genus. For instance, *Spondias mombin* and *S. pinnata* extracts have been shown to exhibit high levels of antioxidant and cytotoxicity activity [10,38]. It is possible that the presence of these phytoconstituents were responsible for the observed antioxidant activity of *S. venulosa* leaf extract, since most members of this genus are endowed with abundant polyphenols. Similarly, *S. venulosa* leaf extract has been reported to contain flavonoid compounds such as quercetin, rutin and rhamnetin [32,37], and this is in agreement with this present study (Table SM 1). It is however, important to mention at this juncture that, the isolation of a lactone flavonoid compound 6-OH-4a.4.7a-tetrahydrobenzofura-2(4H)-one, which was confirmed by various techniques such as GC-MS, FTIR and NMR spectroscopy (Figure SM2-10), agreed with those of previously isolated flavonoids [32-34].

Many furanones have been reported to exert high degree of cytotoxicity and growth inhibitory activities on MCF-7 and B16F10 melanoma cells by inducing apoptosis in cells during *in vitro* studies [12,19]. The current study has shown that the novel compound 6-OH-4a.4.7a-tetrahydrobenzofura-2(4H)-one possessed high level of cytotoxic effects on MCF-7/S0.5 and OV7 cancer cell lines in *in vitro* study. This activity was further confirmed through the *in vivo* study, where the PC has a MST value of  $0.76 \pm 0.02$  days and an % ILS value of 76.02, when compared to other treatment groups that received various doses of SVME (Table SM2-3). The efficacy of a good anticancer agent is in its ability to increase the lifespan of the organism [43-45]. Similarly, the study showed that the growth of tumor cells in the mice were concentration-dependent. From the study, PC showed the highest % inhibition of tumor cells (73.80%). Furthermore, the study showed that *S. Venulosa* leaf extract possessed anti-inflammatory and analgesic activities with EF showing the highest anti-inflammatory after 3 h of induction with values of  $0.26 \pm 0.01$  to  $2.1 \pm 0.01$  reduction of paw edema volumes in dose-dependent fashion, while the percentage inhibition of writhing in mice was 64.21% (Fig. 3-4).

The study demonstrated that SVME possessed remarkable anti-inflammatory and analgesic activities as shown by the EF and the isolated compound (PC), which is related to the inhibition of mediators of inflammation by the blockage of signal pathways regulating swellings and pains [26,29]. This NSAID-like characteristics might likely be due to high contents of phenolic and flavonoid compound in the leaf extracts. These findings further validate the ethno-medicinal use of *S. Venulosa* in management of pain. This study was the first scientifically documented study carried out on the ethno-pharmacology of *S. Venulosa* leaf extract as antioxidant, anti-inflammatory, analgesic and cytotoxicity medicinal agent. Further studies are needed to validate other uses of the plant in traditional medicine.

## Conclusion

From the study, the ethyl acetate fraction (EF) of *S. Venulosa* leaf extracts possessed pronounced antioxidant, anti-inflammatory, analgesic and cytotoxicity activities. The anti-inflammatory and analgesic activities could be related to the inhibition of pro-inflammatory mediators and pain signaling pathways, which is in semblance to the pharmacology of NSAIDs. This present study was the first time an attempt was made to isolate the bioactive compound responsible for the observed biological activities, and evaluating these claims scientifically. GC-MS, FTIR and NMR data also revealed that a lactone flavonoid (6-OH-4a.4.7a-THBF) was the bioactive compound responsible for the observed bioactivities. It is therefore, necessary to carry out further studies on the leaf extract with a view to understanding the mechanism of these observed biological activities.

## Authors' contributions

C.A.U., I.E.O., T.S.M., and M.Y.D., designed the study, conduct the literature search, performed experiments, interpret the spectra and analyzed the data; C.A.U., and A.C.F., coordinated the study, prepared the manuscript for publication; H.R.J., and A.U.S., supervised the study, performed the statistical analysis, while H.H.M.; interpret the spectra, edited the grammar, and revised the manuscript before submission. All authors read the revised manuscript and approved it for final submission.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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## Supplementary materials

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