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Original Research Article

Evaluation of Antimicrobial Activity and Phytoconstituents of Stem Bark Extract and Vacuum Liquid Chromatographic Fractions of *Spondias mombin* (Linn)

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ABSTRACT

Microbial infection is one of the leading causes of death globally with about five million deaths annually. The mortality rate is projected to double by 2050 if the current effort, such as plant-based therapies, to combat multi-drug resistance is not sustained. The study evaluated the antimicrobial properties of the extract and vacuum liquid chromatographic (VLC) fractions of *Spondis mombin*. The susceptibility of clinical isolates of eight bacteria and two fungi to different concentrations of extracts and fractions and the minimum inhibitory concentration (MIC) were evaluated using agar diffusion and agar dilution techniques respectively. Alkaloids, flavonoids, tannins and saponins were detected in the *n*-butanol fraction. *Candida albicans* and *P. aeruginosa* were the most susceptible to the extract with MICs of 1.50 mg/mL while the MICs of 2.5 mg/mL (*E. coli*, *S. aureus*, *K. pneumoniae*, *S. paratyphi* and *B. cerus*), 3.5 mg/mL (*B. subtilis* and *S. dysentriae*) and 6.5 mg/mL (*A. niger*) were also recorded for the respective isolates. The *n*-butanol fraction showed significant inhibition of *E. coli*, *B. subtilis* and *B. cerus* with MIC of 0.35 mg/mL; *C. albicans* with MIC of 0.15 mg/mL and 0.25 mg/mL for *A. niger*. Two VLC fractions showed equipotent inhibitory activity with MIC of 0.085 mg/mL against *S. aureus*, and 0.95 mg/mL against *C. albicans* and *K. pneumoniae*. The broad spectrum of activity recorded and the emerging categorization of *S. aureus*, *K. pneumoniae* and *S. dysenteriae* by the WHO among the priority pathogens list could place *S. mombin* as a source of lead antibiotic compounds.

Keywords: Antimicrobials, *Spondias mombin*, microorganisms, bioactivity, metabolite

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Introduction

The recent WHO reports showed that infectious diseases have caused about 14 million deaths worldwide with many individuals at high risk. [1] It was also reported that bacterial infections ranked as the second leading cause of mortality globally. [1] These epidemiological data are disturbing and have been attributed to bacterial antimicrobial resistance which have also been reported to cause about 2 million deaths directly and 5 million deaths indirectly. [2] This is projected to hit annual deaths of 10 million, higher than malignant cancers by 2050. Unfortunately, the low- to middle-income economies have remained the worst affected by this challenge due to poor hygiene, poverty, abuse and misuse of antibiotics and lack of accessibility to quality healthcare. [3]
The majority of people in low-income countries rely on complementary and alternative medicines for their healthcare needs. This has led to the advancement of homoeopathy, naturopathy, Siddha, Unani and Yoga as well as African and European traditional medicines. [4] It is speculated that diseases that are endemic to a particular region are closely associated with the abundance of specific plant species that are used for the management of those diseases. One important plant species found in the rainforest and coastal regions of sub-Saharan Africa as well as the northern and southern parts of America is *Spondias mombi* Linn. [5]

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Its distribution within these regions has contributed to the understanding of its ethnopharmacological relevance in traditional and alternative medicines.
There are documented pieces of evidence that *Spondias mombin* of the Anacardiaceae is widely used in ethnomedicine. [6-10] The edible fruit possesses diuretic and febrifuge properties. [6] In some cultures, the decoction of the stem bark has emetic and astringent properties and is reputed for its usefulness in the treatment of leukorrhea, diarrhoea, haemorrhoids, dysentery and gonorrhoea. [7] The aerial parts of the plant are usually ground to a fine powder and taken as a tea for its anti-inflammatory, anti-cystitis and anti-urethritis activities. [8] Studies have revealed an abundance of secondary metabolites in *S. mombin*, including saponin, alkaloids and flavonoids in the leaves; as well as flavonoids, alkaloids and tannin in the stem bark. There is also a rich presence of vitamins such as vitamins B1, C, A, and E as well as mineral elements. [9] Mineral elements such as sodium, potassium, iron, calcium, magnesium, manganese, zinc, selenium and phosphorus have been found and the leaves contain more vitamins C and E than the stem bark. [10]

Several pharmacological activities of *S. mombin* have been documented, most of which originated from their ethnomedicinal uses. [11] Of significant importance is the antimicrobial property of the plant which has complemented other pharmacological activities. [12,13] However, the previous antimicrobial activity studies of *S. mombin* were limited to crude extracts using narrow spectra of microorganisms. [14,15] In one of the previous studies, only *Enterococcus faecalis* and *Pseudomonas aeruginosa* were utilized as test microorganisms which represented a narrow spectrum when compared with the etiological agents of microbial infections endemic in sub-Saharan Africa, the northern and southern parts of America and some Asian regions. [14] This study, therefore, evaluated the antimicrobial activity of the stem bark extracts and vacuum liquid chromatographic (VLC) fractions of *S. mombin* on the extended spectrum of microorganisms.

Materials and Methods

Plant sample

The *S. mombin* stem bark was collected in September 2019, at Umuogba-Ihe in Awgu Enugu, Nigeria (GPS location: 6° 4' 0.00"E; 7° 28' 60.00"E) and was identified and authenticated by Mr. Alfred Ozioko, a taxonomist in the Department of Plant Science and Biotechnology, University of Nigeria Nsukka, Nigeria. The voucher specimen of the sample (ID: PCG/2019/ASM13) was deposited at the herbarium of the Department for future reference.

Media

Mannitol salt agar, Mueller-Hinton agar, nutrient broth, eosin methylene blue agar, centrimide agar, MacConkey agar and nutrient agar were obtained from Titan Biotech, India.

Test microorganisms

The test microorganisms used (*Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae*. *Bacillus subtilis, Salmonella paratyphi, Bacillus cerus, Shigella dysenteriae, Pseudomonas aeruginosa, Candida albicans*, and *Aspergillus niger*) were clinical isolates obtained from the Department of Pharmaceutical Microbiology and Biotechnology Laboratory, University of Nigeria, Nsukka.

Preparation of extracts

The dried stem bark was reduced to a coarse powder using a mechanical grinder (MF 10 basic), with cutting edge and 1 mm sieve (IKAWerke GmbH & Co KG, Staufen). A 400 g of the coarse powder was cold-macerated in 2 L of methanol (95%v/v) in a flask first batch for 48 h with intermittent agitation. The sample was filtered and the pooled filtrate was concentrated to dryness using a rotary evaporator (Rotavapor R-210, BÜCHI, Flawil, Switzerland) at reduced pressure and 40 ℃ to obtain *S. mombin* stem bark methanol extract (SMM). The marc was re-dispersed in a fresh 2 x 1 L of methanol and the process was repeated until the extraction was deemed complete. [16] The total extract (SMM) was weighed and stored at 4 ℃ until future use.

Solvent partition of extract

Twenty grams (20.0 g) of SMM was dispersed in 200 mL of 10 %v/v methanol, sonicated for five minutes and made up to 1 L with the same solvent. The mixture was successively partitioned using *n*-hexane (2 x 1 L), ethyl acetate (3 x 1 L) and *n*-butanol (2 x 1 L) respectively. [17] Each fraction was combined and evaporated to dryness using a rotary evaporator (Rotavapor R-210, BÜCHI, Flawil, Switzerland) at reduced temperature and pressure to obtain *n*-hexane (SMH), ethyl acetate (SME) and *n*-butanol (SMB) fractions. The fractions were preserved in a refrigerator (Haier, China) at 4 ºC until required for the experiment. All the solvents used for the extraction and partitioning were of analytical grade and were obtained from Sigma-Aldrich, Germany

Vacuum liquid chromatographic fractionation

Three grams (3.0 g) of SMB was dissolved in 15 mL of methanol and triturated with 15 g of silica gel (Merck KGaA GmbH, Darmstadt, Germany) of mesh size (200 – 400) and allowed to dry completely before loading. [18] The dried sample was loaded into a glass column (150 x 1.5 cm) containing silica gel (200 – 600 mesh) and eluted with a gradient of dichloromethane (Sigma-Aldrich, Germany) in methanol (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9) each 500 mL to obtain nine sub-fractions (SMB-1 to SMB-9). The fractionation process was achieved using Speedivac Edward High Vacuum Pump, ES50 (Edwards and Co., London). The eluates were collected in a 500 mL beaker, concentrated to dryness and stored in sample bottles.

Qualitative phytochemical screening

The qualitative phytochemical analysis of six secondary metabolites (alkaloids, terpenoids, flavonoids, tannins, saponins and steroids) was carried out using standard methods for each of the tests. [19]

Preparation of media

A 0.5%w/v glucose-enriched agar (Java Bicolloid, Surabaya), 11.4 g of nutrient agar powder was suspended in 400 mL of distilled water and was allowed to stand for 10 mins while swirling. The agar suspension was melted by heating in a water bath (IKA, Corning) at 100 ℃. Then, 2 g of glucose was added into the molten agar and mixed well; followed by dispensing 20 mL aliquot of the molten agar into dried and sterilized bijou bottles, cocked and autoclaved for standard sterilization at 121°C for 15 min. The sterile molten nutrient agar was stored at a steady temperature of 60 ℃ until needed for use. The test microorganisms were standardized using the McFarland turbid equivalent.

Antibiotic susceptibility profile of test isolates

The susceptibility patterns of the microorganisms were evaluated using the agar well diffusion technique. [20] Varying graded concentrations of the SMM, SMH, SME and SMB (1 – 10 mg/mL) and SMB-1 to SMB-9 (0.50 – 0.06 mg/mL) were prepared with dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany) in different test tubes using appropriate dilution to obtain the final concentrations for the plant extract samples. The prepared agar plates were properly seeded with the different bacteria (one organism for each agar plate); with the aid of a sterile cotton wool swab. After the inoculum, wells measuring 8 mm in diameter were bored in the plates with the aid of a sterile cork borer. To each well, 50 µL of working solution, standards (ciprofloxacin and fluconazole) and DMSO (100%) were added using a micropipette to serve as the treatments, positive and negative controls respectively. [21] The plates were allowed to stand at room temperature for 30 minutes for the extract to diffuse into the agar properly in the laminar flow to avoid contamination before incubating at 37 ℃ for 24 h.

Determination of minimum inhibitory concentration (MIC)

The MICs were determined by the agar dilution method on Mueller Hinton agar using the extracts or fractions that inhibited the microorganisms and considering the lowest concentration that inhibited the growth. [22] Stock solution of each extract and fraction was prepared by dissolving in DMSO. The stock solutions were diluted to obtain 9.0 – 1.0 mg/mL for SMH, SME and SMB, 5.0 – 0.5 mg/mL for SMB-1 to SMB-9 and 1.0 – 0.25 mg/mL for control. The MIC of the control drug (vancomycin) and DMSO was determined in another agar plate. Agar plates were prepared by dispensing 9 mL of MH agar into sterile Petri dishes containing 1 mL of the various dilutions of each extract and control drug. The final plate concentrations were 0.9 – 0.10 mg/mL for SMH, SME and SMB, 0.50 – 0.05 mg/mL for SMB-1 to SMB-9 and 0.1 – 0.025 mg/mL for the control. The plates were incubated at 37 ℃ for 18 – 20 h and the inhibition was recorded. [23]

Data analysis

Data were expressed as mean ± standard error of the mean (n=3). One-way analysis of variance (ANOVA) was performed using GraphPad Prism v.5.0 (GraphPad Software Inc., San Diego, CA, USA)

Results and Discussion

Extraction and fractionation of extracts

The extraction of the coarsely powdered sample of *S. mombin* and fractionation of the extracts yielded extract and fractions of varying compositions of phytoconstituents. A 400 g of dried coarse sample extracted with methanol (95%v/v) yielded 21.215 g of crude extract designated as SMM, representing 5.303 %w/w of dry weight of coarse powder. On subsequent partitioning of the extract in solvents of increasing polarity, 21.215 g of SMM yielded 1.0564 g (SMH, 0.264%w/w), 3.0906 g (SME, 0.773%w/w), and 5.579 g (SMB, 1.395%w/w) of *n*-hexane, ethyl acetate, and *n*-butanol soluble fractions. The most active fraction, SMB was subjected to further separation using the VLC technique in graded combinations of dichloromethane and methanol starting from 90% dichloromethane/10% methanol to 10% dichloromethane/90% methanol to furnish 0.280 g (SMB-1, 0.070%w/w), 0.5141 g (SMB-2, 0.1285%w/w), 0.4688 g (SMB-3, 0.117%w/w), 0.4384 g (SMB-4 0.1096%w/w), 0.1760 g (SMB-5,

0.044%w/w), 0.0096 g (SMB-6, .0024%w/w), 0.5608 g (SMB-7, 0.140%w/w), 0.3413 g (SMB-8, 0.085%w/w) and 0.305 g (SMB-9, 0.076%w/w). All the yields were expressed as the percentage weight of the extract/fraction per 400 g of the dry coarse powder used for the extraction.

Several plants that have antimicrobial activities have been documented. These plants contain several secondary metabolites that are responsible for their pharmacological activity which can be extracted using solvents of different polarities. [13-15] In this study, the methanol extract of *S. mombin* was separated using *n*-hexane, ethyl acetate and *n*-butanol representing low, moderate and high polar solvents. The SMB, which represented the polar fraction was further separated using solvent combination. It was observed that the distribution of the phytochemical constituents in the fractions represented the polarity differences of the extracting solvent. The 95%v/v methanol was used as a macerating solvent for plant extraction and it was similar with other phytochemical works. In general, hydroalcoholic co-solvents such as 80-96% methanol seems to possess the optimum solubility characteristics for initial crude extraction and provide a high extraction yield. [24] However, despite the several works and interests in phytochemical extraction, there is no single solvent which may be considered standard because it is usually different for different plant matrices. [25] Moreover, the mode of extraction which also reflects the method of preparation of the plant material in ethnomedicinal uses is also an important factor. [3]

Phytochemical constituents of S. mombin

The qualitative phytochemical analysis of the methanol extract of *S. mombin* stem bark revealed the presence of alkaloids, tannins, saponins, flavonoids, steroids and terpenoids as tested (Table 1). The result also showed that SMH is rich in steroids and terpenes. The SME is rich in tannins, terpenoids, steroids, tannins and alkaloids while alkaloids, flavonoids, tannins and saponins were detected in SMB.

Phytochemical analysis of plant extracts and the identification of active components therein have helped to explain the mechanism of antimicrobial activity. [26] Identification of plants with verified activity is the first phase in the discovery of lead compounds that may be developed into novel therapeutic agents. The SME contains the bulk of the moderately polar secondary metabolites such as alkaloids, flavonoids, saponins and tannins while the SMH contains the non-polar constituents such as steroids, and terpenoids. The qualitative phytochemical analysis of the methanol extract, fractions and subfraction shows that the plant is rich in phytochemicals. [5-10] Although the active compound responsible for the activity is yet to be identified, the antimicrobial activity of *S. mombin* could be attributed to a single or a combination of its secondary metabolites such as the presence of alkaloids, flavonoids, terpenoids, steroids, glycosides, tannins, saponins, reducing sugars, phenols in high concentrations, which agrees to the report that most compounds isolated from plants are rich in phytoconstituents and has antimicrobial activity. [8-10]

Table 1: Phytochemical constituents of *S. mombin*

Phytochemical	SMM	SMH	SME	SMB
Alkaloids	+	-	+	+
Flavonoids	+	-	_	+
Terpenes	+	+	+	-
Steroids	+	+	+	-
Tannins	+	-	+	+
Saponins	+	-	-	+

(-) = Absent; (+) = Present

Antimicrobial activity of S. mombin

Susceptibility profiles of isolates to S. mombin

The susceptibility of bacterial (*E. coli*, *S. aureus*, *K. pneumoniae*, *B. subtillus*, *S. paratyphi*, *B. cerus*, *S. dysenteriae* and *P. aeruginosa*) and fungal (*C. albicans* and *A. niger*) isolates to the extract, fractions and sub-fractions of *S. mombin* was evaluated by agar diffusion method. The results showed varying susceptibility patterns of the isolates to the treatments. The results showed that SMM (3-10 mg/mL) inhibited the growth of all the bacterial isolates and *C. albicans*. The *A. niger* was only susceptible to SMM at a concentration of 7-10 mg/mL. On partitioning SMM in solvents of different polarities and subjecting the fractions to a susceptibility test, the SMB inhibited the growth of both bacterial and fungal isolates at the tested concentration of 1-10 mg/mL. The SME also was found to possess varying activity as the growth of all the bacterial isolates was inhibited at 1-10 mg/mL while the fungal isolates were inhibited at a concentration of 7-10 mg/mL. However, SMH (1-10 mg/mL) did not inhibit the growth of any microbial isolate tested.

Since SMB was found to possess broader spectrum of activity, the fraction was subjected to VLC separation by gradient elution method using a dichloromethane-methanol mobile phase system. The nine VLC fractions were also subjected to susceptibility tests. The results showed that VLC fractions, SMB-3 and SMB-4 (0.5 – 0.05 mg/mL) showed significant inhibitory activity against the tested isolates compared with other fractions which did not record any growth inhibition. A pairwise comparison of the two fractions showed that SMB-4 (≥ 0.10 mg/mL) inhibited all the tested microbial isolates while SMB-3 elicited varying growth inhibitory activities against the tested isolates. The growth of *E. coli*, *B. subtilis* and *B. cerus* was significantly inhibited by SMB-3 (≥ 0.40 mg/mL) while the growth of *S. paratyphi* and *A. niger* was inhibited at a concentration of 0.30 mg/mL. A stronger inhibitory activity of SMB-3 (≥ 0.20 mg/mL) was observed against *S. dysenteriae and P. aeruginosa*. Like SMB-4, the inhibitory effect of SMB-3 against *K. pneumoniae* and *C. albicans* was observed at a minimal concentration of 0.10 mg/mL while the highest activity of SMB-3 was recorded against *S. aureus* at a minimal concentration of 0.090 mg/mL.

MICs of S. mombin extracts on microbial isolates

The MICs of the extracts and fractions were determined by the agar dilution method on Mueller Hinton agar considering the lowest concentration that inhibited the microbial growth (Table 2). *Candida albicans* and *P. aeruginosa* were the most susceptible to SMM with MICs of 1.50 mg/mL while the MICs of 2.5 mg/mL (*E. coli*, *S. aureus*, *K. pneumoniae*, *S. paratyphi* and *B.cerus*), 3.5 mg/mL (*B. subtilis* and *S. dysentriae*) and 6.5 mg/mL (*A. niger*) were also recorded for the respective isolates. In comparison with the controls, *S. mombin* showed higher MIC than ciprofloxacin (0.010 mg/mL) and fluconazole (0.030 mg/mL). On separation in solvents of graded polarities, SME and SMB showed significantly improved inhibition of the microbial isolates. Both of them showed equal potential in the inhibition of *E. coli*, *B. subtilis* and *B. cerus* with MIC of 0.35 mg/mL; *C. albicans* with MIC of 0.15 mg/mL and 0.25 mg/mL for *A. niger*. However, SMB showed stronger inhibition of *P. aeruginosa* (MIC of 0.25 vs 0.35 mg/mL) while SME showed stronger inhibition of *S. paratyphi* (MIC of 0.35 vs 0.45 mg/mL).

Table 2: MIC of *S. mombin* extract on different microbial isolates

Clinical isolates	MIC at 10 – 1 mg/mL				MIC at 0.9 – 0.1 mg/mL	
	SMM	SMH	SME	SMB	SME	SMB
E. coli	2.5	n.d	n.d	n.d	0.35	0.35
S. aureus	2.5	n.d	n.d	n.d	n.d	n.d
K. pneumoniae	2.5	n.d	n.d	n.d	n.d	n.d
B.subtillus	3.5	n.d	n.d	n.d	0.35	0.35
S. paratyphi	2.5	n.d	n.d	n.d	0.35	0.45
B.cerus	2.5	n.d	n.d	n.d	0.35	0.35
S. dysenteriae	3.5	n.d	n.d	n.d	0.15	n.d
P. aeruginosa	1.5	n.d	n.d	n.d	0.35	0.25
C.albicans	1.5	n.d	6.5	n.d	0.15	0.15
A.niger	6.5	9.5	6.5	n.d	0.25	0.25

n.d = not determined within the concentration range

MICs of S. mombin VLC fractions on microbial isolates

The MICs of the VLC fractions were determined by the agar dilution method considering the lowest concentration of SMB-1 to SMB-9 that inhibited the microbial growth in the susceptibility assays. The results (Table 3) showed that both SMB-3 and SMB-4 showed equipotent inhibitory activity with MIC of 0.085 mg/mL against *S. aureus* and 0.95 mg/mL against *C. albicans* and *K. pneumoniae*. In other cases, SMB-4 showed higher inhibition potential than SMB-3 with a narrow MICs range of 85 – 95 µg/mL and a wide MICs range of 85 – 350 µg/mL respectively.

Phytochemical compounds have remained the mainstay of drug discovery for the management of several ailments. This is necessitated by the wide distribution, unique chemo-diversity and ease of accessibility. [27] Specifically, their role in the discovery of antibacterial agents lies in the diversity of bioactivity and perceived clinical effectiveness. The study showed that two VLC fractions strongly inhibited the growth of all the clinical isolates tested suggesting the broad spectrum of antibacterial and antifungal activities of *S. mombin*. Emerging trends in the present study elucidated the significant antimicrobial effect of *S. mombin* extract; especially SMB against *S. aureus*, *K. pneumoniae* and *S. dysenteriae* in addition to the antifungal effect on *C. albicans*. The fractions (SMB-3 and SMB-4) have the potential to furnish a drug candidate that is capable of ameliorating the challenges of these microbes since they are part of multi-drug resistant microorganisms categorized by the WHO among the priority pathogens list for research and development of new antibiotic drugs. [28]

Table 3. MIC of VLC fractions on different microbial isolates

Clinical isolates/ fractions	MIC of VLC fractions at 0.5 – 0.05 mg/mL)								
	1	2	3	4	5	6	7	8	9
E. coli	n.d	n.d	0.350	0.095	n.d	n.d	n.d	n.d	n.d
S. aureus	n.d	n.d	0.085	0.085	n.d	n.d	n.d	n.d	n.d
K. pneumoniae	n.d	0.45	0.095	0.095	0.45	0.45	n.d	n.d	n.d
B. subtillus	n.d	n.d	0.350	0.095	n.d	n.d	n.d	n.d	n.d
S. paratyphi	n.d	n.d	0.250	0.095	n.d	n.d	n.d	n.d	n.d
B. cerus	n.d	n.d	0.350	0.095	n.d	n.d	n.d	n.d	n.d
S. dysenteriae	n.d	0.45	0.150	0.095	n.d	n.d	n.d	n.d	n.d
P. aeruginosa	n.d	n.d	0.350	0.095	n.d	n.d	n.d	n.d	n.d
C. albicans	n.d	n.d	0.095	0.095	n.d	n.d	n.d	n.d	n.d
A. niger	0.45	0.35	0.250	0.095	n.d	0.45	0.45	n.d	n.d

n.d = not determined within the concentration range; 1-9 represents VLC fractions, SMB-1 to SMB-9 respectively

Conclusion

The microbial susceptibility screening revealed that *S. mombin* extracts and VLC fractions inhibit the growth of bacterial and fungal isolates tested. Two VLC fractions (SMB-3 and SMB-4) showed strong inhibitory activities against *S. aureus*, *K. pneumoniae*, *S. dysenteriae* and *C. albicans*. The broad spectrum of activity recorded and the emerging categorization of *S. aureus*, *K. pneumoniae* and *S. dysenteriae* by the WHO among the priority pathogens list could place *S. mombin* as a source of lead antibiotic drugs. Isolation and characterization of the bioactive constituents of SMB-3 and SMB-4 are currently in progress.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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