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Short Communication

AntiMtb activity of triterpenoid-rich fractions from *Spondias mombin* L.

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***Spondias mombin* L. used in traditional medicine because of its antimicrobial properties was found to contain cardiac glycosides, flavonoids, tannins, and anthraquinones in the stem bark. Bioassay-directed fractionation of the methanol extract of *S. mombin* was carried out with VLC and HPLC. Isolates were evaluated for antimtb activity which led to the isolation of a series of potential molecules. A semi pure triterpenoid that demonstrated potency of 92.8% inhibition against *Mycobaterium tuberculosis* (Mtb) at a concentration of 64 µg/ml was further purified by HPLC.**

Key words: *Spondias mombin*, *Mycobacterium tuberculosis*, triterpenoids.

INTRODUCTION

Spondias mombin L. plant tree is erect, stately, to 65 ft (20 m) tall, with trunk somewhat buttressed and thick bark; often, in young trees, bearing many blunt-pointed spines or knobs. The fruit is aromatic, ovoid or oblong, golden-yellow; with thin, tough skin, and medium-yellow, clinging to the white, fibrous or "corky" stone. The tree is widely cultivated and naturalized in tropical Africa. Ripe fruits are eaten out-of-hand, or stewed with sugar. The extracted juice is used to prepare ice cream, cool beverages and jelly. Young leaves are cooked as greens. The fruit juice is drunk as a diuretic and febrifuge. The decoction of the astringent bark serves as an emetic, a remedy for diarrhea, dysentery, hemorrhoids and a treatment for gonorrhea and leucorrhea; and, in Mexico, it is believed to expel calcifications from the bladder. The powdered bark is applied on wounds (Morton, 1987).

A tea of the flowers and leaves is taken to relieve stomachache, biliousness, urethritis, cystitis and eye and throat inflammation. The juice of crushed leaves and the powder of dried leaves are used as poultices on wounds and inflammations. The gum is employed as an expectorant and to expel tapeworms.

Our antimtb screening of plant products from Nigeria resulted in *S. mombin* with a high potency *in vitro* activity against mycobacterium tuberculosis. Tuberculosis is a common and often deadly infectious disease caused by *Mycobacterium tuberculosis* (Kumar et al., 2007). Antibiotic resistance remains a growing problem in multi-drug resistant tuberculosis (WHO, 2006). The emergence of multidrug-resistant tuberculosis (MDRTB) represents a large and growing threat to TB control programs. In 2004, mortality and morbidity statistics included 14.6 million chronic active cases, 8.9 million new cases, and 1.6 million deaths, mostly in developing countries (WHO, 2006). In addition, a rising number of people in the developed world are contracting tuberculosis because their immune systems are compromised by immunosuppressive drugs, substance abuse, or AIDS. According to Centers for Disease Control, 8 million people become ill with tuberculosis annually, and 2 million people die from the disease worldwide (CDC, 2006). Tuberculosis Research Scientists tackle important problems in the development of resistance to specific drugs such as pyrazinamide, isoniazid, and PA-824. So, there is an urgent need for drugs to combat the multidrug-resistant tuberculosis.

MATERIALS AND METHODS

General

All chemicals were of analytical grade. Millipore water was collected

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from a Millipre Millipack® Express, 0.22 µm water purifier (Millipore; Billerica, MA, USA). Phytochemical screening of methanol extract was carried out by the standard procedures (Harbone, 1998). Triterpenoids were detected by Liebermann-Burchard test which gave a characteristic blue-green colour. Vacuum liquid chromatography (VLC) was performed on Si gel (230-400 mesh-Merck). VLC on Si gel was carried out with gradient elution using hexane, EtOAc, MeOH and water in the order of increasing polarity. Preparative HPLC was performed with a waters Prep LC system. High resolution mass spectra were run on the Bruker ESI-micrOTOF in positive mode coupled with an Agilent HPLC.

Plant material

The fresh plant material was collected from Ibadan, Oyo State, Nigeria in August, 2007. Voucher specimen has been deposited in the Forestry Research Institute of Nigeria, Ibadan (FHI no. 107896).

Extraction and Isolation

Plant cold extraction was carried out with MeOH and the extract was evaporated *in vacuo* (yield: 3.7%). Bioassay guided fractionation of the crude extract was done by VLC using normal phase conditions. VLC on Si gel was carried out with gradient elution using hexane, EtOAc, MeOH and water in the order of increasing polarity. The active portion was further fractionated by HPLC (Luna C8 column 21.2 x 250 mm) using water, acetonitrile and methanol as eluents with flow rate 15 ml/min. A total of 22 fractions were collected and submitted for antiMtb assay. One of the active fractions: SMi15 (ACN/MeOH 78:22) was subjected to further purification by reversed-phase HPLC with C18 10 x 250 mm; ACN/MeOH (90:10-100) flow rate 3 ml/min which gave rise to 5 metabolites. Isolates SMi-15-4 and SMi-15-5 were selected for characterization.

Antibacterial assay

A microplate-based assay which uses Alamar blue reagent for determination of growth was used. Percent inhibition of fraction agents against *M. tuberculosis* H37Rv was determined in the microplate Alamar blue assay (MABA) (Collins and Franzblau, 1997; Franzblau et al., 1998).

Antimicrobial susceptibility testing was performed in black, clear-bottomed, 96-well microplates (black view plates; Packard Instrument Company, Meriden, Conn.) in order to minimize background fluorescence. Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. Initial drug dilutions were prepared in either dimethyl sulfoxide or distilled deionized water, and subsequent twofold dilutions were performed in 0.1 ml of 7H9GC (no Tween 80) in the microplates. BACTEC 12B-passaged inocula were initially diluted 1:2 in 7H9GC, and 0.1 ml was added to wells. Subsequent determination of bacterial titers yielded 1 x 10^6 CFU/ml in plate wells for H37Rv. Frozen inocula were initially diluted 1:20 in BACTEC 12B medium followed by a 1:50 dilution in 7H9GC. Addition of 1/10 ml to wells resulted in final bacterial titers of 2.0 x 10^5 CFU/ml.

Wells containing drug only were used to detect autofluorescence of compounds. Additional control wells consisted of bacteria only (B) and medium only (M). Plates were incubated at 37°C. Starting at day 4 of incubation, 20 ml of 103 alamarBlue solution (Alamar Biosciences/Accumed, Westlake, Ohio) and 12.5 ml of 20% Tween 80 were added to one B well and one M well, and plates were reincubated at 37°C. Wells were observed at 12 and 24 h for a color change from blue to pink and for a reading of ≥50,000 fluorescence units (FU). Fluorescence was measured in a Cytofluor II microplate fluorometer (PerSeptive Biosystems, Framingham, Mass.) in bottom-reading mode with excitation at 530 nm and emission at 590 nm. If the B wells became pink by 24 h, reagent was added to the entire plate. If the well remained blue or ≤50,000 FU was measured, additional M and B wells were tested daily until a color change occurred, at which time reagents were added to all remaining wells. Plates were then incubated at 37°C, and results were recorded at 24 h post-reagent addition.

Percent inhibition was defined as 1 - (test well FU/mean FU of triplicate B wells) x 100. The lowest drug concentration effecting an inhibition of ≥90% was considered the MIC.

Table 1. *In vitro* activity of *Spondias mombin* against *Mycobacterium tuberculosis*.

Test agents	*M. tuberculosis* % Inhibition
SMi8-9	94.9
SMi10-12	87.4
SMi13	85.7
SMi14	98.3
SMi15	92.8
SMi16	55.7
SMi17	49.1
SMi18	46.2
SMi19-20	36.5
SMi21	23.5
SMi22	20.2
SMJET	60.0
Rifampin	99.7
Isoniazid	91.4
Mox	99.3
streptomycin sulfate	99.7
PA824	98.8

Mycobacterium tuberculosis strain: H37RV.
inhibition of ≥90% was considered active.
Assays were run using 64 µg/ml *Spondias mombin*.

RESULTS AND DISCUSSION

The results of the phytochemical screening of the MeOH extract of plant sample revealed the presence of tannins, flavonoids, cardenolides, and anthraquinones while alkaloids were found absent. The semi-pure SMi15 gave positive Liebermann-Burchard test for a triterpene. Table 1 shows the observed anti-Mtb activity of *S. mombin* column fractions in the in the microplate Alamar blue assay (MABA). SMi8-9, SMi14 and SMi15 demonstrated good potency of 94.9, 98.3 and 92.8% Inhibition, respectively, against *M. tuberculosis* in a dose dependent manner compared with controls. The results reveal *S. mombin* as a promising natural product agent that can provide useful antitubercular drugs.

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