Original Article

In vitro Activities of Methanol Extracts of Some Plants Used as Herbal Remedies

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ABSTRACT

Seven different plants from Nigerian herbs were investigated for anti-infective properties. The plant crude extracts, obtained by maceration with methanol, were subjected to array of antimicrobial screening tests. Antimycobacterial susceptibility of *M. tuberculosis* (H37Rv strain) was performed by Alamar Blue Assay. The results showed *Spondias mombin* and *Anacardium occidentale* to have 68 and 63% inhibition respectively against *P. aeruginosa* (ATCC 27853). The secondary test on the *S. mombin* extract against *P. aeruginosa* had IC_{50} of 37.32µg/ml. Two EtOAC-MeOH soluble fractions of exhibited good antimicrobial activities. One fraction (AOF9) exhibited antifungal activity against *Candida glabrata* with IC_{50} value of 9.0µg/ml while the other fraction (AOF8) showed antibacterial activity against *Pseudomonas aeruginosa* with IC_{50} value of 28.3µg/ml. The result of the antimycobacterial screening tests proved *Spondias mombin* most potent for providing antiturbercular compounds and was further investigated by HPLC-based activity profiling. The HPLC fractions revealed SM8-9, SM14 and SM15 to be effective (94.9, 98.3 and 92.8% Inhibitions respectively) against *M. tuberculosis* H37RV as compared with reference drugs. The findings show *Spondias mombin* and *Anacardium occidentale* to possess good anti-infective potentials and these support the folkloric uses of the plants for the treatment of infectious diseases.

Keywords: Anti-infective, antimicrobial screening, antimycobacterial, antitubercular, *Spondias mombin*, *Anacardium occidentale*.

INTRODUCTION

A key factor in the discovery of new drugs is not only on the layman information collected from traditional medicine but much also on the rigorous investigation of

the plant extracts for various biological activities regardless of their uses or folkloric claims and of their activity in traditional medicine[1]. We are faced with ever-increasing health threats from "notorious" and opportunistic infections, multidrug resistant pathogens and cancers. Diseases such as tuberculosis, gonorrhea, malaria, and childhood ear infections are now more difficult to treat than they were decades ago[2].

In order to combat these important health challenges, discovery of new medicinal agents with novel modes of activity is imperative. Screening for new natural products through an ethnobotanical approach is an effective method of drug discovery that focuses on medicinal plants used by indigenous peoples[3]. Higher plants can provide compounds with new structural features and novel mechanisms of biological activity. As prototype medicinal agents, they may provide starting points for synthetic or semisynthetic modifications aimed at enhancing their potency or therapeutic potential[4,5]. Therefore, the present study was carried out to determine the anti-infective properties of some plants commonly used as herbal helps in South-West Nigeria. The selected plant materials were screened for antibacterial, antifungal, and antimycobacterial properties via activity-guided assays.

MATERIALS AND METHODS

Plant Materials

The plant samples were collected at various locations in Ibadan, Nigeria in February 2008; and identified by Dr. O. A. Ugbogu of Forestry Research Institute of Nigeria (FRIN), Ibadan. Voucher specimens (Table 1) were deposited in the herbarium.

Preparation of extracts

The oven-dried (45^0C) plant samples were powdered and macerated with methanol at room temperature. Extracts were strained and filtered over anhydrous sodium sulphate and then evaporated in vacuo at 45^0C. The concentrated extracts (yields: Table1) were kept in refrigerator for antimicrobial tests. Only the very active crude extracts were subjected to fractionation by vacuum liquid chromatography (VLC) and HPLC successively.

VLC and HPLC conditions

Spondias mombin and *Anacardium occidentale* extracts was fractionated by VLC and evaluated for antimicrobial activity in cell-based assays. The VLC analysis was run on Si gel 230-400 mesh (Merck) with gradient elution in normal phase. The portion of *S. mombin* which exhibited significant antimycobacterial property was further investigated by HPLC-based activity profiling. Reversed-phase HPLC was performed on a waters Prep LC system 400 (column: Luna C_8 21.2 x 250 mm, flow rate 15ml/min; detector wavelength, 254nm)

Microorganisms and inoculums

All organisms were obtained from the American Type Culture Collection (Manassas, VA. USA). The following pathogens were used for antimicrobial study: *Mycobaterium tuberculosis* (H37Rv), *C. albicans* (ATCC 90028), *C. neoformans* (ATCC 90113), *C. glabrata* (ATCC 90030), *A. fumigatus* (ATCC 90906), MRSA (ATCC 43300), *E. coli* (ATCC 35218), *P. aeruginosa* (ATCC 27853), and *M. intracellulare* (ATCC 23068).

Preliminary bioactivity screening of plant samples

The crude methanol extracts of the selected plants were subjected to general preliminary bioactivity screening. The antimicrobial evaluation was conducted against the following pathogens: *Mycobaterium tuberculosis* (Mtb), *C.*

albicans (ATCC 90028), *C. glabrata* (ATCC 90030), *C. neoformans* (ATCC 90113), *A. fumigatus* (ATCC 90906), MRSA (ATCC 43300), *E. coli* (ATCC 35218), *P. aeruginosa* (ATCC 27853), and *M. intracellulare* (ATCC 23068).

Susceptibility testing was performed using a modified version of the NCCLS methods[6,7]. *Mycobaterium intracellulare* and *A. fumigatus* growth were monitored using Alamar Blue™ (BioSource International; Camarillo, CA, USA)[6-8]. Sample dissolved in DMSO were serially diluted using 20%/0.9% DMSO/saline and transferred in duplicate to 96-well flat bottom microplates. Microbial inocula were prepared by diluting saline suspensions of colonies with assay broth to afford recommended colony forming units/ml. Growth (saline only), solvent and blank (media only) controls were included on each test plate. Drug controls [ciprofloxacin, ICN Biomedicals; Aurora, OH, USA) for bacteria and amphotericin B (ICN Biomedicals) for fungi] were included in each assay. All organisms were read at either 630 nm using the EL-340 Biokinetics Reader(Bio-Tek Instruments; Winooski, VT, USA) or 544 ex/590 em, (*M. intracellulare, A. fumigatus*) using the Polarstar Galaxy Plate Reader (BMG LabTechnologies; Offenberg, Germany) prior to and after incubation. The MIC (minimum inhibitory concentration) was defined as the lowest test concentration that allowed no detectable growth.

Antimycobacterial assay

A microplate-based assay which uses Alamar blue reagent for determination of growth was used. Percent inhibition of fraction agents against *Mycobacterium tuberculosis* H37Rv was determined in the microplate Alamar blue assay[6-9].

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. Inocula were initially diluted 1:2 in 7H9GC and 0.1 ml was added to wells which resulted in bacterial titers of 1 x 10^6 CFU/ml in plate wells for H37Rv.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, 20µl of 10 x alamar blue solution (Alamar Biosciences/Accumed, Westlake, Ohio) and 12.5 µl 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.

RESULTS & DISCUSSION

The experimental approach engaged in the present study involved an initial primary screening of the crude extracts of selected plants (Table 1) against some pathogenic microorganisms. The results (Table 2) of the general biological screening of the selected plant samples showed *Spondias mombin* and *Anacardium occidentale* to have 68 and 63% inhibition respectively against *P. aeruginosa* ATCC 27853. The secondary screening test on the methanol extract of *S. mombin* against *P. aeruginosa* revealed moderate activity with IC_{50} of 37.32µg/ml (Table 3). The EtOAC-MeOH soluble fractions of *A. occidentale* exhibited antifungal activity (Table 4) against *Candida glabrata* with IC_{50} value of 9.0 µg/ml (AOF9) and antibacterial activity against *Pseudomonas aeruginosa* with IC_{50} value of 28.3µg/ml (AOF8). The remaining plants were inactive against array of pathogens: *C. albicans* ATCC 90028, *C. neoformans* ATCC 90113, *C. glabrata* ATCC 90030, *A. fumigatus* ATCC 90906, MRSA ATCC 43300, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853, and *M. intracellulare* ATCC 23068. *In vitro* activities of the screened plant extracts against *Mycobacterium tuberculosis* (Table 5) revealed moderate potency of *Spondias mombin* against *Mycobacterium tuberculosis* H37RV. The crude extract of the stem bark of *Spondias mombin* that demonstrated most promising antitubercular activity (27% inhibition) in the primary screening was subsequently fractionated using VLC and HPLC techniques respectively. Fractions VL2 and VL3 (Table 6) showed good activities against Mtb (91% and 69% inhibition respectively) compared to other fractions. VL2 had MIC of 61.1µg/ml (Table 7) at a concentration of 64µg/ml. Subsequently, VL2 and VL3 were pooled together to afford VLF which was later purified by HPLC. The fluorometric microplate alamar blue assay (MABA) of the HPLC fractions (Table 8) revealed SM8-9, SM14 and SM15 to be effective (94.9, 98.3 and 92.8% Inhibition respectively) against *M. tuberculosis* H37RV as compared with reference drugs. The chromatogram (Figure 1) of HPLC analysis of the VLC active portion of *Spondias mombin* portrayed the retention time areas of the anti-Mtb active column fractions of SM8-9, SM14 and SM15.

The use of the selected plants is popular in folkloric medicine for the treatment of infectious diseases[10,11], *Anacardium occidentale* is used in traditional medicine for the treatment of diabetes mellitus. Water extract of the bark is used for the treatment of dysentery, fever, pains, amenorrhea and diarrhea[10-12]. *Spondias mombin* has been reported to have antibacterial, antifungal, and antiviral[13-15]. Phytochemical screening (Table 9) of the stem barks of both *Spondias mombin* and *Anacardium occidentale* revealed the presence of the following classes of natural products-tannins, phenols, flavonoids, saponins, saponins glycosides, cardenolides, anthraquinones and alkaloids (traces in *S. mombin*) while phlobatannins were found absent. An anti-Mtb active fraction of *S. mombin* (SM15) was positive for Liebermann-Burchard test of triterpenoids.

Antimicrobial activities are some of the cited biological properties displayed by phenolics and triterpenoids[4-16] and these classes of natural products are well accumulated in *S. mombin* and *A. occidentale* which may be responsible for the anti-infective properties of the two plant agents.

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Table 1. Selected plants for anti-infectives

S/n	Species	Parts	FHI NO.	% Yield
1	*Spondias mombin*	Stem bark	107896	5.1
2	*Flabellaria paniculata*	Leaf	106122	8.2
3	*Anacardium occidentale*	Stem bark	107898	16.6
4	*Allium sativum*	Bulb	107900	30.0
5	*Acanthus montanus*	Leaf	107897	3.6
6	*Acanthus montanus*	Root	107897	16.4
7	*Spathodea campulata*	Stem bark	107899	12.7

Table 2. Antimicrobial activities of the screened plant extracts - Primary assay % Growth inhibition

Sample	E. coli	P. aerug	MRS	M. intracellulare	C. neoformans	C. albicans	A. fumigatus
S. mombin stem bark	39	68	0	0	0	1	5
F. paniculata leaf	11	0	0	0	0	0	0
A. occidentalis stem bark	51	63	26	4	0	35	7
A. sativum bulb	6	0	5	0	17	8	0
A. montanus leaf	19	5	0	0	23	7	2
A . montanus root	3	5	0	0	0	4	0
S. campulata stem bark	6	2	4	0	15	13	0

1**.** Sample % Growth inhibition < 50 are considered inactive Assays run @50µg/ml

Table 3. Antimicrobial activities of MeOH extract of *S. mombin* - Secondary assay

Sample/control drug	E. coli	P. aerug	MRS	M. intracellulare	C. neoformans	C. albicans	A. fumigatus
S. mombin	>50	37.32	-	-	-	>50	-
Amphotericin B	-	--	-	-	0.55	0.25	0.70
Ciprofloxacin	0.006	0.075	0.10	0.30	-	-	-

$IC_{50} \leq 20\mu g/ml$ was considered active *The concentration (µg/ml) that affords 50% inhibition of growth

Table 4. Antimicrobial activities of the VLC fractions of *Anacardium occidentale*

Sample/control drug	C. glabrata	S. aureus	MRS	*P. aeruginosa*
AOF6	>50	NA	NA	>50
AOF7	28.7	NA	NA	34.2
AOF8	12.6	NA	NA	28.3
AOF9	9.0	NA	NA	34.2
AOFI0	26.8	NA	NA	32.2
Amphotericin B	0.71	-	-	-
Ciprofloxacin	-	-	-	0.08

*The concentration (µg/ml) that affords 50% inhibition of growth
MRS= Methicillin-resitant *S. aureus;* NA= Not active

Table 5. *In vitro* activities of the screened plant extracts against *Mycobacterium tuberculosis*

S/n	Sample/control drug	Parts	% Inhibition
1	*Spondias mombin*	Stem bark	27
2	*Flabellaria paniculata*	Leaf	14
3	*Anacardium occidentale*	Stem bark	19
4	*Allium sativum*	Bulb	13
5	*Acanthus montanus*	Leaf	18
6	*Acanthus montanus*	Root	20
7	*Spathodea campulata*	Stem bark	20
	RMP		**99**
	INH		**94**

Assays run @ 64µg/ml INH= isoniazid, RMP= rifampicin

Table 6. Antimycobacterial activities of the VLC column fractions of MeOH extract of *S. mombin*

S/n	Sample	% Inhibition
1	VL1	2
2	VL2	**91**
3	VL3	69
4	VL4	32
5	VL5	26
6	VL6	27
7	VL7	27
8	VL8	13
9	VL9	24
10	VL10	8
11	VL11	22
	Drug control	
	RMP	99
	INH	94

M.TB strains: H37RV, inhibition of ≥90% was considered active, Assays run @ 64 µg/ml
INH= isoniazid, RMP= rifampicin.

Table 7. MIC Results of the active VL2 fraction of *S. mombin* stem bark against *M. tuberculosis*

Sample	% Inhibition	MIC (ug/ml)
VL2	91	61.1
Drug control		
RMP	99	0.07
INH	94	0.61
PA824	-	0.42

INH= isoniazid, RMP= rifampin, PA824= is an experimental anti-tuberculosis drug
% Inhibition assays run @ 64 µg/ml

Table 8. Antimycobacterial activities of HPLC column fractions of VLF against *M. tuberculosis*

S/n	Sample	% Inhibition
1	SM8-9	94.9
2	SM10-11	87.4
3	SM13	85.7
4	SM14	98.3
5	SM15	92.8
6	SM16	55.7
7	SM17	49.1
8	SM18	46.2
9	SM19-20	36.5
10	SM21	23.5
11	SM22	20.2
12	SMJET	60.0
	Drug control	
13	RMP	99.7
14	INH	91.4

M.TB strains: H37RV, INH= isoniazid, RMP= rifampin;
inhibition of ≥90% was considered active, Assays run @ 64µg/ml

Table 9. Results of phytochemical screening of *Spondia mombin* stem bark and *Anacardium occidentale*

Class of Natural Product	*S. mombin*	*A. occidentale*
Tannins	+++	+++
Phlobatannins	--	--
Phenols	+++	++
Flavonoids	+++	++
Saponins	+++	+++
Saponin glycosides	+++	+++
Cardenolides	+++	+++
Anthraquinones	++	+++
Alkaloids	+	++

Key: +++ = High, ++ = Moderate, + = Mild, -- = Absent

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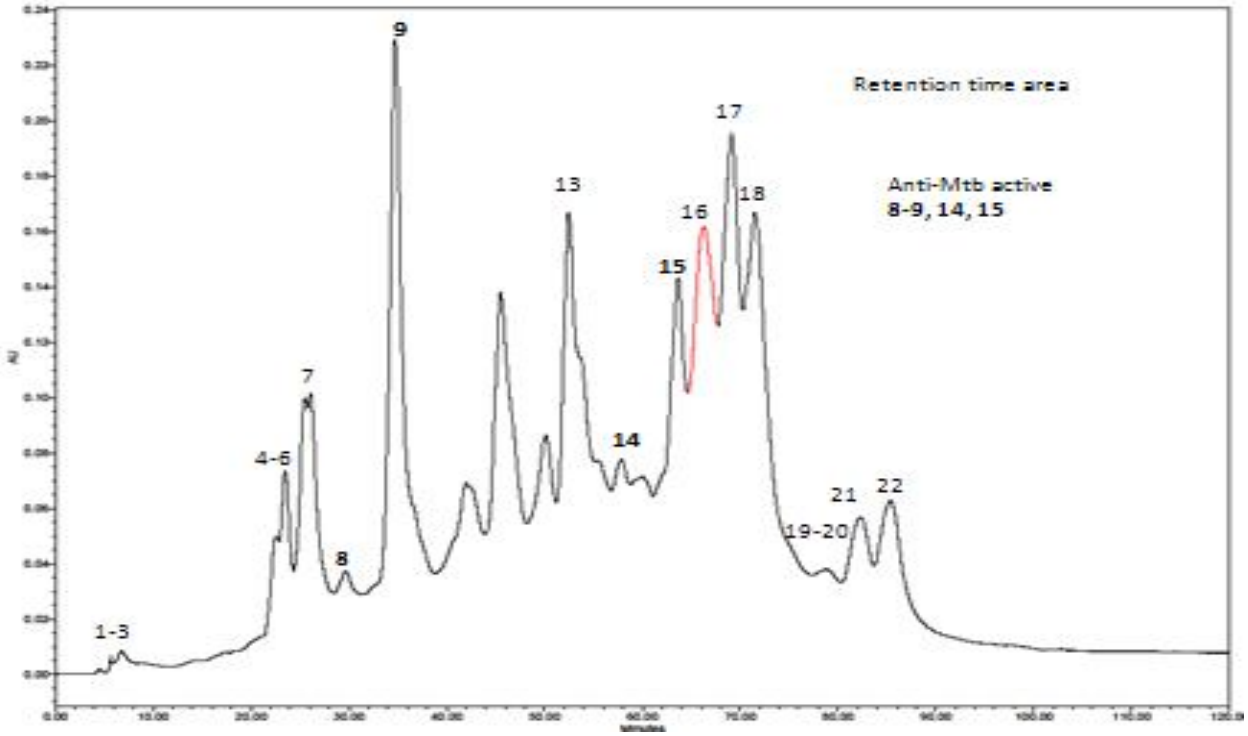

Figure.1. HPLC chromatogram of the anti-Mtb active column fractions (SM8-9, SM14 and SM15) of *Spondias mombin.*