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Contents:

Antimicrobial effects of components of the bark extract of neem (*Azadirachta indica A.* Juss)

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Preliminary phytochemical analysis showed that the leaves and bark of *A.indica* possess saponins, tannins and phenolic compounds. The antimicrobial spectrum of the aqueous, methanol and acetone extracts of leaves and bark of the plant was determined by agar diffusion method. The methanol and acetone extracts of the bark at 10mg/ml concentration showed significant antimicrobial activities against *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Pseudomonas aeruginosa, Candida albicans* and *Trichophyton mentagrophytes.* The minimum inhibitory concentration (MIC) values of the methanol extract of the bark (0.3-0.4 mg/ml) were same or lower than those of ampicillin, erythromycin and griseofulvin (0.2-0.5 mg/ml). The minimum bactericidal concentration (MBC) values (0.3-0.9 mg/ml) and the minimum fungicidal concentration values (MFC) (0.3-2.0 mg/ml) of the methanol extract were comparable to those of ampicillin, erythromycin (0.2-0.6 mg/ml) and griseofulvin(0.8-1.0 mg/ml). Isolation of antimicrobial substances from the methanol extract of the bark was studied by thin layer chromatography (TLC) developed by using benzene and methanol mixture (4:6). Two fractions (A, B) were obtained. Fraction A (the upper band in TLC plate) showed significant activity (diameter of zone 4.0-5.0 mm at 2.0 mg/ml concentration) against *C. albicans* and *T. mentagrophytes* when compared to crude extract (diameter of zone 3.0-5.0 mm at 10 mg/ml concentration). So it may be concluded that there is a scientific basis for traditional use of extracts of bark and leaves of *A. indica* in skin infection and the active principles in the extracts may be purified by using TLC method.

Key words: *A.indica, MIC, MBC, TLC. Plants-microbiology; skin-infections; neem (Azaddirachta A. Juss) products*

I. INTRODUCTION

Antimicrobial compounds are known to be present in the extracts of different tissues of some medicinal plants (Akpata and Akinrimisi, 1977). Emeruwa (1982) showed that antibacterial substances from *Carica papaya* fruits were bactericidal against *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa* and *Shigella flexnari* and these antibacterial substances were appeared to be protein in nature. Marston *et al.* (1993) isolated xanthones from the root of *Polygala nyikensis* which possess antifungal activity. Different parts of *Azadarchta indica*, a common tree plant in Nigeria, are used medicinally (Adeserrano, 1982). A study was made here to compare the antimicrobial spectrum of the extracts of *A. indica* to those of griseofulvin, erythromycin and ampicillin which are conventional antibiotics used against some pathogenic fungi and bacteria.

II. MATERIALS AND METHODS

II.1 *Materials*

The leaves and bark of *A.indica* were collected at School of Agricultural and Agricultural Technology Farm, Federal University of Technology, Minna. The plant was identified and authenticated by Dr. M.I.S. Ezenwa, School of agriculture and Agricultural Technology, FUT, Minna (Keay and Onochie, 1960 and Ivens *et al.*, 1978). After collection, leaves and bark of *A. indica* were sun dried to a constant weight over seven days and then crushed into powder using a blender (National MX 391M;

Matushita Electric). The powdered samples (50-60 gm) were separately extracted with 200 ml water, acetone and methanol using Soxhlet in vacuo to concentrate the extracts. The concentrated extracts were kept refrigerated pending further use. Each of the dried extracts was separately reconstituted in minimum amount of the extracting solvent and further diluted with glycerol to get the desired concentrations (10 mg/ml for susceptibility test and 0.2 –2.0 mg/ml for MIC and MMC tests). The standard antibiotics used for MIC and MMC determination were erythromycin, griseofulvin and ampicillin. The antibiotic solutions were made using sterile distilled water.

II.2 *Organisms*

The fungi used were *Candida albicans* and *Trichophyton mentagrophytes* while the bacteria used were *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli* and *Pseudomonas aeruginosa*. All the cultures were obtained from National Veterinary Research Institute, Vom, Jos, Nigeria. The cultures were maintained on appropriate media- Potato dextrose agar (PDA) for fungi, MacConkey agar (MA) for *E. coli* and Nutrient agar (NA) for other bacterial isolates.

II.3 *Preliminary phytochemical analysis*

The analysis was done to screen the plant parts for phytochemicals using the method of Sofowora (1982). The phytochemicals tested for include sesquiterpenes, anthraquinones, phenolic compounds, tannins and saponins.

II.4 *Determination of antimicrobial activity of the extracts*

The fungal isolates were inoculated into PD broth and the cultures obtained after 48 hours were adjusted to a concentration of 10^6 cells per ml by serial dilution and used for the susceptibility test. The bacterial isolates except *E. coli* were inoculated into nutrient broth and the cultures after 24 hours were adjusted to 10^6 cells per ml. In case of *E. coli*, the same procedure was followed except that MacConkey agar surfaces in plates were inoculated with the respective cell suspension and the extracts were tested for their effect on the organisms by cup plate method of Garrod *et al.* (1981).

II.5 *Determination of minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) of the methanol extract of the bark*

The methanol extract of the bark of *A. indica* which showed significant antimicrobial activity against *T. mentagrophytes, C. albicans, S. aureus, S. pyogenes* and *P. aeruginosa* was selected for MIC and MMC test.

a)MIC test

0.5 ml of varying concentrations of the reconstituted extract was added to two ml broth (potato dextrose for fungi, nutrient broth for bacteria except in case of *E. coli* where MacConkey broth was used). Then the broth containing extract was inoculated with a loopful of the organism at 10^6 cells/ml concentration. For each of the selected bacterial and fungal species the same procedure was followed using either the extract or standard antibiotic. After incubation (for fungi, 48 hours and for bacteria, 24 hours) the highest dilution of the extract or standard antibiotic that prevented visible growth of the test organism was taken as the MIC (Rotimi and Mosadonmi, 1987).

b)*MMC (MFC and MBC) test*

For each of the test tubes in the MIC determination which did not show any visible growth, a loopful of broth was aseptically inoculated on sterile agar surface by the streak method. After incubation, the minimum microbicidal concentration (MMC) was determined by the highest dilution at which there was no visible growth on the media.

II.6 *Isolation of antimicrobial substances from the methanol extract of the bark*

Thin layer chromatography was used for this purpose.Glass TLC plates (20x5 cm), each coated with 25 g of silica gel, were used. Different solvent mixtures were used for the separation of the antimicrobial components in the extract. The solvent mixtures used were (a) chloroform:

Methanol (5:5); (b) chloroform : benzene (5:5); (c) petroleum ether : methanol (5:5) (d) petroleum ether : chloroform (5:5) ; (e) petroleum ether : benzene (5:5); (f) benzene : methanol : water (1:4:5). Using a capillary tube, 10 mg/ml solution of the methanol extract of the bark was spotted on a TLC plate. The plate was then placed in a chromatographic jar containing 30 ml of appropriate solvent mixture. The plate was run for 90 minutes, removed, allowed to dry and put into an iodine jar for 2 minutes to locate the spots. Benzene : methanol solvent mixtures (5:5, 4:6, 3:7) were further used to ensure proper separation .The chromatography procedure was repeated for a broad band (5 ml of 10 mg/ml bark extract) using benzene: methanol (4:6) solvent mixture. The resulting bands (fraction A and fraction (B) and the residue (C) at the base were cut out and each washed with 10 ml of ethanol in a test tube. Using a capillary tube, 0.5 ml of the filtrate was applied on mini TLC plates (microscopic slides coated with 2 g of silica gel) to ensure the purity of these bands.The remaining filtrate obtained from the resultant bands were dried and then reconstituted with 5 ml glycerol. The reconstituted solutions were kept for future use (Vogel, 1980).

II.7 *Determination of the antimicrobial activity of the isolated bands from TLC plate*

The antimicrobial activity of the reconstituted fractions (A, B and C) at 2.0 mg/ml against some selected microorganisms was then determined by cup plate method and compared with that of the crude extract.

III. RESULTS

The phytochemical analysis showed the presence of phenolic compounds, tannins and saponins in both bark and leaves of *A. indica*. The results are shown in Table 1.Table 2 shows the amount of samples obtained by using 100 g of the different plant parts. The aqueous extracts of all plant parts did not exhibit any antimicrobial activity against the test organisms at the concentrations tested. The zones of inhibition produced by acetone and methanol extracts of the leaves against the organisms were not significant. However, they were quite significant for the bark (Table 3). The MIC and MMC of the methanol extracts of the bark were comparable to those of some antibiotics generally used for the treatment of infections caused by these organisms. The results are shown in Table 4. The diameter of zones produced by fraction A at 2.0 mg/ml was comparable to the diameter of zones produced by crude extract at 10.0 mg/ml against *S. aureus, S. pyogenes, P. aeruginosa, C. albicans* & *T. mentagrophytes*. The results are shown in Table 5.

IV. DISCUSSION

The preliminary phytochemical analysis showed that the leaves and bark of *A. indica* contain phenolic compounds and tannins.These phytochemical compounds have been reported by Farnsworth (1990) to inhibit bacterial growth and are capable of protecting certain plants against bacterial infections. The aqueous extracts did not produce any measurable zone of inhibition against the organisms at the tested concentration. When plant materials are ground in water or plant cells are damaged a number of phenolases and hydrolases are released. These enzymes might modulate the activity of active components in the extract. Also it is possible that some phytochemical components are not extracted with water (Akpata and Akinrimisi, 1977). The methanol and acetone extracts of the bark produced significant antimicrobial activity against *S. aureus, S. pyogenes, P.aeruginosa, C. albicans* and *T. mentagrophytes*. The solubility of tannins and phenols in methanol might have been the factor responsible for the significant antimicrobial activity (Wolinsky and Sote, 1982). The extracts of the leaves did not possess any antimicrobial properties at 10 mg/ml against the organisms probably because substances extracted from the leaves had no antimicrobial properties. The separation of the methanol extract of the bark into two distinct components (Fractions A and B) by thin layer chromatography shows that this plant part contains more than one active component. The varying susceptibility of each bacterial and fungal species may be a function of the available binding sites on the bacterial and fungal cells, these are probably surface structures like proteins. Tannins have been shown to form

TABLE 1: Preliminary phytochemical analysis of bark and leaves of *A. indica*

Phytochemical components	Bark	leaves
Sesquiterpenes	-	-
Anthraquinones	-	-
Phenolic compouns (including tannins)	++	++
Saponins	++	++

'++' indicates the presence of component in high amount, '-' indicates the absence of component

TABLE 2: Crude extracts obtained from leaves and bark of *A. indica* using water, methanol and acetone (mean ± SEM; n=3).

Extracting medium	*A. indica* (plant part)	extract (g/100g of plant part)
Water	leaf	7.0±0.21
	bark	8.0±0.17
Methanol	leaf	6.4±0.21
	bark	6.0±0.21
Acetone	leaf	3.2±0.08
	bark	4.0±0.20

TABLE 3: Zones of inhibition produced by acetone and methanol extracts of parts of *A. indica*

plant extracts	Zones of inhibition(mm)					
	A	B	C	D	E	F
Methanol extract						
leaf	1.0	1.0	1.0	-	-	-
bark	4.0	5.0	3.0	4.0	4.0	2.0
Acetone extract						
leaf	-	-	-	-	-	-
bark	2.0	2.0	2.0	3.0	2.0	1.0
Aqueous extract						
leaf	-	-	-	-	-	-
bark	-	-	-	-	-	-

Key: A- *S. aureus*, B- *S. pyogenes*, C- *P.aeruginosa*, D- *T. mentagrophytes*, E- *C. albicans*, F- *E.coli.*
"-" in Table 3 means no activity.

TABLE 4: Determination of MIC and MMC of the methanol extract of the bark of *A. indica* and some standard antibiotics.

Extract & Antibiotics	MIC (mg/ml)					MMC(mg/ml)				
	A	B	C	D	E	A	B	C	D	E
Extract	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	2.0	0.9
Erythromycin	0.3	0.2	0.4	NA	NA	0.6	0.6	0.2	NA	NA
Ampicillin	0.3	0.3	0.2	NA	NA	0.5	0.4	0.3	NA	NA
Griseofulvin	NA	NA	NA	0.3	0.4	NA	NA	NA	1.0	0.7

Key: A- *S.aureus*, B- *S. pyogenes*, C- *P.aeruginosa*, D- *C. albicans*, E- *T.mentagrophytes*, NA- non-applicable.

TABLE 5: Comparison of antimicrobial activity of the separated fractions A, B and the residue C and the crude extract

Fractions & Crude extract	Diameter of zone (mm)				
	A	B	C	D	E
Fraction A (2.0 mg/ml)	4.0	5.0	3.0	4.0	4.0
	-	4.0	2.0	2.0	3.0
Fraction B (2.0 mg/ml)	-	4.0	2.0	2.0	3.0
Residue C (2.0 mg/ml)	-	-	-	-	-
Crude extract (10.0 mg/ml)	4.0	5.0	3.0	3.5	4.0

Key: A- *S. aureus*, B- *S. pyogenes*, C- *P.aeruginosa*, D- *C. albicans*, E- *T. mentagrophytes*

irreversible complexes with proline rich proteins. This could interfere with cell function. Table 4 showed that the MIC and MMC of the methanol extract of the bark was comparable to those of erythromycin, griseofulvin and ampicillin. Griseofulvin, an antifungal drug, inhibits cell division and is active against dermatophytes *Trichophyton* and *Candida* sp. Erythromycin and ampicillin are broad spectrum antibiotics.

Ampicillin is known to inhibit sensitive strains of *P. mirabilis* and *E. coli*. Erythromycin act by affecting ribosome function by selective binding to bacterial 50S subunit (Russel and Hugo,1977).The comparable activity of the upper band (A), lower band (B), residue (C) at the base and the crude extract shows that the antimicrobial activity of the fraction A was quite high (zones of inhibition 4-5 mm at 2.0 mg/ml concentration) compared to the crude one (zones of inhibition 4-5 mm at 10.0 mg/ml concentration). Purification may have eliminated substances that reduce the activity of the active components. Therefore, the information obtained from this study have proved scientifically that the components of the bark of *A. indica* possess significant antimicrobial activity against *S. aureus, S. pyogenes, P. aeruginosa, C. albicans* and *T. mentagrophytes.*

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