Antimicrobial Activities Of Methanolic Extracts Of *Trema guineensis* (Schumm And Thorn) And *Morinda lucida* Benth Used In Nigerian Herbal Medicinal Practice

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Abstract

Extracts obtained from the leaves, stem-bark and roots of two ethnomedicinal plants: *Morinda lucida* Benth and *Trema guineensis* Schumm and Thorn were screened for antimicrobial activities against eleven test organisms (five bacteria and six fungi) namely: *Escherichia coli*, *Staphylococcus aureus* , *Pseudomonas aeruginosa* ,*Salmonella typhi* ,*Bacillus subtilis* ,*Candida albicans* ,*Trichopyton tonsurans, T. rubrum* ,*T. mentagrophytes, Basidobolus haptosporus* and *Aspergillus niger.* The agar plate, cup-plate and broth dilution methods were used for the antimicrobial analysis. The minimal inhibitory concentrations (MIC) and the minimum biocidal concentrations (MBC) of the extracts were determined in vitro.The results showed that most of these extracts posses in vitro antimicrobial activities against the tested clinical isolates of bacteria and fungi. These antimicrobial activities have been attributed to the presence of some or all of the following secondary plant metabolites: alkaloids, tannins, saponins, flavonoids, steroids and glycosides.

Key words: Plant extracts, antimicrobial activities, medicinal plants, methanol.

Introduction

The use of plants in Nigerian traditional medicinal practice as either extracts or infusion is a widespread practice in the treatment of common infections (Iwu, 1982). There are ethnobotanical texts describing the species most frequently used by the population to cure respiratory, urinary, skin, gastrointestinal and other types of infections (Iwu, 1982, 1993). However, there are few reports on scientific studies confirming the possible antimicrobial potencies of a great number of these plants .The present report is a series of studies performed in our laboratory for screening plants with acclaimed antibiotic properties. The two botanical species used in this study belong to different families of plants and are reported to be used for illnesses recognised by traditional medicine, and in which according to the symptomatology described, includes gastrointestinal, respiratory and skin infections. The information about the use of these plants in Nigerian traditional medicinal practice was complimented with ethno botanical data obtained by interviews with (people in the rural areas especially) herbalists who always use these plants to treat their patients. The aim of this study therefore is to scientifically establish the antimicrobial potentials of these two species of plants.

Materials and Methods

Plant collection and identification: Fresh leaves, stem-bark and roots of *Morinda lucida* (Rubiaceae) and *Trema guineensis* (Ulmaceae) were collected from Olido, Igbo-Eze-North local government area of Enugu state Nigeria. They were identified by a plant taxonomist in the Department of Botany, University of Nigeria Nsukka. Voucher specimens were deposited at the Herbarium of the Department. The plant parts were sun-dried ground into fine powder and stored for one week before extraction.

Extraction of active principle: About 100 gm of each of the powered plant extract was separately extracted by macerating in 500 ml of methanol for 2-4 days. The solutions were subsequently filtered using Whatmann filter paper. The filtrate was evaporated to dryness by forced air pressure. A 500 mg quantity of each methanolic extract was reconstituted with 5 ml of 10 % dimethylsulphoxide to achieve a concentration of 100 mg/ml. The liquid extracts were then stored at 4 ^{0}C in sterile bottles and used subsequently for other tests.

Test microorganisms: Stock cultures of some clinical isolates of *S. aureus, P. aeruginosa, E. coli, S. typhi,* and *B. subtilis* were collected from the culture collection center, Department of Microbiology, University of Nigeria, Nsukka. Also stock cultures of *A. niger, T. tonsurans, T. mentagrophytes, B. haptosporus*, and *C. albicans* were obtained from the Medical mycology laboratory, Department of Microbiology, UNN.

Preparation of inocula: All fungal isolates were inoculated onto sabaraud glucose agar (SDA) slants and incubated at 28±1^{0}C for 4 days to obtain young actively growing cultures consisting of mycelia and conidia/ arthrospores / blastospores. The fungal growth on each agar slant was aseptically scrapped off and placed in a sterile bottle containing 10 ml of sterile saline and shaken vigorously using a vortex mixer until the fungal filaments were broken into small colony forming units (CFU's). Each suspension was standardized using a haemocytometer to obtain 10^4 to 10^6 cfu/ml and used as the inoculum and tested by streaking a loop full of each suspension onto SDA plates. For the bacterial isolates, the inocula were prepared according to the methods of National Committee for Clinical Laboratory Standards (NCCLS, 1993).

Agar plate method: Eleven Petri dishes were set up for each organism and 2 ml of each plant extract was added serially to 10 plates. Approximately, 18 ml of molten SDA (for the fungi/bacteria respectively) was poured onto each of the 10 plates containing plant extracts as well as the eleventh plate, which contained no plant extract and thus served as the control. The extracts and the molten agar were thoroughly mixed by rotating the plates before allowing the agar to set. A loop full of the inoculum to be tested was then inoculated onto each of the 11 plates and all the plates finally incubated at 28° C for the fungi and 35° C for the bacteria. As soon as growth was observed in the control plate, the other 10 plates were checked for the presence or absence of growth.

Broth dilution method: The macro broth method of Shadomy and Espineal Ingroff (1984) was used. For detecting each isolate, a total of eleven sterile test tubes were placed on a test tube rack. One ml of each plant extract was placed on the first tube while 4.5

ml of sabaraud glucose broth (SDB) for fungi and nutrient broth (NB) for bacteria was added to each of the remaining ten tubes using a sterile pipette. Each plant extract was then serially diluted two- fold; 0.5 ml of extract was pipetted from the first into the second tube and after shaking thoroughly, the same quantity was transferred to the third tube and so on until the tenth tube. The eleventh tube served as the control tube. Each tube was then inoculated with 20µl of the standardized inoculum of each test organism and then incubated for 7 days at 28° C for fungi and two days at 37° C for bacteria. Similar dilutions were also made with an antifungal, ketoconazole and an antibacterial,ampicillin, which served as the standard. The minimum inhibitory concentration (MIC) of each plant extract was determined. This was the lowest concentration at which no visible growth was observed as compared with the growth in the control tube. Subsequently, those tubes showing no growth (without turbidity) were vigorously shaken and 0.1 ml of the SDB/NB withdrawn and separately introduced onto a freshly prepared SDA and NA plates respectively and incubated for 3-7 days at room temperature. The minimum biocidal concentration (MBC) of each plant extract was determined. This was the concentration of the extracts in the tubes with the highest dilution that gave no growth on the agar plates after incubation.

Phytochemical and Proximate Analysis: Phytochemical tests were done by established methods as described by Iwu (1989) and Iwu and Chiori (1984). Proximate analysis was as described by the Association of Official Agricultural Chemists (AOAC) (1975).

Chromatographic separation of the TLC bands: Two of the plant extracts with significant activity; *Morinda lucida* root-extract possessing mainly antifungal activities and *Trema guineensis* stem- bark extract having mostly antibacterial activity were each separated into different constituents by thin layer chromatography (TLC) using chloroform/methanol in the ratio of 9:1 v/v and 10:1 v/v respectively. Thereafter, 0.005 g of each of the bands aseptically scrapped with a sterile spatula was dissolved in 5 ml of dimethylsulphuroxide (DMSO) to achieve a 5 mg/ml dilution and used for the antimicrobial tests using the cup- plate method as described by Iwu and Chiori (1984). DMSO was used as the control. Individual bands of *T. guineensis* stem-bark extract were combined and tested for antibacterial effects when the individual bands failed to show any activity singly.

Ultraviolet spectroscopy of the TLC bands: The resultant powder from each of the bands of the chromatographic separation was dissolved in methanol. About 10 milligram of the powder was dissolved and made up to 100 ml and a little quantity of this was transferred into a silica cell. Another matched silica cell containing equal volume of only methanol was also prepared and both cells were placed in the appropriate positions in a Vis/UV Spectrophotometer SP 8-100 of Pye Unicam, which automatically showed the reading in terms of absorbance and absorbance wavelengths. The process was repeated for each of the chromatographic bands that showed antimicrobial activity.

Results and Discussion

The result of the preliminary screening (Table 1) showed that all the plant

Table 1: Inhibitory concentration (mg/ml) of the plant extracts against fungal isolates

Plant extract in agar medium 1:10 dilution	*Suppression of fungal growth in days*					
	A. niger	*T. tonsurans*	*T. rubrum*	*T. mentagrophytes*	*B. haptosporus*	*C. albicans*
T .guineensis leaf extract (T1)	4	>12	>12	>12	4	0
T. guineensis stem-bark extract (T2)	4	>12	>12	10	4	>12
T. guineensis root extract (T3)	4	>12	>12	>12	4	4
M. lucida leaf extract (M1)	7	>12	>12	>12	6	>12
M. lucida stem-bark extract (M2)	>12	>12	>12	>12	>12	10
M. lucida root extract (M3)	>12	>12	>12	>12	>12	9
Ketoconazole	>12	>12	>12	>12	>12	>12

Table 2: Inhibitory concentration (mg/ml) of the plant extracts against bacterial isolates

Plant extract in agar medium 1:10 dilution.	*Suppression of bacterial growth in days.*				
	S. aureus	*P. aeruginosa*	*E. coli*	*S. typhi*	*B. subtilis*
T .guineensis leaf extract (T1)	0	1	1	1	1
T. guineensis stem-bark extract(T2)	11	10	>12	>12	8
T. guineensis root extract (T3)	5	4	0	0	0
M. lucida leaf extract (M1)	4	4	5	6	0
M. lucida stem-bark extract (M2)	0	1	1	2	1
M. lucida root extract (M3)	0	0	1	1	0
Ampicillin	0	>12	>12	0	>12

Table 3: Minimum inhibitory concentration (MIC) (mg/ml) of the plant extracts for the fungal isolates

Plant extracts	Suppression of fungal growth in days.					
	A. niger	*T. tonsurans*	*T. rubrum*	*T. mentagrophytes*	*B. haptosporus*	*C. albicans*
T. guineensis stem-bark extract(T2)	-	12.5	25	12.5	-	-
T. guineensis root extract (T3)	-	12.5	12.5	12.5	-	-
M. lucida leaf extract (M1)	100	6.25	12.5	12.5	-	3.13
M. lucida stem-bark extract (M2)	12.5	6.25	12.5	12.5	25	6.25
M. lucida root extract (M3)	12.5	3.13	6.25	6.25	12.5	50
Ketoconazole	0.625	0.078	0.078	0.078	0.63	0.078

Table 4: Minimum inhibitory concentration (MIC) (mg/ml) of the plant extracts for the bacterial isolates

Plant extracts	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. typhi*	*B. subtilis*
T. guineensis stem-bark extract(T2)	1.56	3.13	12.5	3.13	12.50
T. guineensis root extract (T3)	12.50	-	100	-	-
M. lucida leaf extract (M1)	6.25	3.13	12.5	3.13	-
Ampicillin	Resistant	0.002	0.02	Resistant	0.02

Key: -, not determined inhibition < 6 days

Table 5: Minimum biocidal concentration (MBC) of the plant extracts (mg/ml) against the fungal isolates

Plant extracts	*A. niger*	*T. tonsurans*	*T. rubrum*	*T. mentagrophytes*	*B. haptosporus*	*C. albicans*
T. guineensis (T2)	-	50	50	25	-	25
T. guineensis root extract (T3)	-	25	25	50	-	-
M. lucida leaf extract (M1)	100	25	25	25	-	12.5
M. lucida stem-bark extract (M2)	25	25	25	25	50	12.5
M. lucida root extract (M3)	25	12.5	12.5	12.5	50	50

Table 6: Minimum biocidal concentration (MBC) (mg/ml) against bacterial isolates

Plant extracts	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. typhi*
T. guineensis root extract (T3)	50	-	100	-
M. lucida leaf extract (M1)	25	12.5	25	12.5

extracts had inhibitory activities on all the fungal isolates tested. Inhibition however was more pronounced with the dermatophytes of the *Trichophyton* species than in *A. niger; B. haptosporus* and *C. albicans. M. lucida* extract generally appeared to exhibit greater inhibitory effect than *T. guineensis* because it prevented the growth of all the six species of fungi tested for longer periods. The MIC result for the extracts on the fungal isolates is shown in Table 3. The root extracts of *M. lucida* exhibited the least MIC value of 3.125 mg/ml for *T. tonsurans* while the leaf extract had an MIC value of 3.125 mg/ml on *C. albicans* .The standard conventional antibiotic, ketoconazole exhibited MIC values lower than any of the extracts on the fungal isolates. (This may be because ketoconazole is a pure substance).

The screening for antibacterial activities (Table 2) showed that stem-bark and leaf extracts of T. *guineensis* and *M. lucida* respectively performed better than the roots obtained from same species of plants. It is noteworthy that ampicilin-resistant species of *S. aureus* and *S. typhi* were inhibited by some of these plants extracts. This could offer a better solution to problems posed by ampicillin-resistant strains of bacteria in clinical therapy. Although there was a previous study (Iwu, 1993) on *Morinda lucida* plant extracts, we are not aware of any such studies carried out with a view to screening the antimicrobial potentials of the methanolic extracts. Considering that in this study only crude methanolic extracts were employed, we considered a strong response to exist when the extracts produced an effect at concentrations of 25 mg/ml or less for fungal isolates and between 3.125 - 25 mg/ml for the bacterial isolates (Table 4). The results on fungal isolates compare favourably with the in vitro effects of some conventional drugs used to treat zygomycotic infections (Yangco *et al*, 1984; Kelly *et al*, 1980). All the extracts showed MIC levels that fell within the 'active range' on *Trichophyton* species. Extracts from *Morinda* were predominantly antifungal in nature in contrast to extracts from *Trema, which* were broader spectrum in nature. However, the result showed that extracts from *Trema* were more antibacterial than antifungal. The case for *Morinda* appears to be directly

opposite to *Trema*. Results of the minimum biocidal concentrations are shown in Tables 5 and 6 for bacterial and fungal isolates respectively .The MBC values of those extracts that did not show a strong response on the isolates were not determined. Some compounds already established to have a wide range of antimicrobial and pharmacological activities were found to be contained in these extracts (Table 7 and 8).

Table 7: Proximate analysis of the plant extracts

Plant extracts	*Crude protein*	*Oil*	*Ash*	*Fibre*
T. guineensis	1.23	5.26	2.00	0.52
M. Lucida	0.66	2.00	3.00	0.48

Table 8: Phytochemical analyses of the plants

Tested for	M. lucida	T. guineensis
Alkaloids	+	-
Tannins	+	-
Saponins	+	+
Protein	+	+
Steriodal aglycone	+	+
Cardiac glycosides	+	+
Anthracene glycosides	+	-
Cyanogenic glycosides	+	+

Key: +, present; -, absent.

Table 9: Antimicrobial activities of the TLC bands of *M. lucida*

TLC bands	*T. rubrum*	*C. albicans*	*T. tonsurans*	*S. aureus*	*E. coli*	*S. typhi*
M1	+	+	+	NT	NT	NT
M2	+	++	+	NT	NT	NT
M3	+	+	+	NT	NT	NT
M4	++	+	++	NT	NT	NT

Thus, their antimicrobial activities are either due to the single or combined effects of these chemical compounds. (Cimanga *et al*, 1991; Paulo *et al* 1994; Oyekan and Okafor, 1989). Some of the compounds like alkaloids, tannins and saponins from plants have been associated with antimicrobial activity singly (Trease and Evans, 1983, Frel *et al*, 1998). An attempt to characterize the antimicrobial constituents in two of the plant extracts were carried out in a preliminary study via a bioassay guided separation employing thin layer chromatography (TLC) . The antimicrobial activities of the TLC bands of these extracts are shown in Tables IX & X. The inability of two out of the eight bands obtained from *T. guineensis* stem-bark extract to inhibit any of the susceptible organisms which in (a combined form of) the whole extract was inhibitory to such organism were of interest. However, pair combination of these inactive bands showed activity suggesting a possible synergy between these two bands. This is in line with the earlier observations (Dhar *et al*; 1968) with respect to the loss of activity by individual bands of a hitherto antimicrobial plant extract upon fractionation. Other possible factors such as elimination of inorganic constituents during fractionation which stabilize and activate potentially antimicrobial substances in the plant extracts and loss of some labile constituents of the extracts during separation could have led to a loss of activity in these two fractions of *T. guineensis*. Unlike *T. guineensis* stem-bark extract, all the TLC bands from *M. lucida* root extract showed antimicrobial activity against the organisms initially susceptible to the whole extract. The maximum absorption wavelength of between 232 nm and 272 nm for *M. lucida root* extract and between 228 nm and 279 nm for *T. guineensis* stem-bark extract (Fig. III & IV) obtained after ultraviolet spectroscopy (UV) of the active bands

Table 10: Antimicrobial activities of the TLC bands of T. *guineensis*

Plant extracts	*S. aureus*	*E. coli*	*C. albicans*	*S. typhi*	*T. tonsurans*	*T. rubrum*
T^1	++	++	+	+	+	+
T^2	+	+	+	+	+	+
T^3	+	+	+	+	+	+
T^4	+	+	+	+	+	+
T^5	+	+	+	+	+	+
T^6	+	+	+	+	+	+
T^7	-	-	-	-	-	-
T^8	-	-	-	-	-	-
$T^7 + T^8$	++	+	+	+	+	+

Key (For Tables IX & X) Inhibition zone diameter (IZD) 8-11mm, +; 12-14mm, ++; No inhibition -; Not tested, NT. All figures rounded up to the nearest whole number

will serve as a steeping stone to further studies. Specific compounds could not therefore be identified as contained in these plant extracts under study. Huneck (1968) pointed out that UV-spectroscopy is an excellent aid for assigning an unknown lichen substance to its corresponding group just like Scoth (1964) earlier indicated this for other groups of plants. Thus, peaks indicated by different TLC bands of the two plant extracts assayed by UV spectroscopy could be enough to assign groups to these bands but popular opinion will disagree with this. Instead, they are of the view that other fractionation /purification procedures involving other chromatographic techniques will be necessary to confirm the result from UV – spectra.

Hence future research in this direction will solve this problem by identifying specific constituent in each band obtained after UV spectroscopy .The similarity in the UV peaks among some of the TLC fraction 4 and 5 with similar peaks at 308 λ and 228λ was probably due to a spill over from the proceeding fraction.

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