Afr. J. Traditional, **Complementary and Alternative Medicines**
www.africanethnomedicines.net

Research Paper

ISSN 0189-6016©2009

ANTIBACTERIAL ACTIVITY OF *TABERNAEMONTANA STAPFIANA* BRITTEN (APOCYNACEAE) EXTRACTS

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Abstract

Antibacterial and phytochemical screening of methanolic, sequential extracts (hexane, dichloromethane, ethyl acetate and methanol) and alkaloid rich fractions of *Tabernaemontana stapfiana* Britten was carried out. The phytochemical screening showed the presence of alkaloids, flavonoids, coumarins, tannins and saponins that have been associated with antimicrobial activity. The stem and root bark methanolic extracts showed good activity against the bacterial strains used including the multiple drug resistant *Staphylococcus aureus* strain with minimum inhibitory concentrations ranging from 15.6 to 500 µg/ml and minimum bactericidal concentrations ranging from 31.25 to 500 µg/ml. The sequential extracts of the root and stem bark had high antimicrobial activity with minimum inhibitory concentrations (MICs) ranging between 3.9 and 250 µg/ml and minimum bactericidal concentrations (MBCs) ranging between 7.8 and 500 µg/ml against the tested microorganisms. The dichloromethane extract of the alkaloid rich fractions however exhibited reduced antibacterial activities as compared to methanol and sequential extracts but the dichloromethane:methanol (4:1) mixture showed high activity with MICs ranging between 15.6 and 250 µg/ml. These antibacterial efficacy studies suggest that *Tabernaemontana stapfiana* Britten could be a source of antibacterial agents.

Key words: *Tabernaemontana stapfiana*; Antibacterial; MICs; MBCs.

Introduction

The resistance of bacteria and fungi to antimicrobial agents is a world-wide medical problem (WHO, 2004). Currently there are emerging multiple drug resistant (MDR) typhoid outbreaks, especially in developing countries. Tuberculosis is also becoming an unstoppable epidemic due to MDR strains that have emerged. In addition to these, there are many bacteria that have developed drug resistance including *Staphylococcus aureus* and most of the Enterobacteriaceae, such as *Klebsiella pneumoniae* (WHO, 2004). More than 70% of the bacteria causing infections are resistant to at least one of the drugs commonly used to treat them (WHO, 2004). This situation has been worsened by HIV/AIDS pandemic, poverty, an upsurge of new and re-emerging infectious diseases, high costs and side effects of available drugs (Humber, 2002). This has resulted in increased severity of infectious diseases and high mortality rates from certain infections. All these have necessitated studies on potential sources of effective, safe and

cheap antimicrobial alternatives, and plants are one of these sources that have not been exhaustively utilized (Thangadurai et al., 2004).

The genus *Tabernaemontana* comprises of about 100 species distributed throughout the tropical regions of the world (Grover, et al., 2002). *Tabernaemontana stapfiana* Britten is a tree of up to 21 meters high and commonly found in disturbed forests (Beentje, 1994). Information obtained from Keiyo traditional medical practitioners in Kenya indicate that this plant is locally known as "mobonet" and its roots and stem barks are used in the treatment of abdominal problems, sexually transmitted infections and upper respiratory tract infections (Omino and Kokwaro, 1993). The fruits of this plant are edible.

There has been no systematic study on *T. stapfiana* with no information reported on chemical and biological studies. In this study, the methanolic extracts of leaves, root, and stem barks and the sequential (hexane, dichloromethane, ethyl acetate and methanol) extracts were assayed for antibacterial activity and screened for phytochemicals. The alkaloid rich fractions were also assayed against selected bacterial test organisms.

Materials and methods

Plant material

Plant samples which included the stem bark, root bark, fruits and leaves were collected from Kaptagat Forest in Keiyo district about 40 Km East of Eldoret town, Kenya in March 2006. The sample was authenticated by a plant taxonomist, Mr Lucas Karimi of Kenyatta University. A voucher specimen (ER/001/06) was deposited at the herbarium of the Botany Department of Kenyatta University. The samples were air-dried in the shade and ground into a fine powder using a grinding mill (Christy and Norris Ltd, England). The ground materials (100 g) were soaked in 250 ml of methanol for 72 h with occasional swirling. The solutions were there after filtered and concentrated using a rotary evaporator at 45^0C under reduced pressure. This yielded gummy extracts.

The powdered leaves, root and stem barks (100 g) of *T. stapfiana* were each sequentially extracted three times with solvents of increasing polarity starting with hexane, dichloromethane, ethyl acetate and methanol for 48 h, with occasional swirling to ensure thorough extraction. The filtrates were concentrated at 45°C under reduced pressure to obtain gummy extracts.

The methanol (MeOH) extract of *T. stapfiana* was dissolved in distilled water and acidified with 5% HCl to pH 3-4 and the acidic mixture filtered. The solution was then partitioned with diethyl ether several times to remove the neutral materials. The resulting acidic solution was basified with 5% NH_4OH solution to pH 8-9. The basic solution was extracted with dichloromethane (CH_2Cl_2) several times and, finally with CH_2Cl_2–MeOH (4:1) until the solution was free of alkaloids (Machocho, 2000). The extract was combined and concentrated by evaporating under vacuum at 45^oC to yield (brown) gummy extracts.

Bioassay of culture strains

The microorganisms used in the bioassay tests were obtained from Kenya National Public Health Laboratories (KNPHL) and Kenya Medical Research Institute (KEMRI). These included standard and local clinical isolates. The microorganisms that were used included: three Gram-positive bacteria; *Staphylococcus aureus* (ATCC 20591), *Enterococcus faecalis* (ATCC 29212) and *Bacillus subtilis* (Type K [11]), three Gram-negative bacteria; *Salmonella typhi* (Type K [1]), *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella pneumoniae* (KNPHL 002). The Multiple Drug Resistant (MDR) strains used were *Staphylococcus aureus* (KNPHL 003) and *Klebsiella pneumoniae* (KNPHL 001). The test strains of bacteria were kept refrigerated (4^oC) on Muller-Hinton (Merck, Germany) agar slants during the experimental period and were sub cultured and incubated for 24 h at 37^oC then tested biochemically for purity before use as described by Elgayyar et al. (2000).

Screening for antibacterial activity

Antibacterial efficacies were tested using the disc diffusion method (Elgayyar et al., 2000). A solution of each extract was prepared by dissolving 200 mg in 1 ml of methanol and 10 µl of the solution were aseptically dispensed onto sterilized 6 mm filter paper discs and dried (2 mg/disc). The Muller-Hinton was used in the culture of bacteria and the medium was prepared using the manufacturers' instructions.

Each plate was inoculated with 0.1 ml of a bacterial culture directly from the 24 h broth culture diluted to match 0.5 MacFarland standard (10^8 Colony Forming Units (CFU)/ml). Inoculums on the plates were aseptically spread using a sterile glass rod to avoid contamination and allowed to stand for 2-3 mins in a refrigerator at 4^0C. The

discs loaded with the extracts were then placed onto the seeded plates. The bacterial cultures were incubated at 37^0C for 24hr. After the incubation period, the zones of inhibition were measured and recorded in mm as described by Elgayyar et al. (2000). Negative controls were done by using sterile 6mm discs soaked in 10 µl of methanol and dried.

Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC)

The active extracts from the antibacterial screening were tested for Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The MICs of the active extracts were determined using a two-fold serial dilution method in a peptone water solution for bacteria. Each tube was then inoculated with 0.1 ml of bacteria directly from the 24h broth cultures adjusted to match 0.5 MacFarland standards to give a final extract concentration of between 0.975 and 8000 µg/ml. The cultures were incubated at 37^0C for 24 h. The first tube of each inoculum showing no growth was taken as the MIC.

The MBC were determined by sub-culturing 0.1 ml of all the tubes showing no growth on Nutrient Agar (NA). After 24 h incubation at 37^0C, the first plate showing no growth was recorded as the MBC (Michael et al., 2003). The MICs accuracy was checked against standard antibiotics, gentamycin. Sterility of the media was controlled by use of broth only in a negative control tube, and growth of the organism was checked by broth plus microorganism in question in a positive control tube. All the controls were subjected to the same conditions as the tests.

Standard antibiotics

Antimicrobial sensitivity and resistance were confirmed by use of standard discs containing ampicilin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamycin (10 µg), ciprofloxacin (10 µg), tetracycline (30 µg), amikacin (30 µg) and an additional oxacilin (1 µg) (oxoid, London) for *S. aureus*. Gentamycin was used as a positive control for antibacterial activity in performing the MIC assay.

Phytochemical screening

Standard qualitative procedure was used to detect classes of compounds present in the extracts as described by Chhabra (1989) and Harborne (1973). The classes of compounds that were screened are alkaloids, flavonoids, anthocyanins, anthraquinones, tannins, coumarins and saponins. These classes of compounds were chosen as they have been associated with antimicrobial activities in previous studies.

Data analysis

Results were analyzed by use of Student's 't' test to compare antimicrobial activity of the extracts from different plant parts.

Results and discussions

Phytochemical screening

The results obtained showed that both the stem and root methanol extracts were particularly rich in alkaloids (Table 1). The leaf also had alkaloids in moderate quantities but the fruit did not have any alkaloids. Other *Tabernaemontana* species that have been studied, for example *T. chipii* and *T. bovina* Lour, had over 40 alkaloids isolated (Van Beek et al., 1985, Medeiros et al., 1999). All the parts of *T. stapfiana* contained coumarins. The root and stem bark extracts exhibited the presence of saponins and flavonoids while the leaf and fruit showed negative results but contained tannins that were absent in the stem and root.

The phytochemical screening results of the sequential extracts (Table 1) indicated high presence of alkaloids in both the ethyl acetate and methanol extracts of the root and stem. There were no alkaloids in hexane and dichloromethane extracts. There was moderate presence of coumarins also in the ethyl acetate and methanol extracts of the root and stem. The methanol extracts of the root and stem exhibited high quantities of saponins while the ethyl acetate extracts of the root and stem had moderate quantities. There were low concentrations of flavonoids in the methanol extracts of both the stem and root. The sequential extracts of the root and stem did not indicate any presence of tannins.

Table 1: Phytochemical screening results of *Tabernaemontana stapfiana* Britten Extracts

Extract	Plant part	1	2	3	4	5
Methanol Extracts	Fruit	-	+++	-	+++	-
	Leaves	-	++	++	++	-
	Stem	+	+++	+++	-	+++
	Root	+	+++	+++	-	+++
Sequential extracts						
Hexane	Stem	-	-	-	-	-
	Root	-	-	-	-	-
Dichloromethane	Stem	-	-	+	-	-
	Root	-	-	+	-	-
Ethyl acetate	Stem	-	++	+++	-	+++
	Root	-	++	+++	-	+++
Methanol	Stem	+	++	+++	-	+++
	Root	+	++	+++	-	+++

Key; 1 = Flavonoids, 2 = Coumarins, 3 = Alkaloids, 4 = Tannins, 5 = Saponins
+ = low concentration, ++ = moderate, +++ = high and - = not detected

Table 2: Antibacterial activity results for *Tabernaemontana stapfiana* Britten methanol extracts

Microorganisms	Root			Stem		
	Zone (mm)	MIC µg/ml	MBC µg/ml	Zone (mm)	MIC µg/ml	MBC µg/ml
S. aureus (MDRS)	18	15.6	31.25	19	15.6	31.25
S. aureus (MDRSA)	17	31.25	62.5	17	31.25	125
E. faecalis (Std.)	18	31.25	62.5	14	62.5	250
B. subtilis (L)	17	31.25	62.5	18	31.25	62.5
P. aeruginosa (Std.)	-	-	-	-	-	-
S. typhi (L)	9	500	500	9	500	500
K. pneumoniae (L)	-	-	-	-	-	-

Key; L = drug sensitive local strain, MDRS = multiple drug resistant local strain and Std. = standard strain.

Table 3: Antibacterial activity results of the root sequential extract of *Tabernaemontana stapfiana* Britten

Microorganisms	**EtOAc**			**MeOH**		
	Zone (mm)	MIC µg/ml	MBC µg/ml	Zone (mm)	MIC µg/ml	MBC µg/ml
S. aureus (MDRS)	18	3.9	15.6	21	7.8	7.8
S. aureus (MRSA)	18	3.9	31.25	20	7.8	31.25
B. subtilis (L)	15	7.8	500	20	15.6	31.25
E. faecalis (Std)	16	7.8	125	20	15.6	62.5
S. typhi (L)	-	-	-	9	250	500

Key; L = drug sensitive local strain, MDRS = multiple drug resistant local strain, Std. standard strain, EtOAc = ethyl acetate and MeOH = methanol.

Table 4: Antibacterial activity results of the stem sequential extract *Tabernaemontana stapfiana* Britten

Microorganism	EtOAc			MeOH		
	Zone (mm)	MIC µg/ml	MBC µg/ml	Zone (mm)	MIC µg/ml	MBC µg/ml
S. aureus (MDRS)	18	3.9	15.6	20	3.9	15.6
S. aureus (MRRSA)	18	3.9	31.25	20	7.8	31.25
B. subtilis (L)	14	31.25	500	20	31.25	250
E. faecalis (Std.)	17	7.8	125	20	31.25	125
S. typhi (L)	-	-	-	9	125	500

Key; L = drug sensitive local strain, MDRS = multiple drug resistant local strain, Std. = standard strain, C_6H_{14}= hexane, CH2Cl2= dichloromethane, EtoAc = ethyl acetate and MeOH = methanol

Table 5: Antibacterial activities of alkaloid rich fractions

Solvent/Organism	Root			Stem		
	Zone (mm)	MIC µg/ml	MBC µg/ml	Zone (mm)	MIC µg/ml	MBC µg/ml
CH_2Cl_2						
S aureus (MDRS.)	11	1000	1000	11	2000	2000
S. aureus (MRSA)	10	1000	1000	10	2000	2000
B. subtilis (L)	10	1000	1000	10	2000	2000
E. faecalis (Std.)	13	1000	1000	10	2000	2000
S. typhi (L)	-	-	-	-	-	-
CH_2Cl_2 – MeOH (4:1)						
S. aureus (Std.)	13	15.6	250	11	15.6	250
*S. aureus (*MRSA)	13	31.25	250	11	31.25	250
B. subtilis (L)	14	31.25	31.25	13	31.25	31.25
E. faecalis (Std.)	14	31.25	250	11	31.25	250
S. typhi (L)	-	-	-	-	-	-

Key; L = drug sensitive local strain, MDRS = multiple drug resistant local strain, Std. = standard strain, CH_2Cl_2= dichloromethane and MeOH = methanol

Table 6: Results for the standard antibiotics

Microorganisms	**Standard antibiotic discs/Zones of inhibition in mm**								**MIC in µg/ml**
	Amp	Chl	Eryt	Gen	Cip	Tet	Ami	Oxa	Gen
S. aureus (Std.)	28	22	24	22	24	20	23	22	1.95
S. aureus (MDRS)	6	6	6	6	6	6	6	8	nd
E. faecalis	23	18	24	21	23	18	21	nd	15.6
B. subtilis	23	20	22	20	25	17	24	nd	7.8
P. aeruginosa (Std.)	17	19	20	18	27	18	20	nd	1.95
S. typhi (L)	19	20	23	21	26	14	18	nd	1.95
K. pneumoniae (L)	18	20	23	20	24	15	16	nd	1.95
K. pneumoniae (MDRS)	6	6	6	6	6	6	6	nd	nd

Key; Amp = ampicilin, Chl = chloramphenicol, Eryt = erythromycin, Gen = gentamycin, Cip = ciprofloxacin, Tet = tetracycline, Ami = amikacin, Oxa = oxacacilin, nd = not done, L = drug sensitive local strain, MDRS = multiple drug resistant local strain, Std. = standard strain, mm = average zones of inhibition of the 3 replicates

Antimicrobial activity

The methanol extracts from the root bark and stem bark showed activity against 5 out of the 7 bacterial strains screened (Table 2). The two extracts had zones of inhibition of between 9 and 19 mm in diameter with the highest being against Std *Staphylococcus aureus* (19 mm). The multiple drug resistant *Staphylococcus aureus* (MDRSA) had a zone of inhibition of 17 mm. The zones of inhibition are comparable with those of the control Stand

Sequential extract

When the sequential extracts were subjected to antimicrobial screening, only methanol and ethyl acetate fractions showed high activity against the tested organisms. The antibacterial activity of the methanol extract from sequential extracts of the root was slightly higher (21 mm) than when only methanol was used in the extraction (19 mm) against Std *Staphylococcus aureus* (Table 3) while that of the stem (Table 4) increased from 19 mm to 20 mm against the same organism. In general there was a slight increase in activity against all the bacteria that had activity in crude methanol extracts except against *Salmonella typhi* that remained constant. The active principle(s) of high polarity is likely to be responsible for the observed antibacterial activity.

Alkaloid rich fractions

The alkaloid rich fraction was extracted from the original methanol extract and it resulted in 3 extracts, diethyl ether, dichloromethane and dichloromethane-methanol (4:1). The antimicrobial results are presented in Table 5. CH_2Cl_2-MeOH and CH_2Cl_2 extracts showed reduced antibacterial activity, while the diethyl ether extract exhibited no activity. CH_2Cl_2-MeOH (4:1) extract had the highest zone of inhibition of 13 mm against both the Std *S. aureus* and MDRSA, while both CH_2Cl_2 and CH_2Cl_2-MeOH extracts had no activity against *S. typhi*. All the extracts that showed activity in the screening stage were further assayed for MICs and MBCs.

MICs and MBCs
Methanol extracts

The MICs of the extracts that showed antimicrobial activity in the screening stage were carried out using a two-fold broth serial dilution method (Elgayyer et al., 2000). The MIC and MBC results are presented in Table 2. The antibacterial MICs ranged from very high (15.6 µg/ml) to high activity (500 µg/ml) with the lowest MIC of both the root and stem bark of 15.6 µg/ml against Std *S. aureus* while the highest was 500 µg/ml against *S. typhi*. The activity against all the Gram-positive bacteria tested was very high with MICs below 100 µg/ml. The methanol extract had an MIC against MDRSA at 31.25 µg/ml for both root and stem bark extracts. For *S. typhi*, the only Gram-negative microorganism which was susceptible in the screening assay had a high MIC (500 µg/ml) for both root and stem bark extracts. There was no significant difference between the activity of the root and stem barks ($p>0.05$) but there was significant difference between the activity of the root and leaf ($p<0.05$), and stem and leaf ($p<0.05$) against the microorganisms tested.

The tubes that had no growth in the bacterial MIC tests were assayed for MBCs. Both root and stem extracts of *T. stapfiana* showed strong bactericidal activity. The MBCs against Std *S. aureus* were 31.25 µg/ml for both the root and stem bark extracts, while that of MDRSA were 62.5 and 125 µg/ml in the root and stem barks, respectively. The activity against *Enterococcus faecalis* had MBCs of 62.5 and 250 µg/ml for the root and stem barks, respectively, *Bacillus subtilis* had 62.5 µg/ml for both the root and stem. The MBC for *S. typhi* was 500 µg/ml for the both root and stem bark extracts. There were no significant differences between the MICs and MBCs of all the tested bacteria ($p>0.05$). The MICs were the same as the MBCs or within two-twofold dilutions of the MIC which is an indication that the extracts are bactericidal.

The results obtained in this study indicate significant differences ($p<0.05$) in antibacterial activity against Gram-positive and Gram-negative bacteria, and the activity was higher against Gram-positive than Gram-negative bacteria. The reason for the different sensitivity could be ascribed to the morphological differences between these microorganisms. Gram-negative bacteria have an outer phospholipid membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, while the porins constitute a selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da (Nikaido and Vaara, 1985). On the other hand the Gram-positive bacteria are susceptible because of having only an outer peptidoglycan layer which is not an effective permeability barrier (Nostro et al., 2000).

The high activity of the root and stem bark methanolic extract may be due to presence of alkaloids and saponins as these classes of compounds have been associated with antibacterial activity in the previous studies (Van Beek et al., 1985). There is also a possibility of synergistic or additive interaction of two or more active components in the extracts. These components could include flavonoids and coumarins which were detected in the methanol root and stem bark extracts of the plant.

From the literature, the substances responsible for antimicrobial activity have different polarities ranging from polar to non-polar. In this regard, to identify the type of compounds responsible for the antimicrobial activity (whether polar or non-polar), and also to check if the activity could be increased, antimicrobial activities of sequential extracts were evaluated.

Sequential extracts

There was an increase in activity of both MeOH and EtOAc extracts of root and stem barks of the sequential extracts against all the bacteria that had showed activity in the MeOH extracts. Between the two different extracts of root and stem (MeOH and EtOAc), the EtOAc extract was found to exhibit growth inhibition at the lowest concentration of 3.9 µg/ml against both the Std *S. aureus* and MDRSA. This was an increase from the previous 15.6 µg/ml and 31.25 µg/ml, respectively. This represents a significant change in potency of antibacterial activity of the extracts. The lowering of the MIC of *Enterococcus faecalis* to 7.8 µg/ml brought the activity to within the activity of gentamycin (4-16 µg/ml, NCCLS standards) against this species of bacteria.

The MBCs were also lowered with the lowest, at 31.25 µg/ml, for the MeOH extracts against Std *S. aureus* going down to 7.8 µg/ml for root MeOH extract from the sequential extract while the highest MBC, which was against *S. typhi*, remained constant at 500 µg/ml in MeOH extract of both the root and stem barks.

From the literature, the family Apocynaceae and in particular *Tabernaemontana* species are rich in alkaloids (Van Beek et al., 1985). Most alkaloids are also known to have pharmacological activity and therefore the activity of *Tabernaemontana stapfiana* could be due to these as the presence of alkaloids was confirmed (Table I) in phytochemical screening. For this to be ascertained, alkaloid rich fractions of the root and stem barks were subjected to antibacterial assays.

Alkaloid rich fractions

In the CH_2Cl_2-MeOH fraction of the root and stem bark extracts, most of the MICs were maintained at the same concentration but the MBCs were tremendously raised. The lowest MBC, which was 31.25 µg/ml against Std *S. aureus*, was raised to 250 µg/ml. There was only one improvement, against *B. subtilis* from an MBC of 62.5 µg/ml to 31.25 µg/ml. Since 3 out of 4 bacteria tested had their MBC raised (lowered activity), this suggests that the strong bactericidal activity is not only due to presence of alkaloids but probably because of synergistic or additive effects of other compounds present in the plant, probably the saponins.

Standard antibiotics

The bacteria used in the study showed susceptibility to the Std antibiotic discs and Multiple Drug Resistant Strains (MDRS) of *S. aureus* and *K. pneumoniae*, showed resistance to all the standard drugs used (Table 6). This confirmed that the bacteria used in the study were susceptible to the standard drugs and the MDRS, resistant to the drugs as per the NCCLS (2002) recommendations. The standard antibiotic (gentamycin) used as quality control in the determination of MICs behaved in conformity with the patterns determined by NCCLS (2002). The negative controls to check the sterility of the media were negative while the controls of the growth of the organisms were positive.

Conclusion

From this study we can conclude that *Tabernaemontana stapfiana* Britten have antibacterial activity. The active principle(s) from *T. stapfiana* are likely to provide biologically active constituents that will serve as alternatives to presently less effective antimicrobials. These results form a good basis for selection of *T. stapfiana* for further phytochemical and pharmacological investigation. Isolation of these active principle(s) is underway in our research laboratories and will be reported at a later stage.

Acknowledgements

The authors are thankful to the traditional medical practitioners in Keiyo District especially Kimoi Kiplagat and Arap Alwela who gave the ethnobotanical uses and assisted in the plant collection. We also thank the Kenyatta University, Kenya Medical Research Institute and Kenya National Public Health Laboratory Services and their members of staff for the support during the study.

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