Antimicrobial Activity of Compounds from *Tabernaemontana Stapfiana*

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Abstract— The crude extracts of the plant *Tabernaemontana stapfiana* Britten exhibit antimicrobial properties and has been used by traditional healers in Elgeyo Marakwet County in Kenya highlands. The stem and the root extracts of the plant were subjected to isolation and characterization of the compounds using several chromatographic and spectroscopic techniques. The compounds isolated were Stigmasterol (**1**), α-Amyrin (**2**), Lupeol acetate (**3**), Conodurine (**4**) and Gabunine (**5**). They were tested for antibacterial and antifungal activity.

Index Terms— Antimicrobial properties, characterization, chromatography, isolation, spectroscopic techniques

INTRODUCTION

Kenya has four species of *Tabernaemontana; T. elegans* Stapf, *T. pachysichon* Stapf, *T. stapfiana* Britten and *T. ventricosa* A.DC. These plants are found in the central, western and coastal parts of Kenya (Beentje, Adamson, & Bhanderi, 1994). The traditional healers from Keiyo community in Elgeyo Marakwet County use extracts from *Tabernaemontana stapfiana* Britten to treat skin diseases, gonorrhea, syphilis, abdominal problems and cough. The main constituents the *Tabernaemontana* species are indole alkaloids, a class of compounds with wide range of pharmacological activities such as anti-inflammatory, antibacterial, antitumoral, analgesic and cardiotonic action (Pelletier, 1999). Besides the alkaloids, pentacyclic triterpenoids and steroids have also been isolated from the species (Nielsen, Hazell, Hazell, Ghia, & Torssell, 1994; Pereira et al., 2008).

Experimental

Plant material

Stems and root barks of *Tabernaemontana stapfiana* were collected from Kaptagat forest in Keiyo District in Kenya highlands. A taxonomist authenticated these species and a voucher specimen was deposited in the Kenya National Museum Herbarium for future reference. The plant materials (stem and root barks) was dried at room temperature and then separately ground to fine powder using a Christy and Morris laboratory-grinding mill. The powdered plant materials were each weighed using a weighing balance.1.8 kgs of powdered root barks and 2.5 kgs stem barks of *Tabernaemontana stapfiana* was sequentially soaked in hexane, dichloromethane (DCM) ethyl acetate (EtOH) and finally methanol (MeOH) for 48 hours.

Bioassay

Antimicrobial activity of the extracts was done to detect the presence or absence of activity. This was done against selected strains of bacteria and fungi using the agar diffusion technique (Chhabra & Uiso, 1991; McChesney, Clark, & Silveira, 1991). Similar results for crude extracts were reported by Ruttoh *et al* (2009)(Ruttoh et al., 2009).

Anti-bacterial activity test

The antibacterial activity test was carried out *in vitro* using agar diffusion method. Small, sterile discs of filter papers (6 mm) were used as carriers of antibiotics and (or) crude extracts solutions to be assayed or tested. The test organisms used in this study *Staphylococcus aureus* ATCC 25922 and *Escherichia coli* ATCC 25923. These were obtained from KEMRI, Nairobi. The test bacteria were prepared by culturing the required bacterium in nutrient broth medium from stock cultures and later when required transferred on to the nutrient agar in the Petri dishes (Elgayyar, Draughon, Golden, & Mount, 2001; Zaidan et al., 2005).

Nutrients agar (28g, Oxoid, UK) was dissolved in distilled water to make one litre of the solution and placed in an autoclaving instrument at 121-124^{0}C and 15 psi pressure for 20 minutes. Portions of the sterilized nutrient agar medium (15 ml) were dispensed into 90mm diameter pre-sterilized Petri dishes to yield a uniform depth of 4 mm under septic conditions in a laminar flow. The Petri dishes were covered and allowed to cool at room temperature undisturbed until the culture me-

dium hardened. They were then incubated at 37-39^{0}C for 24 hours in an inverted position to test their sterility. Using a sterile wire loop under septic conditions, bacteria cultures from stock cultures were scooped and spread on the nutrient agar surface with 3 fold dilutions incubated aerobically at 37-39^{0}C for 24 hours.

Using a sterile cotton swab, the suspension was evenly spread on another freshly prepared agar surface. 13 g nutrient broth powder (Oxford Ltd. Basingstoke, England) was dissolved in distilled water to make one litre of solution. 25 ml of the solution were dispensed into Erlenmeyer flasks and steamed in an auto claved instrument at 121-124^0 and 15 psi of pressure for 20 minutes. After cooling, one loopful of bacteria strain from the culture was added to sterile nutrient broth medium and incubated at 37-39^0 C for 24 hours in a rotary shaker (Elgayyar et al., 2001; Zaidan et al., 2005). The broth bacteria culture (0.1 ml) was pipetted onto nutrient agar media in the Petri dishes and spread evenly using a sterilized L-shaped glass rod under sterile conditions.

200 mg of each extract was dissolved in 1 ml of DMSO and 10 µl of the solution will be dispensed onto 6 mm filter paper disc and dried (2 mg/disc). Some discs were dipped in DMSO as control. All discs were dried in an oven at 50^{0}C for about 1 hour to expel the solvent. The dry filter paper discs containing 2 mg/disc of extract was firmly placed on the inoculated Petri dishes not later than 15 minutes after inoculation using sterile forceps under sterile conditions. They were the pressed down with a slight pressure to ensure complete contact of the inoculated agar surface and incubated at 37^{0}C aerobically in an inverted position. The zones of inhibition were measured after 24 hours and 48 hours in triplicates. Standard antibiotics were used as positive control (Chhabra & Uiso, 1991; McChesney et al., 1991).

Antifungal activity test

The Agar plate diffusion method was used. The fungal species used were *Candida albicans* ATCC 90028 and *Trichophyton mentagrophytes* (Clinical isolate). They were obtained from KEMRI, Nairobi. Commercial PDA powder (39 g, Himedia laboratories, Pvt. Ltd., Bombay) was dissolved in distilled water to make a litre of solution followed by steam sterilization in an autoclave at 121^{0}C and 15 psi pressure for 20 minutes and allowed to cool to 50^{0}C. 15 ml were dispensed into Petri dishes under sterile conditions and left to solidify. This provided the medium for growing the fungal spores.

Each crude extract (1 mg) as weighed and dissolved in DMSO (50 µl) and the solution made to 1ml using methanol. This gave a stock solution of 1000 ppm used for the test. Pure culture of the concerned fungus was made on the PDA surface in the Petri dishes from stock cultures and incubated at 30^{0}C for seven days to produce a good crop of spores. The fungal inoculum were prepared by harvesting the spores from the crop of spores with a bent spores-harvesting needle in a sterile environment and tranfering them to a sterile tube containing sterile water (Pepeljnjak, Jalsenjak, & Maysinger, 1985). The spore suspension (0.5 ml) was pipetted on to the PDA medium in the Petri dishes. The plate was then tilted several times to spread the inoculum and left still for about 10 minutes.

Using a sterile cork borer (6 mm), 4 agar wells were cut out in the inoculated PDA medium. The (0.1 ml) of known concentration was pipetted into each of these wells in triplicates. The Petri dishes were then covered, sealed and kept aerobically at 30^{0}C for 72-96 hours. At the end of the incubation period, the diameter of zone of inhibition produced around the agar wells if any were measured with a transparent measuring scale. Sterile distilled water and solvent mixture ratio used to prepare the drugs being screened were used as negative control and standard antibiotics were used as positive control.

Extraction and isolation

Separation and purification of the secondary metabolite constituents was carried out using standard chromatographic techniques which includes vacuum liquid chromatography (VLC) on Kieselgel silica gel 60G (0.040-0063 MM, Merck, Germany), Preparative TLC, column chromatography (CC) using silica gel 60G (0.63-0.2 MM, Merck Germany) and sephadex LH20 and thin layer chromatography (TLC).

The different crude extracts of the root and the stem were separately fractioned by VLC and CC using solvent system with gradual increasing polarity starting from n-hexane to 10% MeOH in DCM and eluted with a slow gradient of solvent system. Spots on the chromatograms were detected under UV light at a λ 254 and 366 nm for UV active compounds and visualized upon development by separately spraying Dragendorff and *p*-anisaldehyde and heating for 10 minutes at 110^{0}C in an oven. Fractions that showed homogeneity were combined and concentrated together to give pure compounds or impure compounds for further purification. Sephadex columns were used during the purifications. The Sephadex columns were run using 1:1 ratio of DCM to methanol or in pure methanol.

Compound of hexane extract of *Tabernaemontana stapfiana* (15 g) were separated using column chromatography and the fractions obtained at 7:3 (Hex:DCM). The fraction was not UV active but gave a blue color with *p*-anisaldehyde spray reagent.

The fraction was purified further using column chromatography and at a solvent system of 4:1 (Hex:DCM), 35 mg was obtained. A prep-TLC was done on this fraction using 4:1 (Hex:DCM) solvent system and 31 mg of stigmasterol (**1)** obtained. Vacuum liquid chromatography was used to fractionate 40g of stem bark DCM extract. The fractions obtained at 3:2 (Hex: DCM) (9.55g) were pooled was further fractionated using CC then the resulting fraction was separated using a sephadex column. The fractions that showed two spots on TLC using *p*-anisaldehyde were separated through prep-TLC to yield 22 mg of α-amyrin acetate (**2**) and 18 mg of lupeol acetate (**3**).

The DCM extract also gave a fraction was UV active and turned orange on developing in Dragendorff's spray reagent. The 1.2g of the fraction was separated using a sephadex column to result in 0.7g of a fraction that was further fractionated by a sephadex column then prep-TLC performed to obtain 13.2 mg of a white crystalline solid conodurine (**4**). Column chromatography and sephadex column were used to fractionate 28.7 g of stem bark EtOAc extract to obtain 0.77g fraction. A prep-TLC was done twice with 7:3 (DCM: EtOAc) solvent system to obtain white crystalline solid 14 mg of gabunine (**5**) were obtained.

^{1}H (1D, 2D, COSY) and ^{13}C spectrum was recorded using Varian Gemini 200 and 400 MHz machine using $CDCl_3$ as solvent. Peak on H-NMR were recorded as a singlet (s), doublet (d), doublet of doublet (dd), triplet (t), quartet (q), multiplet (m) and/or broad (b) using TMS as reference. DEPT analysis was also perfomed. The chemical shifts were recorded in δ (ppm) and coupling constants, J, in hertz (Hz). A known weight of the sample was dissolved in $CDCl_3$ in a silget sample tube and mixed thoroughly. The solution was transferred into NMR tube and the spectrum recorded. The uncorrected melting points were recorded with open capillary tube using Gallenkamp melting apparatus (Sanyo, West Sussex UK).Visualization of spots on a developed TLC plate was done using long and short wave lengths (λ 366 and 254 nm) on an ENF-240 C/F UV lamp (Spectronics Co., Westbury).

Stigmasterol (**1)**

White powder, MP169-173^{0}C, IR (KBr) $V_{max}cm^{-1}$: 3595-3510, 3425, 3240, 3163, 2916, 2862, 2515, 1728, 1465-1404, 1265, 1157-1026, 802. ^{1}H-NMR (Acetone-d_6, 200 MHz, δ ppm): 5.32 (1H, m), 5.20 (1H, m), 5.09 (1H, m), 3.38 (1H, m), 1.29 (3H, s), 1.07 (3H, s), 1.02 (3H, s), 0.75 (3H, s), 0.73 (3H, s). ^{13}C-NMR: 140.7, 138.2, 129.2, 121.6, 71.7, 56.8, 56.0, 51.16, 50.1, 42.2, 40.4, 39.7, 39.6, 37.2, 36.4, 33.9, 31.8, 31.8, 31.6, 28.8, 24.2, 25.3, 21.1, 21.0, 20.0, 19.3, 18.9, 12.1, 11.8.

α-Amyrin acetate (**2**)

White crystalline solid, Mp 200-205^{0}C, IR (KBr) $V_{max}cm^{-1}$: 3448, 2970-2877, 2723 1735, 1658, 1465, 1365 1249, 1141, 1095, 1018, 902, 825. ^{1}H-NMR (Acetone-d_6, 200 MHz, δ ppm); 5.19 (m), 4.47(m), 2.00(s), 1.95 (d), 1.19(s), 1.14(s), 1.06(s), 1.02(s), 0.93(s), 0.89(d), 0.83(d). ^{13}C-NMR; 170.1, 139.8, 124.7, 80.4, 59.1, 55.3, 47.8, 47.5, 42.2, 41.6, 40.2, 39.8, 38.5, 37.7, 36.9, 33.8, 32.9, 31.2, 27.70, 26.9, 26.7, 23.7, 23.4, 23.0, 21.0, 20.4, 18.3, 17.3, 16.7, 16.5, 15.4.

Lupeol acetate (**3**)

White crystalline solid, Mp125-130^{0}C, IR (KBr) $V_{max}cm^{-1}$: 3903, 3448, 3301-3085, 2970-2885, 2715, 1736, 1635, 1465, 1373 1250, 1018, 879. ^{1}H-NMR ($CDCl_3$, 400 MHz, δ ppm) 4.68 (1H,d), 4.56 (1H,s), 4.46 (1H, dd), 2.03 (3H,s), 1.68 (3H,s), 1.02 (3H,s), 0.93(3H,s), 0.85 (3H,s), 0.84 (3H,s), 0.83 (3H,s), 0.78 (3H,s)., ^{13}C-NMR; 168.9, 150.9, 109.3, 81.0, 55.4, 50.4, 48.3, 43.0, 42.8, 40.0, 38.4, 38.1, 37.8, 35.6, 34.2, 29.8, 29.7, 27.9, 27.4, 25.1, 23.7, 21.3, 20.9, 19.3, 18.2, 18.0, 16.5, 16.1, 16.0, 14.5.

Conodurine (**4**)

White crystals, $CDCl_3$, 400 MHz, ^{1}H-NMR; 7.67 (d, 7.2), 7.22 (s), 7.11 (ddd, 7.2, 6.6, 1.4), 7.09 (dd 7.5, 6.5), 7.03 (d, 7.5), 6.82 (d, 10.8), 5.33 (d, 3.6), 5.30 (q, 6.5), 4.37 (ddd, 9.5, 8.0, 3.0), 3.97 (s), 3.69 (s), 3.61 (d 12.5), 3.46 (ddd, 12.0, 6.5, 2.4), 3.45 (dd, 15, 10.0), 3.35 (s), 3.35 (m), 2.96 (m), 2.87 (m), 2.77 (dd, 3.6, 2.4), 2.69 (brd 12.0), 2.69 (m), 2.63 (s), 2.51 (s), 2.47 (d12.0, 8.4),1.9 (m), 1.75 (d), 1.67 (d, 6.4), 1.46 (m),1.45 (m), 1.1 (m), 0.9 (m), 0.80 (t, 7.4), 0.68 (br d). ^{13}C-NMR; 174.9, 171.7, 152.0, 136.8, 136.1, 136.0, 135.1, 129.5, 124.5, 122.1, 119.4, 118.8, 118.0, 114.4, 110.0, 109.8, 109.1, 105.0, 59.6, 57.6, 56.9, 54.6, 52.4, 52.3, 51.3, 51.2, 50.2, 47.30, 42.4, 38.9, 35.2, 34.7, 33.8, 33.5, 31.8, 27.0, 26.5, 22.0, 19.6, 12.3, 11.6

Gabunine (**5**)

White crystals, $CDCl_3$, 400 MHz, ^{1}H-NMR; 7.71 (d, 7.2), 7.11 (ddd, 7.2, 6.0, 1.2), 7.23 (d, 8.4), 7.09 (dd 7.0, 6.5), 7.00 (d, 7.2), 6.81 (d, 7.2)5.32 (dd, 14.4, 3.6), 5.28 (s), 4.37 (ddd, 10.8, 7.2, 3.0), 3.97 (s), 3.69 (s), 3.67 (d, 13.6), 3.60 (m), 3.59 (m), 3.24 (ddd, 13.8, 7.6, 5.6), 3.33 (s), 2.96 (m), 2.68 (ddd, 15.3, 12.5, 12.0), 2.70 (br d, 12.0), 2.68 (m), 2.53 (s), 1.58 (m), 1.69 (d, 13.5),1.67 (d, 6.4), 1.48 (m), 1.42(m), 0.9 (m), 0.79 (t, 7.4). ^{13}C-NMR; 174.5, 170.8, 152.0, 136.8, 136.1, 135.9, 134.9, 129.2, 124.4, 122.2, 119.5, 119.3, 118.1, 117.1, 114.0, 109.8, 109.7, 106.0, 104.8, 57.4, 56.7, 54.4, 53.0, 52.8, 52.4, 51.2, 50.1, 48.8, 43.4, 38.7, 35.3, 34.7, 33.8, 31.7, 29.6, 27.0, 26.5, 24.4, 21.9, 12.1, 11.4.

Results and discussion

The stem-bark of *Tabernaemontana stapfiana* gave Stigmasterol (**1**), α-Amyrin (**2**), Lupeol acetate (**3**), Conodurine (**4**) and Gabunine (**5**). The alkaloids, Conodurine (**4**) and Gabunine (**5**) were UV active characteristic of indole chromophore. The proton and carbon NMR spectrum with COSY, NOESY, DEPT, HSQC and HMBC experiments were used to identify the compounds.

Antibacterial tests

The crude extracts were subjected to antibacterial test using *Escherichia coli* and *Staphylococcus aureus* in agar diffusion assay method. The 1000 µg/disc were loaded on a sterile filter paper of diameter 6 mm and incubated at 37^0C for 24 h. The zones of inhibition were measured and the results were tabulated in the table 1 below. A zone of 6 mm indicates failure by the extract to inhibit the growth of bacteria.

Table 1: Zones of inhibition results for antibacterial activity of crude extract of T. stapfiana

	Extract	*E. coli* (in mm)	*S. aureus* (in mm)
Root bark	Hexane	6	6
	DCM	6	14
	EtOAc	15	18
	MeOH	20	21
Stem bark	Hexane	6	6
	DCM	6	14
	EtOAc	20	18
	MeOH	20	20
Positive control		6	6
Tetracycline		22	22
Ampicillin		23	24

The crude extracts showed increasing activity as the polarity of the solvent increased. The hexane extract showed the lowest inhibition zones followed by the DCM extract. The EtOAc and MeOH extracts had the highest activity indicating that the active ingredients are in high concentration in the EtOAc and MeOH extracts. The root bark extract showed slightly higher activity against Gram-positive bacteria and Gram-negative bacteria than the stem bark. The standard antibiotics gave inhibition zones almost similar to the results obtained with crude extract of the plant under investigation.

The isolated compounds were subjected to the antibacterial assay against the selected bacteria *Stapylococcus aureus* and *Escherichia coli*, and the inhibition zones (in mm) measured. The activity results were recorded in the table 2 below. A zone of 6 mm indicates failure by the extract to inhibit the growth of fungus.

Table 2: Antibacterial activities of isolated compounds in the diffusion method assay after 24 h

Compound	*S. aureus* (in mm)	*E. coli* (in mm)
Stigmasterol (**1**)	8	6
α-Amyrin acetate (**2**)	6	6
Lupeol acetate (**3**)	14	6
Conodurine (**4**)	6	6

Stigmasterol (**1**) showed low activity (8 mm) against *S. aureus* and no activity against *E. coli*. α-Amyrin acetate (**2**) and conodurine (**4**) had no activity against the test organisms. Lupeol acetate (**3**) showed moderate activity against *S. aureus* with an inhibition zone of 14 mm and had no activity against *E. coli*. The four compounds exhibited no activity against *E. coli* and had low activity against *S. aureus*. The moderate activity of lupeol acetate (**3**) justified the use of *T. stapfiana* in traditional medicine as antibacterial.

Antifungal tests

The antifungal activity was measured in terms of zones of inhibitions in mm for growth of fungi by the plant extracts using *Candida albicans* and *Trichophyton mentagrophytes*. The zones of inhibition were measured and the results were recorded in table 3 below. A zone of 6 mm indicates failure by the extract to inhibit the growth of fungus.

Table 3: Zones of inhibition results for antifungal activity of crude extract of *T. stapfiana*

Plant	Extract	*C.albicans* (in mm)	*T. mentagrophtes* (in mm)
Root bark	Hexane	6	6
	DCM	9	6
	EtOAc	16	10
	MeOH	20	10
Stem bark	Hexane	6	6
	DCM	9	6
	EtOAc	15	10
	MeOH	20	10
Positive control		6	6

The EtOAc and MeOH extract were very active against the selected fungal strains. The hexane extract showed no activity against the fungi while the DCM extract gave lower inhibition zones. The results indicated the antifungal properties

The isolated compounds were subjected to the antifungal assay against the fungus *Candida albicans* and *Trichophyton mentagrophytes*, and the inhibition zones (in mm) measured. The activity results were recorded in the table 4 below. A zone of 6 mm indicates failure by the extract to inhibit the growth of fungus.

Table 4: Antifungal activities of isolated compounds in the diffusion method assay after 24 h

Compound	*C. albicans* (in mm)	*T. mentagrophytes* (in mm)
Stigmasterol (**1**)	6	8
α-Amyrin acetate (**2**)	6	6
Lupeol acetate (**3**)	6	14
Conodurine (**4**)	6	20

The results showed that the activities of the isolated compounds were generally less than those of the crude extracts from which the compounds were isolated. All the compounds had no activity against *Candida albicans*. Stigmasterol (**1**) had low activity (8 mm) against *T. mentagrophytes.* α-Amyrin acetate (**2**) had no activity while lupeol acetate (**3**) showed moderate activity (14 mm) against *T. mentagrophytes.* Conodurine (**4**) had the strong inhibition zone (20 mm) against *T. mentagrophytes.* The amounts of gabunine (**5**) were not enough for both the NMR analysis and bioassay.

The bacteria used in this study cause diseases such the gastrointestinal tract infection which include diarrhea and intestinal mucosa. The fungi used in the study are known to cause ailments such as candidiasis, vaginal thrush and skin infections. The activities shown by the crude extracts were higher than those of isolated compounds meaning that there were other compound not isolated that are responsible for the high activity of the crude extracts especially in the ethyl acetate and methanol extracts. The high activity of the crude extract might also be a result of synergistic effects of the compounds in the extract.

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