Antimicrobial Compounds from *Terminalia brownii* against Sweet Potato Pathogens

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Abstract: Phytochemical evaluation of *Terminalia brownii* extracts led to the isolation of five compounds namely β-sitosterol, stigmasterol, monogynol A, betulinic acid and arjungenin. Their structures were established by spectroscopic and physical methods as well as by comparison with literature data. The *in vitro* antimicrobial activities of the extracts and isolates were investigated against fungi and bacteria which infect sweet potato. Ethyl acetate extract exhibited the highest ($p \leq 0.05$) antifungal and antibacterial activities compared to *n*-hexane and methanol ones. *Streptomyces ipomoeae* was more susceptible to ethyl acetate extract (inhibition zone, 18.6 mm) than streptocycline used as a positive control. The minimum inhibitory concentration (MIC) for the isolates ranged between 50 and 200 µg/ml with the lowest MIC value of 50 µg/ml being observed with betulinic acid against *Aspergillus niger* and *S. ipomoea.*

Keywords: *Terminalia brownii*, Combretaceae, isolates, sterols, triterpenes, antibacterial, antifungal, MIC.

1. INTRODUCTION

Sweet potato is an important potato food crop worldwide since it is drought tolerant and acts as a famine relief crop [1]. The crop is a rich source of carbohydrates, vitamins, and oligominerals. However, the production of sweet potato is limited by viral, fungal, and bacterial infections [2-4]. Apart from reducing the yield, these infections cause rotting of roots, changes in appearance, texture, and flavor, making the produce unpalatable [2, 4].

Synthetic chemicals such as dichloronitroaniline have been used to protect root and tuber crops against microbial infections [2]. However, the use of such chemicals apart from their potential danger to both for humans and environment [5, 6], are unaffordable by most farmers. Moreover, because of pathogens resistance, most chemicals has become ineffective [6]. In order to fully exploit the potential of the sweet potato crop, there is a need to search for affordable, readily available, sustainable, and environmentally friendly means of managing the problems posed by these pathogens. Plants extracts have been reported to be safe, non-phytototic to humans, but effective against several plant pathogens [7].

Plants of the genus *Terminalia* (Combretaceae) are a rich source of pentacyclic triterpenes and their glycoside derivatives, flavonoids, tannins, and other aromatic compounds [8-10]. Biological activities of *Terminalia* species include antifungal, antibacterial [11-13], antioxidant, antitumor [8], feeding deterrent and growth inhibitor [14]. The antimicrobial principles from the genus include arjunic acid, arjungetin, and arjungenin [13].

Terminalia brownii Fries (Combretaceae) is found in many parts of Africa and is used as a remedy for diarrhoea and stomach ache, ulcers, sexually transmitted diseases, malaria, cough, hepatitis, jaundice and yellow fever [15]. In this communication, we report the first time isolation of β-sitosterol (**1**), stigmasterol (**2**), monogynol A (**3**), butelinic acid (**4**), and arjungenin (**5**) from *T. brownii* and their antimicrobial activity.

2. MATERIALS AND METHODS

2.1. General

Melting points were determined on a Gallenkamp (Loughborough, UK) melting point apparatus and are uncorrected. The UV spectra were run on Pye Unicam SP8-150 UV–vis spectrophotometer (Cambridge, UK) using acetonitrile. IR data were recorded on a PerkinElmer FTIR 600 series spectrophotometer (Waltham, MA, USA) as KBr pellet. The ^{1}H and ^{13}C NMR data were measured in $CDCl_3$ and $CDCl_3$–DMSO-d_6 on a Bruker NMR Ultrashield TM (Darmstadt, Germany) operating at 500 and 125 MHz, respectively. The MS data were obtained on a Varian MAT 8200A instrument (Bremen, Germany).

2.2. Plant Materials

Stem bark of *T. brownii* was collected near Kendu Bay Mission Hospital along Kendu Bay - Oyugis road (latitude 0° 22' 22.00" S and longitude 34° 39' 09.05" E) in November 2008 and voucher specimen (2008/11/15/SAO/CHEMMK)

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was identified at the Kenya National Museum herbarium after comparison with authentic samples. The plant materials were chopped into small pieces, air dried and reduced to fine powder using a mill.

2.3. Extraction and Isolation of Compounds

Powdered plant material (2 kg) was sequentially extracted with 3 liters of *n*-hexane, EtOAc and MeOH in the cold for seven days each with occasional shaking. The macerate was filtered and the filtrate concentrated under vacuum using rotary evaporator to afford 18 g, 75 g and 150 g of *n*-hexane, ethyl acetate and methanol extracts, respectively. Ethyl acetate extract (75 g) was subjected to column chromatography (CC) over silica gel eluting with *n*-hexane-EtOAc (10% increase of ethyl acetate), neat EtOAc and finally with CH_2Cl_2-MeOH (with 10% and 20% increment of MeOH) to give 132 fractions (each 20 ml). Composition of fractions was monitored by TLC and fractions showing similar profiles were combined into five pools (I-V). Pool I (6 g) showed an intense purple color upon spraying with anisaldehyde – conc. H_2SO_4 mixture and heating on TLC. The pool contained mainly fatty acids and was discarded. Pool II (8.5 g) was subjected to further CC eluting with *n*-hexane: EtOAc mixture (95:5, 9:1, 85:15, 4:1) to give β-sitosterol (**1**), 85 mg [16] and stigmasterol (**2**) 65 mg [17]. Pool III (10.5 g) was further fractionated by CC eluting with *n*-hexane: EtOAc (4:1, 75:25, 7:3, 65:35, 3:2) to give monogynol A (**3**) 95 mg [18], and betulinic acid (**4**) 98 mg [18], on recrystalization with CH_2Cl_2 – EtAOc mixture . Pool IV (9.6 g) afforded arjungenin [**5**] 65 mg [14], after repeated CC eluting with EtOAc: MeOH mixture (99:1, 95:5).

2.4. Isolation of Test Organisms

Five fungi and two bacteria isolated from infected sweet potato roots were used in this study. Sterilized pieces of infected sweet potato were incubated in nutrient agar (NA) and potato dextrose agar (PDA) at room temperature for up to 5 days and fungal and bacterial growth associated with rot affected tissues were identified with the aid of the appropriate taxonomic keys [19]. The isolates were maintained on NA and PDA slants.

2.5. Antimicrobial Assay of Crude Extracts

Antimicrobial activities of the methanol, ethyl acetate and *n*-hexane extracts of *T. brownii* were evaluated by the agar diffusion method [20]. The tests were performed in sterile Petri dishes (90 mm diameter) containing 20 ml PDA and NA for fungi and bacteria, respectively. The PDA and NA media were prepared by suspending 39 and 28 g in 1 liter of distilled water and heated to dissolve completely. The media were sterilized by autoclaving at 120 °C for 20 min. Inoculation was done by spreading 0.5 ml of spore suspension (1 x 10^5 cfu/ ml) of the test pathogen on the surface of the solidified agar [21]. Paper disc (Whatmann No. 1, 5 mm diameter) were impregnated with 100 ml of the plant extracts (5 mg/ml) using a sterile micropipette and left for 30 min to dry in the hood. The dried discs were placed on the surface of the solidified inoculated agar and incubated at 28 °C for 48 h for fungi and 37 °C for 24 h for bacteria. Blitox and streptocycline (10 mg/ml) were used as positive controls while DMSO without plant extract was used as a negative control. All tests were done in triplicates. The presence of zones of inhibition around the disc was interpreted as an indication of antimicrobial activity.

2.6. Antimicrobial Assay of Pure Isolates and Minimum Inhibitory Concentration

The minimum inhibitory concentrations (MICs) of pure isolates were determined as previously described [21]. The compounds were dissolved in DMSO and different concentrations ranging between 200 µ/ml and 1 µ/ml were prepared. Sterile paper discs were impregnated with 100 ml of the reconstituted samples in DMSO. The dried discs were transferred aseptically into PDA an NA plates previously inoculated with test fungi and bacteria, respectively and MIC was regarded as the lowest concentration that produced a visible zone of inhibition.

3. RESULTS AND DISCUSSION

ESI-MS spectrum of compound **1** (Fig. **1**) gave a quasi-molecular ion peak at *m/z* 437 $[M+Na]^+$ corresponding to molecular formula of $C_{29}H_{50}O$ and was supported by ^{13}C NMR spectrum which showed presence of 29 distinct carbon peaks resolved into six methyl, eleven methylene, nine methine and three non-protonated carbon atoms by DEPT. The ^{13}C NMR spectrum showed the presence of two olefinic carbon atoms (δ 140.75, 121.70), an oxymethine carbon atom (δ 71.81) and six methyl carbon atoms δ 19.80, 19.39, 19.03, 17.77, 11.97 and 11.85. The ^1H NMR spectrum showed presence of one olefinic proton (δ 5.35 m), thus confirming the carbon-carbon double bond to be trisubstituted. The peak at δ 3.52 which was assigned to the proton at C-3 confirmed the presence of the hydroxyl group while those at δ 1.01(s), 0.92 (d, J = 6.2 Hz), 0.84 (t, J = 7.0 Hz), 0.82 (d, J = 6.5 Hz), 0.81 (d, J = 6.5 Hz) and 0.68 (s) confirmed the presence of two tertiary, three secondary and one primary methyl groups. Comparison of these data with the literature data [16] confirmed the structure of **1** as β-sitosterol.

EIMS spectrum of compound **2** gave a molecular ion peak at *m/z* 412 suggesting a molecular formula of $C_{29}H_{50}O$ and was supported by ^{13}C NMR and DEPT spectra which showed the presence of 29 carbon atoms consisting of six methyl, nine methylene, eleven methine and three quaternary carbon atoms. The ^{13}C NMR olefinic peaks at δ 139.56, 138.09, 127.65 and 117.32 showed the presence of two carbon-carbon double bonds (tri- and di-substituted) while the peak at δ 71.04 showed the presence of oxymethine carbon atom at C-3 [17]. ^{13}C NMR spectrum at δ 12.06, 21.99, 21.38, 12.16, 19.05 and 12.9 showed the presence of six methyl groups. The ^1H NMR spectrum showed the presence of three olefinic protons at δ 5.18 m, 5.15 d (J = 15.3 Hz) and 5.03 dd (J = 15.3, 8.1 Hz); a proton attached to a oxymethine carbon atom (δ 3.54 m) and six methyl groups at δ 0.54 s, 0.84 s, 0.78 d (J = 7.1 Hz), 0.82 d (J = 6.5 Hz), 1.00 d (J = 6.5 Hz) and 0.79 t (J = 8.0 Hz) corresponding to two tertiary, three secondary and one primary methyl groups. Based on the spectral data as well at comparison with literature [17], compound **2** was identified as stigmasterol.

ESI-MS spectrum of compound **3** gave a quasi molecular ion peak at *m/z* 467 $[M+Na]^+$ suggesting a molecular formula

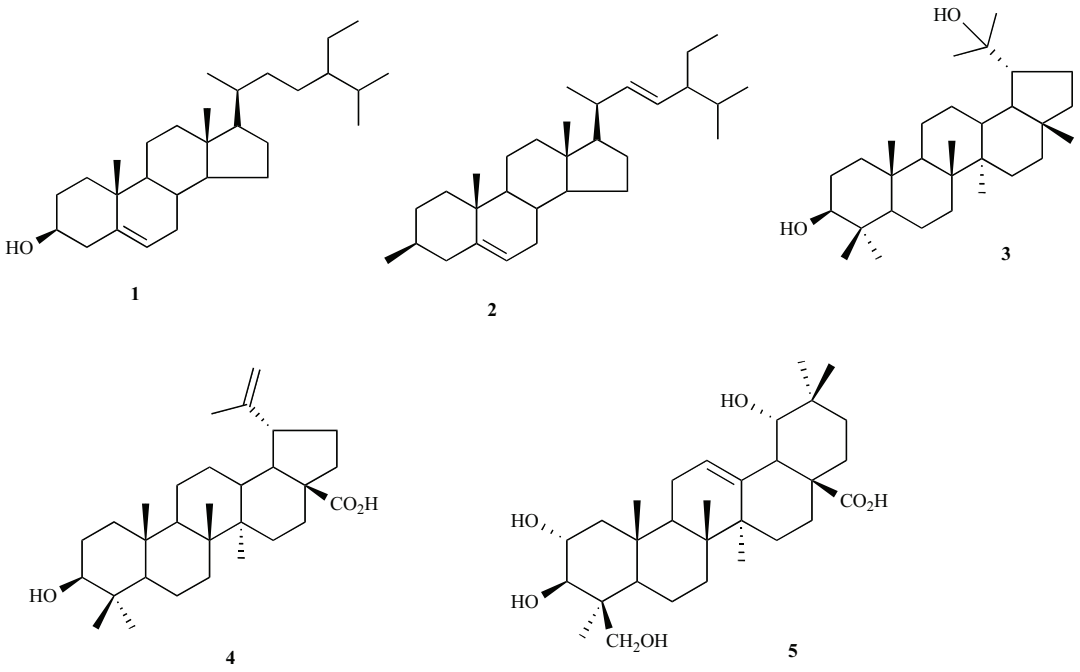

Fig. (1). Compounds isolated from *Terminalia brownii*.

of $C_{30}H_{52}O_2$ and was supported by ^{13}C NMR spectrum which afforded 30 carbon atoms consisting of a carbon bearing a tertiary OH and another carbon bearing a secondary OH at δ 73.59 and 78.77, respectively. The ^{13}C NMR data also showed the presence of eight methyl carbon atoms at δ 30.02, 27.73, 17.90, 17.77, 16.22, 16.16, 15.40 and 14.78. The rest of ^{13}C NMR signals corresponded to five quaternary carbons (δ 43.17, 43.17, 41.39, 38.18 & 35.15), five methine carbons (δ 54.87, 49.37, 47.19, 38.78& 38.55) and ten methylene carbon atoms (δ 38.41, 37.93, 37.50, 35.21, 29.11, 27.12, 26.37, 21.31& 18.07). The presence of the eight methyl group was supported by the eight singlets (δ_C 0.76, 0.84, 0.85, 0.95, 0.97, 1.07, 1.09, 1.18), each integrating for three protons in the ^1H NMR. Comparing these data with the literature data [18] confirmed the structure of **3** as monogynol A.

EIMS spectrum of compound **4** afforded a molecular ion peak at *m/z* 456 for molecular formula of $C_{30}H_{48}O_3$ and was supported by the ^{13}C NMR spectrum which gave 30 carbon atoms consisting of a carbonyl carbon (δ 180.0), a quaternary vinyl carbon (δ 150.40), a terminal methylene carbon (δ 109.67) and a carbon holding a secondary alcohol (δ 79.11). The other ^{13}C NMR signals corresponded to five quaternary carbons (δ 56.95, 42.59, 40.89, 38.56, 37.36), ten methylene carbons (δ 39.89, 37.09, 34.52, 32.28, 30.73, 29.81, 27.54, 25.68, 21.00, 18.40), five methine carbons (δ 55.56, 50.72, 49.51, 46.99, 38.95) and six methyl carbons (δ 28.06, 19.45, 16.14,16.14, 15.35, 14.77). The ^1H NMR spectrum gave two singlets at δ 4.73 and 4.60 corresponding to two protons of a terminal vinyl methylene group while the peak centered at δ 1.69 was assigned to the vinyl methyl group. Comparison of these data with those available in the literature [18] confirmed compound **4** to be betulinic acid.

The ESI-MS spectrum of compound **5** afforded quasi-molecular ion peak at *m/z* 527 $[M+Na]^+$ for a molecular formula of $C_{30}H_{48}O_6$. The ^{13}C NMR spectrum gave 30 showed presence of carbon atoms attributable to a carbonyl carbon (δ 179.72), three carbon atoms bearing secondary OH groups (δ 84.52, 79.76, 67.40), a carbon atom bearing a primary OH group (δ 63.82), a quaternary vinyl carbon (δ 144.13) and a methine vinyl carbon (δ 122.06) [8,14, 21]. The rest of the ^{13}C NMR signals corresponded to six quaternary carbons (δ 43.78, 41.69, 39.37, 39.24, 38.22, 35.49), three methine carbons (δ 43.13, 47.37, 55.48), eight methylene carbons (δ 18.87, 23.39, 27.15, 27.86, 28.41, 32.29, 32.80, 46.80) and six methyl carbon atoms (δ 16.37, 16.59, 23.33, 24.03, 24.67, 28.08). ^1H NMR spectrum showed the presence of a vinyl proton (δ 5.22 *br*. s), five protons on carbon atoms bearing hydroxyl groups at δ 4.48 d (J = 8.1 Hz), 4.01 m, 3.75 d (J = 10.1 Hz), 3.36 d (J = 10.0 Hz) and 3.33 m [14]. Other diagnostic peaks in the ^1H NMR spectrum were the six singlets, each integrating for three protons at δ 1.27, 1.08, 0.89, 0.87, 0.83 and 0.64. Based on the spectral data as well as comparison with literature data, compound **5** was concluded to be arjungenin.

3.1. Antimicrobial Activity of Crude Extracts and Isolates

Results of the antimicrobial activities of MeOH, EtOAc and *n*-hexane extracts of *T. brownii* stem bark are shown in Table **1**. The extracts were tested against five fungi (*Alternaria* spp, *Aspergillus niger*, *Fusarium oxysporum*, *F. solani* and *Rhizopus stolonifer*) and one Gram negative and one Gram positive bacteria namely *Ralstonia solanacearum* and *Streptomyces ipomoeae*, respectively. All the extracts were active against one or more of the tested organisms. Ethyl acetate extract exhibited the highest ($p \leq 0.05$) inhibitory effects against all the microorganisms while methanol extract had the lowest activity. *S. ipomoeae* was the most susceptible

to ethyl acetate extract (inhibition zone, 18.6 mm) while *F. oxysporum* was the least susceptible (5.7 mm) to the extract. Activity of ethyl acetate extract against *S. ipomoeae* was significantly ($p \leq 0.05$) higher than that of streptocycline, used as a positive control.

Fractionation of ethyl acetate extract afforded five compounds which were active against one or more of the microorganisms tested at concentrations ≤ 200 µg/ml except compound **3** (Table **2**).

β-Sitosterol (**1**) was active against *A. niger*, *F. solani*, *R. stolonifer* and *R. solanacearum*; stigmasterol (**2**) was active against *A. niger*, *F. oxysporum*, and *F. solani*; betulinic acid (**4**) was active against *A. niger*, *F. solani*, *R. stolonifer* and *S. ipomoea* while *arjungenin* (**5**) was active against *Alternaria* spp, *A. niger*, *F. solani* and *R. solanacearum*. Betulinic acid was the most active against *A. niger* and *S. ipomoea* with MIC value of 50 µg/ml. The antimicrobial activity of betulinic acid was previously reported [22, 23].

Findings from this study revealed that extracts of *T. brownii* have antimicrobial activity against *F. oxysporum*, *F. solani*, *Alternaria* spp, *R. stolonifer*, *A. niger*, *R. solanacearum* and *S. ipomoeae* which infect sweet potato and other root crops [3]. This suggests that the pathogens can be managed using herbal extracts as had also been observed in other studies [24]. Use of plant extracts to manage plant infections is environmentally safe compared to the synthetic antimicrobial drugs currently used [25, 26]. Extracts and isolates from *T. brownii* have broad spectrum activity since they are also

Table 1. Antimicrobial Activity of Crude Extracts

	*Zone of Growth Inhibition (mm)				
	Extracts			Standard Drugs	
Test Organisms	***n*-hexane**	**EtOAc**	**Methanol**	**Blitox**	**Streptocycline**
Fungi					
Alternaria spp.	9.0	13.1	5.0	22.1	ND
A. niger	10.1	15.5	6.5	28.0	ND
F. oxysporum	5.0	5.7	5.0	16.9	ND
F. solani	6.2	10.8	6.2	25.1	ND
R. stolonifer	5.0	5.9	5.0	18.3	ND
Bacteria					
R. solanacearum	12.4	15.0	9.1	ND	18.8
S. ipomoeae	13.2	18.6	9.8	ND	14.4
Mean	**8.7**	**12.1**	**6.7**	**22.1**	**16.6**

*Values are means of three replicates; ND: Not done.

Table 2. Minimum Inhibitory Concentration (MIC, µg/ml) of Isolated Compounds

Compound	**MIC, µg/ml of Isolated Compounds**						
	Test Fungi					**Test Bacteria**	
	Alter spp	***A. nig***	***F. oxy***	***F. sol***	***R. sto***	***R. sola***	***S. ipo***
β-Sitosterol (1)	>200	100	>200	100	200	100	>200
Stigmasterol (2)	>200	100	200	200	>200	>200	>200
Monogynol A (3)	>200	>200	>200	>200	>200	>200	>200
Betulinic acid (4)	>200	50	>200	100	100	100	50
Arjungenin (5)	100	100	>200	200	>200	200	>200
Blitox	6.25	50	12.5	6.25	12.5	ND	ND
Streptocycline	ND	ND	ND	ND	ND	25	12.5

ND: Not done; *Alter spp: Alternaria spp*; *A. nig*: *Aspergillus niger*; *F. oxy: Fusarium oxysporum*; *F. sol*: *Fusarium solani*; *R. sto*: *Rhizopus stolonifer*; *R. sola*: *Ralstonia solanacearum*; *S. ipo*: *Streptomyces ipomoeae*.

active against fungi and bacteria that cause infection in humans [12]. The extracts and isolates were also active against both gram positive and gram negative bacteria which were tested in this study.

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