



Evaluation of Antimicrobial and Antioxidant Activity of β -Sitosterol-3-O-Glucoside Isolated from *Lannea Kerstingii* Engl. & K. Krause (Anacardiaceae).

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Abstract

*β -sitosterol-3-O-glucoside was isolated using dry vacuum liquid chromatography. It was characterized using ¹H-NMR, ¹³C-NMR and 2D NMR spectra. The antimicrobial activity was determined using agar diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) were determined using broth dilution method. Antioxidant activity was determined through reaction of compound with DPPH radical. The compound was active against *S. aureus*, Methicillin Resistant *Staphylococcus aureus*, *P. mirabilis*, *S. typhi*, *K. pneumoniae*, *E. coli*, *B. subtilis*, *C. albicans* and *C. tropicalis* with zone of inhibition ranging from 22.0 ± 0.4 to 34.0 ± 0.3 mm and inactive against *P. aeruginosa*, *Proteus vulgaris* and *C. krusei*. The MIC ranged from 25.0 to 50.0 $\mu\text{g/ml}$ while the MBC/MFC ranged from 50.0 to 200.0 $\mu\text{g/ml}$. The % DPPH scavenging activity at 4.0×10^3 $\mu\text{g/ml}$ was found to be 81.44 ± 0.41 %. This result shows the high antioxidant and wide spectrum antimicrobial activity of β -sitosterol-3-O-glucoside which can be a potential lead drug for resistant bacteria such as MRSA, and a potential source of natural antioxidants.*

Keywords: *β -sitosterol-3-O-glucoside; *Lannea kerstingii*; liquid chromatography; antioxidant; antimicrobial*

1.0 INTRODUCTION

Infectious disease is one of the leading causes of death worldwide and was reported in 2012 to be responsible for 68% of all deaths globally (WHO, 2014). All these are due to the emergence of multidrug resistant pathogens which contributes to the high causes of death world-wide (Reddy *et al.*, 2009). The screening of plant extracts and plant products for antimicrobial activity has shown that plants represent a potential source of new anti-infective agents (Costa *et al.*, 2008).

Antioxidants, which serve as important nutraceuticals are highly beneficial to our health (Drode *et al.* 2002; Valko *et al.*, 2007). Some natural and effective antioxidants are ascorbic acid, carotenoids and phenolic compounds, etc (Ghani, 2003). A number of natural products, such as flavonoids, coumarins, curcuminoids or terpenes, isolated from plants have shown potent antioxidant activity and low toxicity (Yang *et al.*, 2007)]. Antioxidants have the capacity to remove oxygen derived free radicals effectively either by donating hydrogen or an electron (Pietta, 2000). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical compound with an unpaired valence electron present at one atom of nitrogen bridge (Eklund *et al.*, 2005). Removal of DPPH by any chemical shows its antioxidant activity and it is the basis of DPPH

2.0 EXPERIMENTAL

2.1 General

All analyses of samples using Thin Layer Chromatography (TLC) were performed at room temperature using pre-coated plates (MERCK, silica gel 60 F₂₅₄ 0.2 mm). Detection of spots was by ultraviolet lamp (254 and 366 nm). Silica gel G for TLC (BDH) and SephadexTM LH-20 (GE Healthcare) were used as stationary phases for dry vacuum liquid chromatography and column chromatography respectively to fractionate fractions. Nuclear magnetic resonance (NMR) spectra data were obtained from a JEOL ECX 400 MHz spectrometer. Chemical shifts are expressed in parts per million (ppm). TLC chromatograms obtained were sprayed with a saturated solution of cetric sulphate in 65% sulphuric acid; the plates were

antioxidant assay (Alma *et al.* 2003; Kordali *et al.*, 2005).

Several diseases such as atherosclerosis, ischemic heart disease, aging, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases, etc are majorly caused by free radical mediated oxidative stress (Young and Wood, 2001). Synthetic antioxidants have the ability to form possible toxic or carcinogenic components during their degradation (Barlow, 1990). Thus, identification and isolation of new antioxidants from natural sources has become an active area of research.

Lannea kerstingii has been reported to contain: tannins, flavonoids, alkaloids, steroids and triterpenes (Kone *et al.*, 2011; Njinga *et al.*, 2014). It is a tree with a height of 12 m and 40 cm in diameter, with a wide-spreading and relatively dense crown. The bark is smooth to slightly fissured, fissures spiral around the trunk (spiral grain), pale grey with pinkish, white-striped slash (Arbonnier, 2002). This plant is widely utilized in traditional medicine by various cultures in the world and their applications vary (Le Houerou, 1980; Michel, 2002; Doka and Yagi 2009).

The objective of this research is to isolate and evaluate the antimicrobial and antioxidant activities of phytochemical constituent(s) from *Lannea kerstingii*.

heated at 120 °C for 15 minutes (Schreiber *et al.*, 1963). The absorbance was measured spectrophotometrically at 517 nm using UV-VIS spectrophotometer (DU 800; Beckman Coulter, Fullerton, CA, USA).

2.2 Plant material

The plant was collected in May, 2011 at area BZ, Ahmadu Bello Univeristy, Zaria, Kaduna State, Nigeria and identified by a botanist Mal. Umar Galla of the Department of Biological Science, Ahmadu Bello University Zaria, Nigeria. A voucher specimen (1832) was deposited in the herbarium for future references. After identification, the stem bark was dried under shade for two weeks, after which it was ground in to powder.

2.3 Extraction procedure

The powdered stem bark (580 g) was extracted using maceration (Raaman, 2006) with petroleum ether (3x1.5 L) at room temperature and occasional shaking for 48 h. The mark was subsequently extracted with ethyl acetate (3 x 1.5 L) at room temperature for another 48 h. Both extracts were filtered and concentrated under reduced pressure to yield a pale yellow gel petroleum ether extract (4.32 g) and a greenish brown solid ethyl acetate extract (10.72 g).

2.4 Isolation

The ethyl acetate extract (5 g) was subjected to dry vacuum liquid chromatography on silica gel (Coll and Bowden, 1986) using chloroform – ethyl acetate; 25:75 (210 ml), 50:50, (90 ml), 25:75 (4x90 ml), 0:100 (6x90 ml) successively (Pedersen and Rosenbohm, 2001). This procedure afforded 12 fractions. Fraction 6 (150 mg) from CHCl₃:EtOAc (75:25) contained some white microcrystalline precipitate which was separated from the rest of the solvent by decanting and washing with chloroform. The precipitate was further subjected to chromatography on sephadex LH-20 eluting with a mixture of CH₃OH and CHCl₃ (70:30) to yield 50 mg of compound **1**. The Thin layer chromatography (TLC) using CHCl₃-EtOAc (1:9) showed a spot with R_f value of 0.42. The ¹H and ¹³C NMR spectrum of compound **1** was recorded in DMSO-d₆ at 400 MHz and 125 MHz respectively. 2D experiments (COSY, HMQC and HMBC spectra) were also run on compound **1** using a JEOL ECX 400 MHz spectrometer.

2.5 Test organisms

The bacteria isolates viz; *Staphylococcus aureus*; *Methicillin Resistant Staphylococcus aureus*, *Streptococcus pyogenes*; *Bacillus subtilis*; *Corynebacterium ulcereans*; *Escherichia coli*; *Proteus vulgaris*; *Proteus mirabilis*; *Pseudomonas aeruginosa*; *Salmonella typhi*; *Shigella dysenteriae*; *Klebsiella pneumonia* and the fungi *Candida albicans*, *Candida krusei* and *Candida tropicalis* were gotten from Ahmadu Bello University Teaching Hospital, Zaria, Kaduna state, Nigeria. All the micro-organisms were checked for purity and maintained in slants of agar.

2.6 Assay for antibacterial activity

The test organisms were first inoculated into tubes of nutrient broth separately and incubated at 37°C for 18 h. Each of the cultures was then adjusted to 0.5 McFarland turbidity standard and (0.2 ml) inoculated onto Mueller Hinton Agar (MHA, Oxoid) in petri plates (diameter: 15 cm). A sterile cork borer was then used to make wells (6 mm diameter) on each of the plates containing cultures of the different test organisms. Compound **1** was re-dissolved in Dimethylsulfoxide (DMSO) to obtain stock concentrations of 200µg/ml and 0.1ml was then introduced into the wells using sterile Pasteur pipettes. DMSO (0.1 ml) was introduced in another well to serve as negative control. Wells containing the standard antimicrobials ciprofloxacin and fluconazole (5µg/ml) were included as positive control. The culture plates were allowed to stand on the working bench for 30 min for pre diffusion and were then incubated at 37°C for 24 h (bacteria) and 27°C for 48 hrs (fungi). After 24/48 h, antibacterial/antifungal activity was determined by measurement of diameter zones of inhibition (mm) (against the test organisms) around each of the extracts and the antibiotics/antifungal (Lino and Deogracious, 2006). The zone of inhibition was measured in triplicates.

2.7 Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of the compound was determined for each of the test organisms at varying concentrations of 200, 100, 50, 25, 12.5, and 6.25µg/ml. To obtain these concentrations, varying concentrations (1 ml) of compound **1** containing double strength of the concentrations (200, 100, 50, 25, 12.5, and 6.25 µg/ml) in a test tube, 1 ml of nutrient broth was added and then a loop full of the test organism previously diluted to 0.5 McFarland turbidity standard was introduced to the tubes. The procedure was repeated on the test organisms using the standard antibacterial ciprofloxacin and fluconazole. A tube containing nutrient broth only was seeded with the test organism to serve as negative control. All the tubes were then incubated at 37°C for 24 h (bacteria) and 27°C for 48 h (fungi) and after, examined for growth by observing for turbidity (Lino and Deogracious, 2006). The MIC was recorded as the mean of triplicates.

2.8 Minimum Bactericidal Concentration (MBC)/ Minimum Fungicidal Concentration (MFC)

Bacterial/fungi culture (1 ml) was pipetted from the mixture obtained in the determination of MIC tubes which did not show any growth in MIC and subcultured on to MHA and incubated at 37°C for 24 hrs (bacteria) and 27°C for 2-7 days (fungi) respectively. After incubation the concentration at which there was no single colony growth of bacteria/fungi was taken as MBC/MFC (Ajiyeoba *et al.*, 2003).

2.9 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assay

The method of Yu *et al.*, (2008) was used for the determination of scavenging activity of DPPH free radical. One ml of 0.135 mM DPPH prepared in methanol was mixed with 1.0 ml of the compound at different concentrations (0.125, 0.25, 1, 2 and 4 mg/ml). The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30 min. The control solution

was prepared by mixing methanol (1.0 ml) and DPPH radical solution (1.0 ml). The absorbance was measured spectrophotometrically at 517 nm using UV-VIS spectrophotometer. Tests were carried out in triplicate. The scavenging ability of the compound was calculated using this equation;

$$\text{DPPH Scavenging activity (\%)} = [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})/(\text{Abs}_{\text{control}})] \times 100$$

where $\text{Abs}_{\text{control}}$ is the absorbance of DPPH + methanol;

$\text{Abs}_{\text{sample}}$ is the absorbance of DPPH radical + sample (i.e. compound).

2.10 Statistical analysis

The data were presented as mean \pm standard deviation (SD). Statistical analyses were performed using one way analysis of variance and $P < 0.05$ was taken to be significant. All computations were done by employing the statistical software (SPSS 16.0; Chicago, IL, USA).

3.0 RESULTS

3.1 Isolation of compound 1 (β -Sitosteml-3-O-glucoside)

The TLC plate containing compound 1 showed a red-brown colour when sprayed with a solution

of cetric sulphate in 65% sulphuric acid and plate heated at 120 °C for 15 minutes.

Compound 1 (β -Sitosteml-3-O-glucoside) White microcrystalline powder, soluble in mixture of CH_3OH and CHCl_3 , m.p. 290 °C.

^1H NMR (400 MHz, DMSO- d_6): 0.65 (3H, s, Me-18) 0.80 (3H, m, Me-26), 0.79 (3H, m, Me-27) 0.85 (3H, m, Me-29) 0.96 (3H, s, Me-19) 0.90 (3H, d, $J = 7.2$ Hz, Me-21) 3.37 (1H, m, H-3), 4.24 (1H, H-1'), 5.35 (1H, m, H-6); ^{13}C NMR (125 MHz, DMSO- d_6): ppm 12.16 (C-18) 12.28 (C-29), 20.20 (C-26), 19.10 (C-19) 19.58 (C-27), 21.06 (C-11) 23.05 (C-28), 24.33 (C-15), 25.86 (C-23), 28.27 (C-16) 31.84 (C-25), 29.15 (C-2), 31.80 (C-8), 31.84 (C-7), 33.76 (C-22), 36.68 (C-20), 37.29 (C-10), 38.75 (C-1), 39.55 (C-4), 39.34 (C-12), 42.32 (C-13), 45.59 (C-24), 50.01 (C-9), 56.69 (C-17), 55.92 (C-14), 61.55 (C-6'), 70.54 (C-4'), 74.09 (C-2'), 77.42 (C-5'), 77.42 (C-3'), 77.42 (C-3), 101.41 (C-1'), 121.83 (C-6), 140.90 (C-5).

3.2 Antimicrobial activity of β -sitosterol-3-O-glucoside

Compound 1 was found to be active against *S. aureus*, MRSA, *B. subtilis*, *E. coli*, *P. mirabilis*, *S. typhi*, *S. dysenteriae* and *K. pneumoniae* for the bacteria and *C. albicans* and *C. tropicalis* for

the fungi. It was found to be inactive against *C. ulcerans*, *P. vulgaris*, *P. aeruginosa* and *C. krusei* (Table 1). The zone of inhibition ranges from 22.0 ± 0.4 to 34.0 ± 0.3 mm. The compound was very active against *B. subtilis* with zone of inhibition 34mm followed by *S. dysenteriae* (30mm).

Table 1: Zone of inhibition of β -sitosterol-3-O-glucoside on some disease causing organisms.

Organisms	Zone of inhibition (mm \pm SD)		
	C9 (200 μ g/ml)	Ciprofloxacin (5 μ g/ml)	Fluconazole (5 μ g/ml)
<i>S. aureus</i>	27.0 \pm 0.3	42.0 \pm 0.2	
<i>MRSA</i>	24.0 \pm 0.4	0.0	
<i>S. pyogenes</i>	0.0	47.0 \pm 0.3	
<i>B. subtilis</i>	34.0 \pm 0.3	47.0 \pm 0.2	
<i>C. ulcerans</i>	0.0	37.0 \pm 0.1	
<i>E. coli</i>	27.0 \pm 0.1	34.0 \pm 0.2	
<i>P. vulgaris</i>	0.0	24.0 \pm 0.3	
<i>P. mirabilis</i>	24.0 \pm 0.3	30.0 \pm 0.1	
<i>P. aeruginosa</i>	0.0	40.0 \pm 0.2	
<i>S. typhi</i>	24.0 \pm 0.2	24.0 \pm 0.1	
<i>S. dysenteriae</i>	30.0 \pm 0.1	27.0 \pm 0.2	
<i>K. pneumonia</i>	31.0 \pm 0.2	30.0 \pm 0.1	
<i>C. albicans</i>	23.0 \pm 0.3		32.0 \pm 0.1
<i>C. krusei</i>	0.0		30.0 \pm 0.3
<i>C. tropicalis</i>	22.0 \pm 0.4		29.0 \pm 0.1

The MIC of compound **1** against the organisms ranged from 25.0 to 50.0 μ g/ml (Table 2). The MIC was 25 μ g/ml for *B. subtilis*, *S. dysenteriae* and *K. pneumonia*. The MBC ranges from 50.0 to 200.0 μ g/ml while the MFC was 200.0 μ g/ml each for both *C. albicans* and *C. tropicalis* (Table 2).

Table 2: MIC and MBC/MFC of compound **1**.

Organisms	MIC (μ g/ml)	MBC (μ g/ml)	MFC (μ g/ml)
<i>S. aureus</i>	25.0	100.0	
<i>MRSA</i>	50.0	100.0	
<i>B. subtilis</i>	25.0	50.0	
<i>E. coli</i>	50.0	100.0	
<i>P. mirabilis</i>	50.0	100.0	
<i>S. typhi</i>	50.0	200.0	
<i>S. dysenteriae</i>	25.0	100.0	
<i>K. pneumoniae</i>	25.0	50.0	
<i>C. albicans</i>	50.0		200.0
<i>C. tropicalis</i>	50.0		200.0S

3.3 Antioxidant activity

The DPPH free radical-scavenging activity (%) increased as the concentration increases with

least activity at 250 μ g/ml and maximum activity at 4000 μ g/ml (Figure 1).

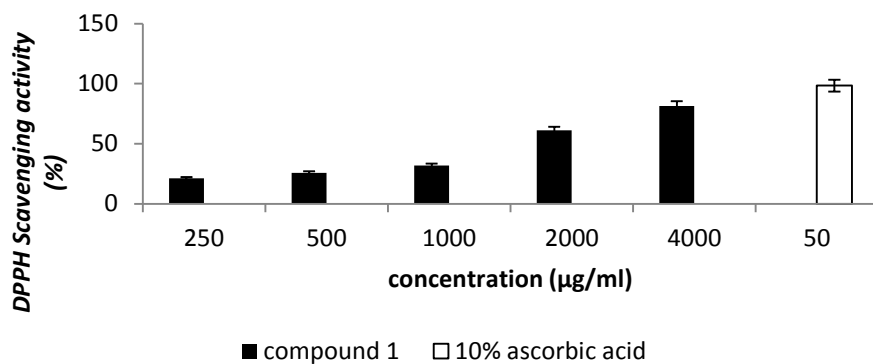


Figure 1. The DPPH free radical-scavenging activity (%) of β -Sitosteml-3-O-glucoside at different concentrations and 10% Ascorbic acid.

4.0 DISCUSSION

4.1 Structure elucidation of isolated compound 1

The red-brown color on the sprayed TLC plate containing compound **1** suggested the presence of a steroidal skeleton. The $^1\text{H-NMR}$ spectrum showed six methylic signals, two singlets (Me-18 and Me-19), three doublets (Me-21, Me-26 and Me-27), and one triplet (Me-29), whose chemical shifts obtained by a HETCOR experiment were in accordance with a Δ^5 sterol. The up field chemical shift at δ 38.75, 29.15, 77.42, 39.55, 121.83, 31.84, 21.06, 39.34, 24.33 and 28.27 were appropriate for the cyclohexyl and cyclopentyl carbons at positions 1, 2, 3, 4, 6, 7, 11, 12, 15 and 16, respectively (Rahmana *et al.*, 2009; Kasal *et al.*, 2010). The chemical shift at δ 56.69 was assigned to the carbon number 17

which was the point of linkage of the side chain to the cyclopentyl ring (Kasal *et al.*, 2010). The olefinic proton (H-6) was a multiplet at δ 5.35, confirmed also by the resonances of the ^{13}C NMR at δ 122 and δ 144 ppm. In addition signals of a glucose were also present. The HMBC correlation of δH 4.2 (H1) of the sugar with C3 (δC 77.42, J3) of the steroidal nucleus indicated that the glycosidic linkage at C-3 in the cyclopentyl ring. On the basis the $^1\text{H-NMR}$, $^{13}\text{C-NMR}$, and 2D-NMR (COSY, HMBC, HMQC) spectral data and comparing with literature (Bilia *et al.*, 1996; Wang *et al.*, 2009; Tagne *et al.*, 2014) shows that the isolated compound **1** was β -sitosterol-3-O-glucoside (Figure 2).

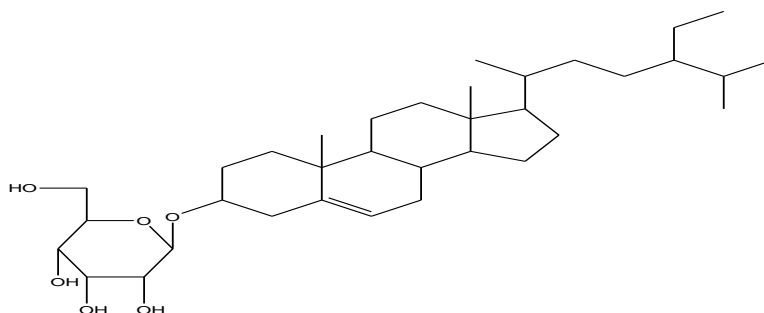


Figure 2. Compound **1** (β -sitosterol-3-O- β -D-glucoside).

4.2 Antimicrobial activity of β -sitosterol-3-O-glucoside

The very low MIC and MBC of β -sitosterol-3-O-glucoside on both gram positive and gram negative bacteria (Table 2) indicates the broad spectrum activity of β -sitosterol-3-O-glucoside. Studies established β -sitosterol-3-O- β -D-glucoside as potent antimicrobial agent at lower concentration against a wide range of bacteria including *E. subtilis*, *P. aeruginosa*, *S. faecalis*, *E. coli*, *S. aureus*, *K. pneumonia* and *N. gonorrhoea* (Tagne *et al.*, 2014) which is in line with the current studies although in the present study, β -sitosterol-3-O- β -D-glucoside was inactive against *P. aeruginosa* and active against the fungi *C. albicans* and *C. krusei* contrary to literature (Tagne *et al.*, 2014) which reported no activity.

The low MIC of β -sitosterol-3-O-glucoside showed the compound's activity against bacteria which are associated with different type of infections including urinary tract infections (Brooks *et al.*, 2002; Usman *et al.*, 2007) typhoid fever, pneumonia, diabetic foot infections (Shorr, 2007), burn wound infections, keratitis, and respiratory tract infections (Marquart *et al.*, 2005). In view of the fact that prevalence of *S. aureus* resistant strains to conventional antibiotics has increased to high levels in some hospitals (Usman *et al.*, 2007), β -sitosterol-3-O-glucoside could serve as a remedy

5.0 CONCLUSION

β -sitosterol-3-O-glucoside isolated for the first time from the stem bark of *Lannea kerstingii* showed a wide spectrum antimicrobial activity at concentration of 200.0 μ g/ml against *S. aureus*, *Mithicillin-Resistant Staphylococcus aureus*, *P. mirabilis*, *S. typhi*, *K. pneumoniae*, *E. coli*, *B. subtilis*, *C. albicans* and *C. tropicalis*.

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to such resistance since it is active against *MRSA*.

4.3 Antioxidant activity β -sitosterol-3-O-glucoside

The DPPH radical-scavenging assay was used to evaluate the ability of β -sitosterol-3-O-glucoside to scavenge free radicals and to promote the formation of the non-radical form DPPH-H by hydrogen-donating action (Chung *et al.*, 2006). Plants are a potential source of natural antioxidants, which act as reducing agents, hydrogen donors, oxidant and free radical scavengers. As shown in figure 2, the DPPH free radical-scavenging activity (%) of this compound increase as the concentration increases with the highest at 4000 μ g/ml though not comparable to the standard Ascorbic acid at 50.0 μ g/ml. The higher the DPPH values, the higher the antioxidant activity. The DPPH radical is a stable lipophilic free radical and is a measure of non-enzymatic antioxidant activity of compounds (Deng *et al.*, 2014). β -sitosterol stimulates antioxidant enzymes by activation of estrogen receptor/PI3-kinase-dependent pathway (Vivancos and Moreno, 2005). These results are in accordance with previous research on the antioxidant activity of β -sitosterol-3-O-glucoside (Badria *et al.*, 2007) and explain the observed antioxidant activity of *lannea kerstingii* (Tagne *et al.*, 2014).

The compound also showed antioxidant activity at 4000 μ g/ml which shows its ability to increase the activities of antioxidant enzymes. This result thus gives scientific base and credence for the claims of the therapeutic capabilities and folkloric usage of *Lannea kerstingii* for the treatment of various ailments.

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