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Original Article

Isolation and Antimicrobial Activity of β-Sitosterol-3-O-Glucoside from *Lannea Kerstingii* Engl. & K. Krause (Anacardiacea)

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Abstract

The emergence of more and more drug resistance bacteria has led to the study of the antimicrobial activity of the compound isolated from *Lannea kerstingii* Engl. & K. Krause (Anacardiacea) since the active principles of many drugs found in plants are secondary metabolites. A compound was isolated using dry vacuum liquid chromatography and eluting with $CHCl_3$-EtOAc and monitored using TLC. The glycoside was characterized using ^{1}H NMR and ^{13}C NMR spectra recorded in DMSO-d6 at 400 MHz and 125 MHz, respectively. The antimicrobial activity of the compound was determined using agar diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal/minimum fungicidal concentration (MBC/MFC) was determined using broth dilution method. The compound isolated was found to be β-sitosterol-3-O-glucoside. The β-sitosterol-3-O-glucoside (200µg/ml) was active against *S. aureus, Methicillin Resistant Staphylococcus aureus, P. mirabilis, S. typhi, K. pneumoniae, E. coli, B. subtilis* with zone of inhibition ranging from 24mm to 34mm and inactive against *P. aeroginosa* and *Proteus vulgaris.* It was also active against the fungi *C. albicans and C. tropicalis* but inactive against *C. krusei.* The MIC ranged from 25 to 50 µg/ml while the MBC/MFC ranged from 50 to 200 µg/ml. These results show the wide spectrum antimicrobial activity of β-sitosterol-3-O-glucoside.

Keywords : sitosterol, glycoside, Lannea kerstingii, isolation

Introduction

Infectious diseases is one of the leading cause of death worldwide. In 2012, it was responsible for 68% of all deaths globally [1]. All these are due to the emergence of multidrug resistant pathogens which contributes to the high causes of death world-wide [2]. The screening of plant extracts and plant products for antimicrobial activity has shown that plants represent a potential source of new anti-infective agents [3].

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Lannea kerstingii has been reported to contain: tannins, flavonoids, alkaloids, steroids and triterpenes [4,5,6]. It is a tree with a height of 12m and 40cm 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 [7]. This plant is widely utilized in traditional medicine by various cultures in the world and their applications vary. In Sudan, a decoction of the back is used to treat swellings [8]. The back, leaves and bud are used for flatulence [9], the fruits are used against rickets and scurvy [10], the stem bark is also used in the treatment of malaria[11], hemorrhage, diarrhoea and epilepsy[4]. Also, in some areas in West Africa, it is prescribed against oedema, rickets, wounds, scurvy, scorbut and epilepsy [7].

Materials and method

Plant collection and identification

The plant was collected in May, 2011 at area BZ, Ahmadu Bello University, 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 shad for two weeks, after which the size was reduced using mortar and pestle, filtered for homogeneity and kept away from light until further use.

Extraction

The stem bark (580g) was extracted (maceration) with petroleum ether (3x1.5L) at room temperature. The mark was subsequently extracted with ethyl acetate (3 x 1.5L) at room temperature. Both extracts were concentrated under reduced pressure to yield a pale yellow gel petroleum ether extract (4.32g) and a greenish brown solid ethyl acetate extract (10.72g).

Isolation

The ethyl acetate extract (5g) was subjected to dry vacuum liquid chromatography [12] on silica gel for TLC (Merck) using chloroform – ethyl acetate; 25:75 (210ml), 50:50, (90ml), 25:75 (4x90ml), 0:100 (6x90ml) successively. This procedure afforded 12 fractions. Fraction 6 (150mg) from $CHCl_3$:EtOAc (75:25) contained some 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 CH_3OH and $CHCl_3$ (70:30) to yield 50mg of compound labeled C9. The Thin layer chromatography (TLC) using $CHCl_3$-EtOAc (1:9) showed a spot with Rf value of 0.42. The ^{1}H and ^{13}C NMR spectrum of C9 was recorded in DMSO-d6 at 400 MHz and 125 MHz, respectively.

TLC chromatograms obtained were sprayed with a saturated solution of cetric sulphate in 65% sulphuric acid; the plates were heated at 120 °C for 15 minutes [13].

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.

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) for the compound on each of the plates containing cultures of the different test organisms. The compound (C9) was re-dissolved in DMSO to obtain concentrations of 200ug/ml. 0.1 ml of the C9 was then introduced into the wells using sterile Pasteur pipettes. 0.1 ml of DMSO only was introduced in another well to serve as negative control. Wells containing the standard antimicrobials ciprofloxacin and fluconazole (5ug/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. After 24 h, antibacterial activity was determined by measurement of diameter zones of inhibition (mm) (against the test organisms) around each of the extracts and the antibiotics/antifungal [14].

Minimum Inhibitory Concentration (MIC)

The MIC of the extracts were determined for each of the test organisms at varying concentrations of 200, 100, 50, 25, 12.5, and 6.25 mg/ml. To obtain these concentrations, varying concentrations (1 ml) of the extracts containing double strength of the concentrations (200, 100, 50, 25, 12.5, and 6.25 mg/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 hrs (fungi) and after, examined for growth by observing for turbidity [14].

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

1 ml bacterial/fungi culture was pipetted from the mixture obtained in the determination of MIC tubes which did not show any growth and subcultured on to MHA and incubated at 37°C for 24 hrs (bacteria) and 27°C for 2-7days (fungi) respectively. After incubation the concentration at which there was no single colony growth of bacteria/fungi was taken as MBC/MFC [15].

Results

The ^{1}H-NMR of compound C9 revealed a series of peaks (singlets) between dH 0.65 to dH 0.99. It also showed a prominent peak at dH 3.37, peaks between dH 2.8 to 3.8 indicates the presence of a glucoside. Some other prominent peaks include dH 5.35 (t) indicating an olefinic proton, dH 4.21(d) indicating an anomeric proton.

The ^{13}C-NMR showed the presence of 35 carbon atoms with some prominent peaks like dc 140.9, dc 121.8, dHc 101.4, dc 77.42 which indicates the presence of olefins, and carbons linked to oxygen. Some other prominent peaks include dc 74.09, dc 70.54, dc 61.55 which also indicates carbons linked to oxygen.

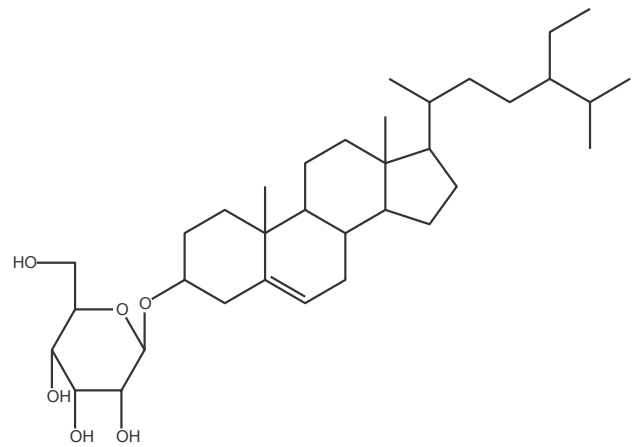
The HSQC was used to connect the protons with their various carbon atoms as summarized in Table 4.3. The HMBC showed correlations between the protons signal at dH 4.2 (H1') and carbon signal dc 77.42 (C3, J3).

Compound C9 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 24mm to 34mm. The compound was very active against *B. subtilis* with zone of inhibition 34mm followed by *S. dysenteriae* (30mm).

The MIC of the isolated compound against the organisms ranged from 25µg/ml to 50µg/ml (Table 2). The MIC of C9 was 25µg/ml for *B. subtilis, S. dysenteriae* and *K. pneumonia.* The MBC ranges from 50µg/ml to 200µg/ml while the MFC was 200µg/ml for both *C. albicans* and *C. tropicals* (Table 2).

ß-Sitosteml-3-O-glucoside (1)

^{1}H NMR (400 MHz, DMSO-d6,): 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 (lH, m, H-3), 4.24 (lH, H-l'), 5.35 (IH, m, H-6); ^{13}C NMR (125 MHz, DMSO-d6): 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).



Compound C9: β-sitosterol-3-O-β-D-glucoside (**1**)

Discussion :

The TLC plate containing C9 showed a red-brown colour when sprayed with a solution of ceric sulphate in 65% sulphuric acid and plate heated at 120 °C for 15 minutes [13]. This suggested the presence of a steroidal skeleton. The protonic 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 ?^{5} sterol. The up field chemical shift at d 38.75, 29.15, 77.42, 39.55, 121.83, 31.84, 21.06, 39.34, 24.33 and 28.27

Table 1: Zone of inhibition of C9 on some disease causing organisms

Organisms	Zone of inhibition (mm)		
	C9 (200µg/ml)	Ciprofloxacin (5µg/ml)	Fluconazole (5µg/ml)
S. aureus	27	42	
MRSA	24	0	
S. pyogenes	0	47	
B. subtilis	34	47	
C. ulcerans	0	37	
E. coli	27	34	
P. vulgaris	0	24	
P. mirabilis	24	30	
P. aeruginosa	0	40	
S. typhi	24	24	
S. dysenteriae	30	27	
K. pneumoniae	31	30	
C. albicans	23		32
C. krusei	0		30
C. tropicalis	22		29

Table 2 : MIC and MBC/MFC of C9

Organisms	MIC (µg/ml)	MBC (µg/ml)	MFC (µg/ml)
S. aureus	25	100	
MRSA	50	100	
B. subtilis	25	50	
E. coli	50	100	
P. mirabilis	50	100	
S. typhi	50	200	
S. dysenteriae	25	100	
K. pneumoniae	25	50	
C. albicans	50		200
C. tropicalis	50		200

were appropriate for the cyclohexyl and cyclopentyl carbons at positions 1, 2, 3, 4, 6, 7, 11, 12, 15 and 16, respectively [16,17]. The chemical shift at d 56.69 was assigned to the carbon number 17 which was the point of linkage of the side chain to the cyclopentyl ring [17]. The olefinic proton (H-6) was a multiplet at d 5.35, confirmed also by the resonances of the ^{13}C NMR at d122 and d144 ppm. In addition signals of a glucose were also present. The HMBC correlation of dH 4.2 (H1) of the sugar with C3 (dc 77.42, J3) of the steroidal nucleus indicated that the glycosidic linkage at C-3 in the cyclopentyl ring. On the basis the ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR spectral data and comparing with literature [18,19] shows that the isolated compound C9 was ß-sitosterol-3-O-glucoside.

The very low MIC and MBC of C9 on both gram positive and gram negative bacteria (Table 2) indicates the broad spectrum activity of β-sitosterol-3-O-glucoside its potential use as antimicrobial agent. Studies established ß-sitosterol as potent antimicrobial agent at lower concentration against a wide range of bacteria including *E. coli, Staphylococcus aureus, Klebsiella pneumonia and Pseudomonas aeruginosa* [20] which is in line with the current studies though compound C9 (β-sitosterol-3-O-β-D-glucoside) was inactive against *P. aeruginosa.*

The low MIC of β-sitosterol-3-O-glucoside (Table 2) showed compound's activity against both Gram positive and Gram negative bacteria which are associated with different type of infections including urinary tract infections and typhoid fever (*S. typhi*). *S. aureus* which is also responsible for a wide variety of diseases including skin and soft tissue infections, pneumonia, and diabetic foot infections [21] can be managed using this compound. Similarly, *P. aeruginosa* is a common pathogen associated with burn wound infections, keratitis, and respiratory tract infections [22]. The compound also showed activity against *E. coli* which is the commonest cause of urinary tract infection and accounts for approximately 90% of first urinary tract infection in young women [23]. Thus this indicates the usefulness of this plant in the treatment of urinary tract infection, respiratory tract infections, diabetic foot infections due to its activity against the organisms causing these infections.

In view of the fact that *S. aureus* is a pyogenic bacterium known to play significant role in invasive skin diseases including superficial and deep follicular lesion [24] and that prevalence of *S. aureus* resistant strains to conventional antibiotics has increased to high levels in some hospitals [23], β-sitosterol-3-O-β-D-glucoside could serve as a remedy to such resistance since it is active against MRSA. The compound has also showed the same high level of activity against *E. coli* which is the commonest cause of urinary tract infection and accounts for approximately 90% of first urinary tract infection in young women [23,25]. Thus this result gives scientific base and credence for the claims of the therapeutic capabilities and folkloric usage of the various

parts of *Lannea kerstingii* for the treatment of various ailments.

Conclusion

The compound ß-sitosterol-3-O-glucoside isolated from *Lannea kerstingii* showed a wide spectrum antibacterial and antifungal activity at concentration of 200µg/ml against *S. aureus, MRSA, P. mirabilis, S. typhi, K. pneumoniae, E. coli, B. subtilis and also* active against the fungi *C. albicans and C. tropicalis.* This shows that the compound's ability to treat a wide range of infectious diseases.

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