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Synthesis and antibacterial activity of 7-deacetoxy-7α-hydroxygedunin

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A convenient synthetic route for bioactive 7-deacetoxy-7α-hydroxygedunin was realized by one-step conversion of 7-deacetoxy-7α-hydroxygedunin potassium salt. The 7-deacetoxy-7α-hydroxygedunin was obtained in 88% w/w yield as a fluffy white precipitate by recrystallization from methanol. The 7-deacetoxy-7α-hydroxygedunin and the starting material were evaluated for antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* using the microbroth dilution assay. It was discovered that 7-deacetoxy-7α-hydroxygedunin potassium salt had minimum inhibitory concentration (MIC) of 1000 µg/ml against *K. pneumoniae*, 2000 µg/ml against *S. aureus*, and *E. coli*. However, *B. subtilis* was not susceptible to 7-deacetoxy-7α-hydroxygedunin potassium salt at 2000 µg/ml. Interestingly, 7-deacetoxy-7α-hydroxygedunin had MIC of 2000 µg/ml against *B. subtilis*, and *E. coli*; and MIC of 1000 µg/ml against *K. pneumoniae*.

Key words: 7-deacetoxy-7α-hydroxygedunin, synthesis, gedunin potassium salt, antibacterial.

INTRODUCTION

Semi-synthetic natural product derivatization continues to be a veritable tool in drug discovery. This trend is sustained by current growing global demand for new therapeutically effective medicines from renewable natural sources. Globally, about 75% of all anti-infective drugs are of natural origin; some had been synthetically modified to improve their therapeutic properties (Newman et al., 2003).

Amit and Shailendra (2006) recommended that the structure activity relationships of gedunin limonoids be pursued to allow safe introduction as a pharmaceutical drug.

In response to the recommendation by Amit and Shailendra (2006) we reported the synthetic modification of gedunin (1) to gedunin potassium salt (2) and antibacterial activity of gedunin potassium salt (Okhale et al., 2012). Mahmoodin a derivative of gedunin have been reported to possess antibacterial activity (Siddiqui et al., 1992).

In 1961, preparation of 7-deacetoxy-7α-hydroxygedunin (3) was reported. The 7-deacetoxy-7α-hydroxygedunin was prepared by hydrolysis of gedunin under mild alkaline condition, followed by acidification and column chromatography of the product on alumina.

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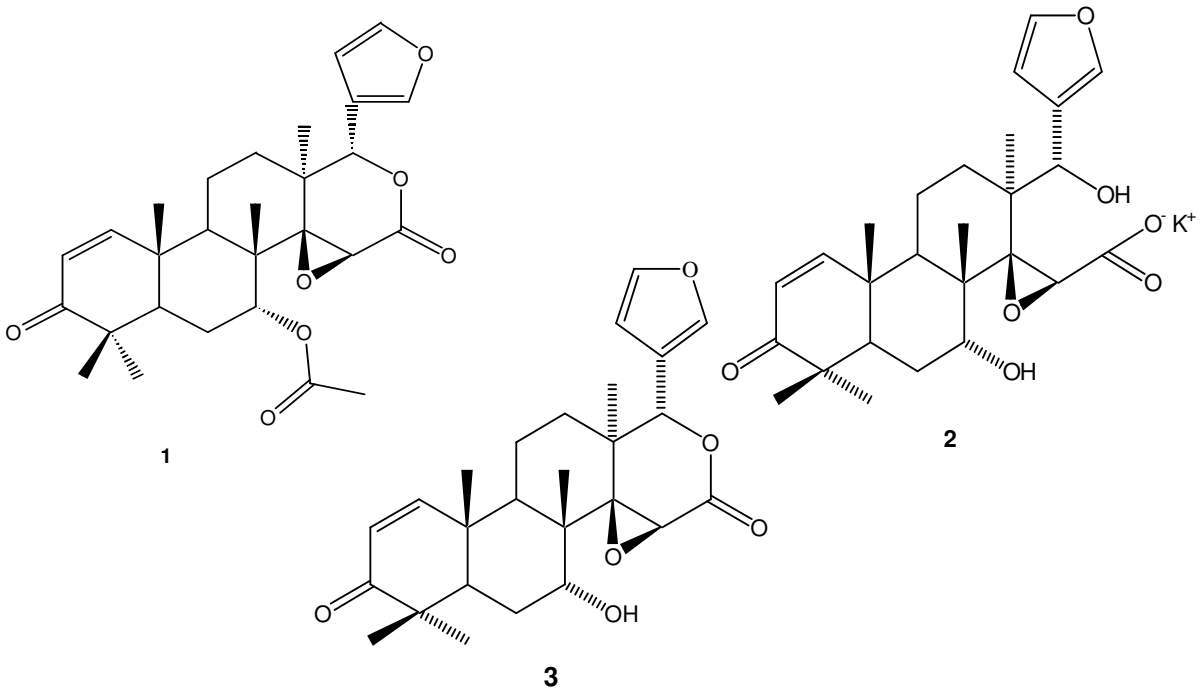

Figure 1. Structure of (1) gedunin; (2) 7-deacetoxy-7α-hydroxygedunin potassium salt; and (3) 7-deacetoxy-7α-hydroxygedunin.

The pure 7-deacetoxy-7α-hydroxygedunin was obtained in 43.4% w/w yield (Akisanya et al., 1961). Naturally occurring 7-deacetoxy-7α-hydroxygedunin along with other hydroxylated gedunin derivatives were isolated from *Cedrela sinensis* (Mitsui et al., 2006) and from the root and leaf of *Cedrela fissilis* (Alessandra et al., 2006). However, the antibacterial activity of 7-deacetoxy-7α-hydroxygedunin had not been reported. In continuation of our search for antibacterial gedunin limonoids, the aim of this work is to make 7-deacetoxy-7α-hydroxygedunin (3) and investigate its antibacterial activity. It also aims to compare the antibacterial activity of gedunin potassium salt (2) and 7-deacetoxy-7α-hydroxygedunin (3) for any possible structure-activity relationship (Figure 1).

MATERIALS AND METHODS

Nuclear magnetic resonance spectroscopy (NMR)

^{1}H and ^{13}C NMR data were obtained using Bruker Avance III 500 and Avance 500 asc with ascend magnet both of 500 MHz at the Columbia University, USA. Gedunin and 7-deacetoxy-7α-hydroxygedunin were dissolved in deuterated chloroform ($CDCl_3$) and gedunin potassium salt was dissolved in deuterated water (D20).

Melting point determination

Melting points of compounds were determined using Barnstead Electrothermal Melting Point Apparatus (Model 9100).

Reagents and chemicals

Hexane (analytical grade), ethyl acetate (analytical grade), methanol (analytical grade), vanillin (analytical grade), $CDCl_3$ (analytical grade) and D20 (analytical grade), were purchased from Sigma Aldrich (Germany).

Extraction and isolation of gedunin

The isolation of gedunin was done as previously reported (Okhale et al., 2012). Briefly, the dried pulverized stem wood of *Entandrophragma angolense* (3 kg) was packed into an aspirator bottled and extracted by percolation, refluxing with n-hexane (6 L) at 60°C for 48 h. The extract was vacuum filtered with Whatman No. 1 filter paper. The filtrate was concentrated under vacuum with rotary evaporator at 40°C to 100 ml and kept in the dark for 24 h during which time gedunin crystals formed. The gedunin crystals were collected by filtration, dried in a desiccator, and recrystallized from methanol in 0.092% w/w yield. The melting point of the crystals was determined to be 219 to 220°C. This melting point was undepressed by the addition of authentic gedunin sample which had a melting point of 219 to 220°C.

Preparation of 7-deacetoxy-7α-hydroxygedunin potassium salt

Gedunin potassium salt was prepared as reported (Okogun and Orisadipe 1996). Briefly, 11 g (0.0225 mol) of gedunin was dissolved in 130 ml of aqueous methanolic potassium hydroxide (5.5 g, 0.0975 mol.) in a 250 ml conical flask. It was then warmed

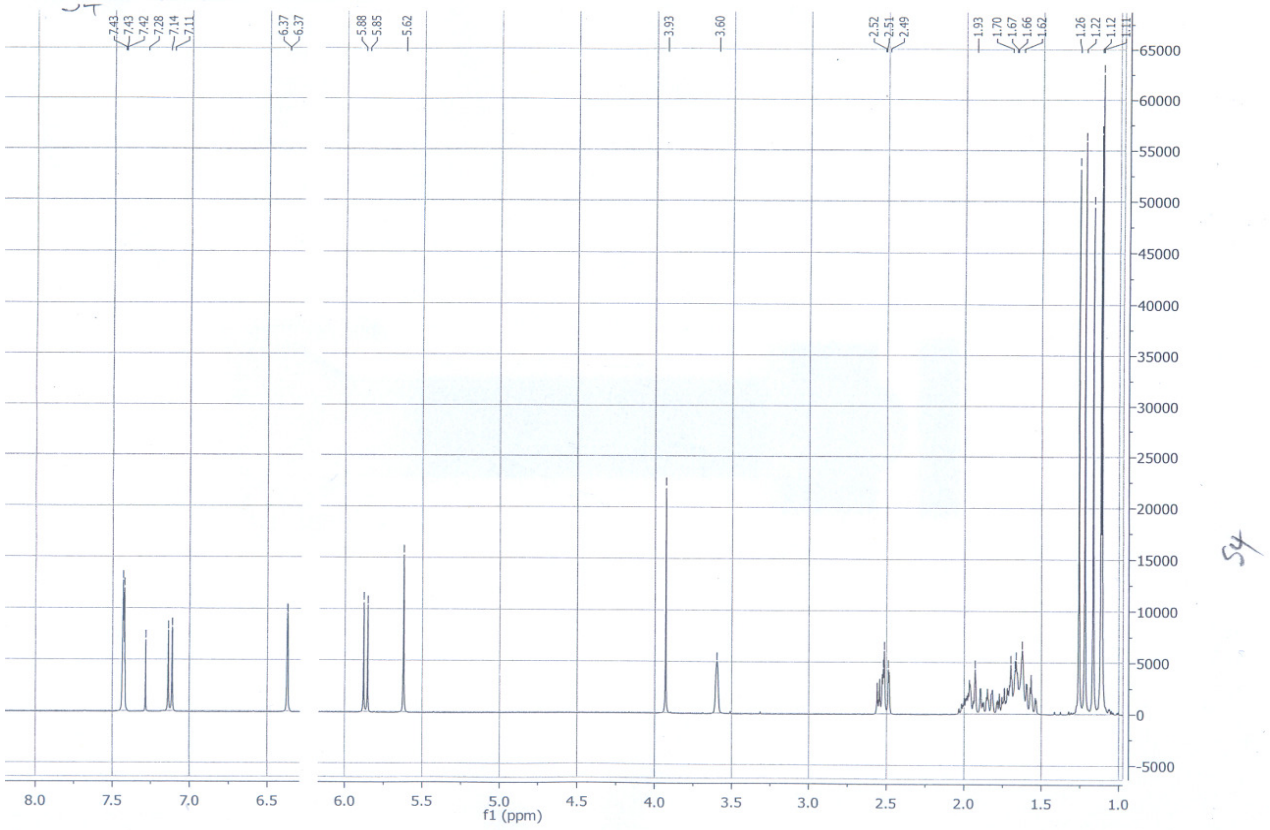

Figure 2. Full ^{1}H NMR Spectra of 7-deacetoxy-7α-hydroxygedunin.

with swirling in a water bath at 55°C until it dissolved. The swirling was continued in the water bath for a further 20 min without bringing the solution to boil. The solution was left to stand at room temperature, crystals began to form within an hour. After four hours at room temperature, white crystals of 7-deacetoxy-7α-hydroxygedunin potassium salt (2) were collected by vacuum filtration using Whatman No. 1 filter paper and sintered glass funnel. The crystals were washed with ice cold methanol and dried in a desiccator. Yield was determined to be 9.9581 g (90.53%).

Acidification of 7-deacetoxy-7α-hydroxygedunin potassium salt

7-deacetoxy-7α-hydroxygedunin potassium salt (4.0 g) was dissolved in distilled water (100 ml) to give a clear solution. Upon acidification of the solution with 10% v/v hydrochloric acid solution to pH 2.0 (pH indicator, Macherey-Nagel, Germany), white fluffy precipitate formed. The precipitate was collected by vacuum filtration and dried in a desiccator. The dried precipitate was recrystallized from methanol as colourless crystals and designated OK-S4. Yield was determined to be 3.52 g (88% w/w). The melting point of OK-S4 was 240-242°C. Figures 2 and 3 show the ^{1}H and ^{13}C nmr spectra of OK-S4 respectively.

Thin layer chromatography

The acidification reaction of 7-deacetoxy-7α-hydroxygedunin potassium salt to form OK-S4 was monitored on thin layer chromatography. The OK-S4 and 7-deacetoxy-7α-hydroxygedunin potassium salt were dissolved in chloroform and spotted on precoated glass normal phase TLC plate (K5, Whatman) stationary phase previously activated at 110°C for 2 h. The mobile phase consisted of hexane and ethyl acetate (3:2). After development, the plate was air dried and sprayed with 1% w/v vanillin sulphuric acid reagent. The sprayed plate was heated at 105°C for 2 min. The chromatogram showed OK-S4 at R_f 0.67 with 7-deacetoxy-7α-hydroxygedunin potassium salt remaining at the origin unmoved.

Bacterial strain

Four bacteria organisms comprising two typed namely *Staphylococcus aureus* (S2014 Sigma-Aldrich), *Escherichia coli* ATCC 25922; and two clinical isolates of *Bacillus subtilis,* and *Klebsiella pneumonia* were subjected to susceptibility test.

Spectroscopic monitoring of bacterial strain

Overnight broth cultures of the test organisms *S. aureus* (S2014 Sigma-Aldrich), *E. coli* ATCC 25922, *B. subtilis,* and *K. pneumoniae* were diluted to10^{7}cfu/ml monitored by spectrophotometric methods described by Dominguez et al. (2001). Two to three colonies of 20 h growth on Mueller-Hinton Agar of the organisms to be studied were suspended on 50 ml pre-warmed (37°C) Mueller-Hinton broth. The suspension was incubated overnight at 37°C, diluted 1/2500 in the same pre-warmed medium and incubated in water bath with agitation (50 rpm). The absorbance of the culture was monitored

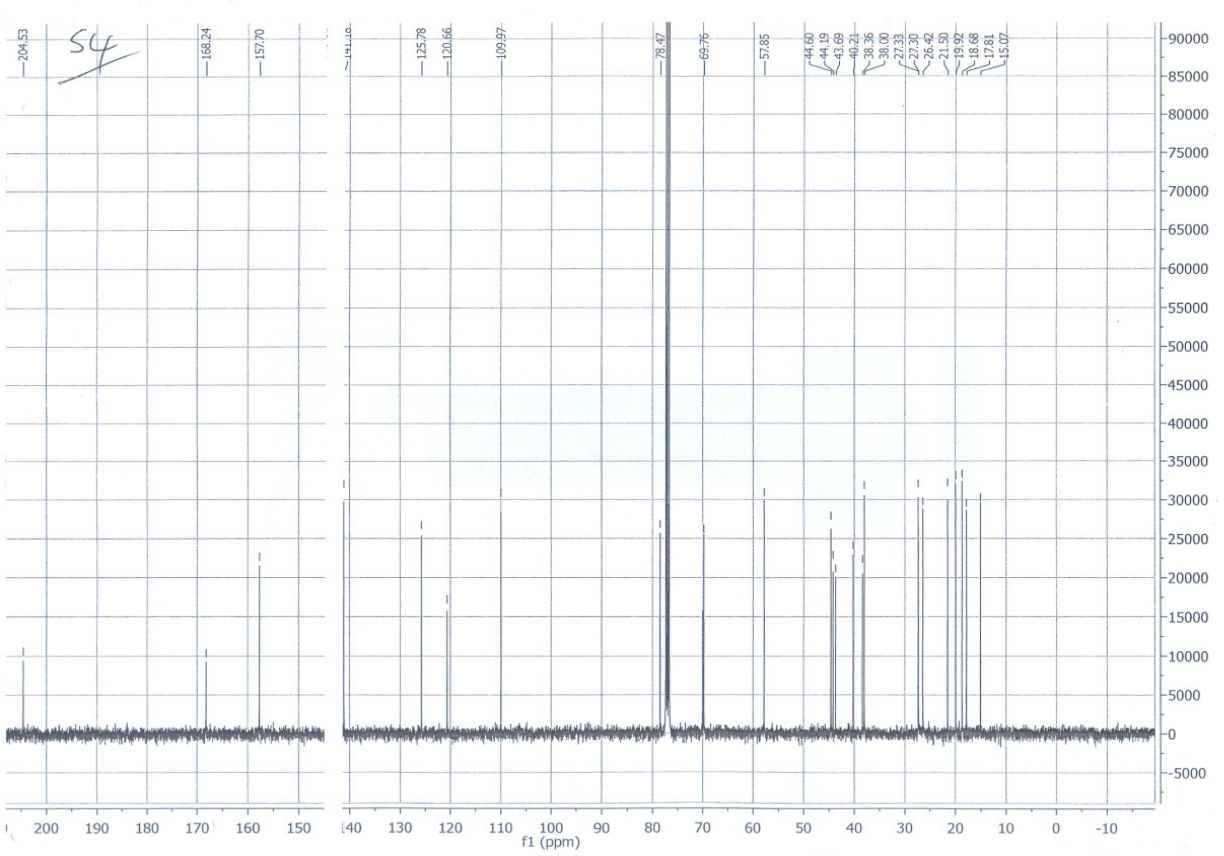

Figure 3. Full ^{13}C NMR Spectra of 7-deacetoxy-7α-hydroxygedunin.

with a spectrophotometer (6405 Jenway, Barloworld Scientific Ltd. Dunmow, Essex CMB 3LB), using 450 nm wavelength and 1 cm cuvette until absorbance of 0.1 was reached and a plotted standard curve (equivalent to 2.5-3.0 × 10^7cfu/ml for *E. coli* and 1.8-2.0 × 10^7cfu/ml for *S. aureus* and *B. subtilis*, respectively). The experimental and controls were done in duplicates.

Antibacterial minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentrations (MICs) of the compounds were determined by broth microdilution method (BMM). Two milligram of each compound was dissolved in 0.2 ml of DMSO and made up to 1 ml with 0.8 ml of sterile Mueller-Hinton nutrient broth to give concentration of 2000 µg/ml. Then, 100 µl of each test compound solution was transferred to the first designated well of a sterile 96 well microliter plate in duplicate. Fifty microliter of the sterile nutrient broth was pipetted into wells 2 to 12. Wells A1 – A12 and B1 – B12 were used for organism control. Wells G1 – G12 and H1 – H12 were used for standard drug control. Using a multi-channel pipette (Finnpipette II, Fisherbrand 50-300 µl), fifty microliter of solution in well 1 was transferred to well 2, mixed thoroughly by pipetting up and down four times, and the process repeated through to well 12 where fifty microliter was withdrawn and discarded. All the wells were inoculated with fifty microliter of overnight diluted cultures of each organisms (10^7cfu/ml) prepared by spectroscopic method as described earlier. The plates were incubated for 24 h at 37°C. Standard drug used was Ciprotab (Fidson, Lagos Nigeria), at concentration of 50 µg/ml. Media sterility control was also set up. MIC was determined as the last concentration where no growth was observed. The presence or absence of growth of organism was observed using macroscopic inverted mirror (Oladosu, 2011). All assays were carried out in duplicate.

RESULTS AND DISCUSSION

Extraction and isolation of gedunin

Gedunin (1) from the hexane extractive of *E. angolense* stem wood and recrystallized from methanol was obtained in 0.92% w/w yield with a melting point of 219 - 220°C. The melting point was undepressed by the addition of authentic gedunin sample. The ^1H and ^{13}C chemical shift δ-values of the crystals were consistent with those reported for gedunin by Gonzalez et al. (1989) and Duddeck and Dietrich (1992).

Alkaline hydrolysis of gedunin

Gedunin was converted to 7-deacetoxy-7α-hydroxy-gedunin potassium salt (2), by methanolic alkaline

hydrolysis as previously reported by Okogun and Orisadipe (1996). The product was obtained in 90.53%yield. It decomposed at 180°C with characteristic effervescence due to evolution of furfural.

Acidification of 7-deacetoxy-7α-hydroxygedunin potassium salt

Acidification of 7-deacetoxy-7α-hydroxygedunin potassium salt with 10% hydrochloric acid solution led to closure of the opened lactone ring to form a white fluffy precipitate, which was recrystallized from methanol as colourless crystals designated OK-S4 in 78% w/w yield. The melting point of OK-S4 was found to be 240 to 242°C. The ^{1}H and ^{13}C nmr chemical shift δ-values of OK-S4, presented in Figures 2 and 3, were consistent with those of 7-deacetoxy-7α-hydroxygedunin as reported in the literature (Mitsui et al., 2006; Alessandra et al., 2006).

^{1}H and ^{13}C NMR spectra analyses of gedunin

The ^{1}H and ^{13}C nmr spectra assignment for the gedunin were in agreement with those reported by Gonzalez et al. (1989) and Duddeck and Dietrich. (1992). ^{1}H nmr spectrum showed the presence of five methyl protons δ 1.09 (3H, s, H-28), 1.10 (3H, s, H-29), 1.18 (3H, s, H-30), 1.25 (3H, s, H-19) and 1.28 (3H, s, H-18), one acetate methyl proton 2.13 (3H, s, H-32) each for three protons; three methylene protons 1.96 (1H, dd, H-6α), 1.84 (1H, dd, H-6β), 2.03 (1H, m, H-11α), 1.87 (1H, m, H-11β), 1.59 (1H, m, H-12α), 1.73 (1H, m, H-1β), ten methine protons 7.12 (1H, s, H-1), 5.89 (1H, s, H-2), 2.21 (1H, d, H-5), 4.55 (1H, s, H-7), 2.52 (1H, dd, H-9), 3.56 (1H, s, H-15), 5.64 (1H, s, H-17), 6.37 (1H, s, H-22) and at δ 7.44 for two accidentally isochronous protons (H-21 and H-23), with total number of signals accounting for 34 hydrogen atoms.

The ^{13}C nmr spectrum of gedunin showed nine quaternary carbons at δ 204.09 (C=O, C-3), 44.06 (C, C-4), 42.65 (C, C-8), 40.04 (C, C-10), 38.75 (C, C-13), 69.75 (C-O, C-14), 167.42 (O-C=O, C-16), 120.44 (C, C-20),169.88 (O-C=O, C-31); ten methine carbons at δ156.91 (HC=C, C-1), 126.03 (HC=C, C-2), 46.06 (CH, C-5), 73.24 (HC-O, C-7), 39.56 (CH, C-9), 56.91 (HC-O, C-15), 78.27 (HC-O, C-17), 143.09 (HC=C, C-21), 109.87 (HC=C, C-22), 141.21 (HC=C, C-23); three methylene carbons at δ 23.28 (CH_2, C-6), 15.00 (CH_2, C-11), 26.03 (CH_2, C-12); five methyl carbons at δ17.75 (CH_3, C-18), 19.74 (CH_3, C-19), 27.18 (CH_3, C-28), 21.07 (CH_3, C-29), 18.32 (CH_3, C-30); and one acetate methyl carbon at δ 21.91 (CH_3, C-32). The molecular formula of gedunin is $C_{28}H_{34}O_7$.

The ^{13}C and ^{1}H chemical shift δ-values assignment for gedunin are identical to those reported by Gonzalez et al. (1989) and Duddeck and Dietrich (1992). The ^{1}H NMR proton resonances at δ 7.44 (lH, s, H-21), δ 6.37 (lH, s, H-22) and δ 7.44 (lH, s, H-23) are characteristic of the β-substituted furan ring protons H-21, H-22 and H-23 of gedunin. From the HSQC spectrum the corresponding furan ring carbon resonances occurred in the ^{l3}C NMR spectrum at δ 143.09 (C-21), δ 109.87 (C-22) and δ 141.21 (C-23). C-20 is a quaternary carbon at δ 120.44 (s). The ^{1}H NMR showed the characteristic epoxidic 15α-H at δ 3.56 for gedunin (Okhale et al., 2012).

^{1}H and ^{13}C NMR spectra of 7-deacetoxy-7α-hydroxygedunin potassium salt

The ^{1}H and ^{13}C nmr spectral data of 7-deacetoxy-7α-hydroxygedunin potassium salt obtained (Okhale et al., 2012) were consistent with that of gedunin potassium salt (2) as reported by Okogun and Orisadipe (1996) with molecular formula $C_{26}H_{34}O_7K$.

^{1}H and ^{13}C NMR spectra analyses of OK-S4

^{1}H nmr spectrum of OK-S4 showed the presence of five methyl protons δ 1.11 (3H, s, H-28), 1.12 (3H, s, H-29), 1.17 (3H, s, H-30), 1.22 (3H, s, H-19) and 1.26 (3H, s, H-18) each for three protons; three methylene protons 1.66 (1H, dd, H-6α), 1.93 (1H, dd, H-6β), 1.98 (1H, m, H-11α), 1.82 (1H, m, H-11β), 1.57 (1H, m, H-12α), 1.70 (1H, m, H-1β), ten methine protons 7.13 (1H, d, J = 15 Hz, H-1), 5.87 (1H, d, J = 15 Hz, H-2), 2.49 (1H, m, H-5), 3.60 (1H, s, H-7), 2.51 (1H, m, H-9), 3.93 (1H, s, H-15), 5.62 (1H, s, H-17), 6.37 (1H, s, H-22) and at δ 7.43 for two accidentally isochronous protons (H-21 and H-23), with total number of signals accounting for 31 hydrogen atoms in the spin system. The ^{13}C nmr spectra of OK-S4 showed eight quaternary carbons at δ 204.53 (C=O, C-3), 44.19 (C, C-4), 43.69 (C, C-8), 40.21 (C, C-10), 38.36 (C, C-13), 70.00 (C-O, C-14), 168.24 (O-C=O, C-16), 120.66 (C, C-20); ten methine carbons at δ157.70 (HC=C, C-1), 125.78 (HC=C, C-2), 44.60 (CH, C-5), 69.76 (HC-O, C-7), 38.00 (CH, C-9), 57.85 (HC-O, C-15), 78.47 (HC-O, C-17), 141.18 (HC=C, C-21), 109.97 (HC=C, C-22), 143.11 (HC=C, C-23); three methylene carbons at δ 27.33 (CH_2, C-6), 15.07 (CH_2, C-11), 26.42 (CH_2, C-12); and five methyl carbons at δ 17.81 (CH_3, C-18), 19.92 (CH_3, C-19), 27.30 (CH_3, C-28), 21.50 (CH_3, C-29), 18.68 (CH_3, C-30).

As shown in Figure 2, the ^{1}H nmr spectra of OK-S4 lacks the characteristic sharp singlet peak at δ_H 2.13 ppm observed in the ^{1}H nmr spectra of gedunin, attributed to the C-32 methyl protons of the 7-acetyl group (Okhale et al., 2012). Hence OK-S4 is 7-deacetoxy-7α-hydroxygedunin (3), with structure as shown in Figure 1 with molecular formula $C_{26}H_{32}O_6$. All the ^{1}H chemical shift values of OK-S4 were comparable with those reported for 7-deacetoxy-7α-hydroxygedunin (3) by Alessandra et al. (2006). The ^{1}H and ^{13}C chemical shift values of OK-S4

Table 1. ^{1}H and ^{13}C NMR Assignment of 7-deacetoxy-7α-hydroxygedunin compared with that reported by Mitsui et al. (2006).

Carbon No.	Carbon chemical shift OK-S4	Carbon chemical shift	Carbon No. to which proton is attached/ orientation	No. of protons	Proton chemical Shift OK-S4	Proton chemical shift
1	157.7	157.7	1	1H	7.13 (d, J = 15)	7.10 (d, J = 10.2)
2	125.78	125.8	2	1H	5.87 (d, J = 15)	5.84 (d, J = 10.2)
3	204.53	204.6	-	-	-	-
4	44.19	44.2	-	-	-	-
5	44.60	44.6	5	1H	2.49 (m)	2.48 (m)
6	27.33	27.3	6α	1H	1.66 (m)	1.63 (m)
			6β	1H	1.93 (m)	1.96 (m)
7	69.76	69.8	7	1H	3.93 (s)	3.57 (br, s)
8	43.69	43.7	-	-	-	-
9	38.00	38.0	9	1H	2.51 (m)	2.53 (m)
10	40.21	40.2	-	-	-	-
11	15.07	15.1	11α	1H	1.98 (m)	1.97 (m)
			11β	1H	1.82 (m)	1.81 (m)**
12	26.42	26.4	12α	1H	1.57 (m)	1.55 (m)
			12β	1H	1.70 (m)	1.72 (m)
13	38.36	38.4	-	-	-	-
14	70.0	70.0	-	-	-	-
15	57.85	57.9	15	1H	3.60 (s)	3.91 (s)
16	168.24	168.2	-	-	-	-
17	78.47	78.5	17	1H	5.62 (s)	5.60 (s)
18	17.81	17.8	18	3H	1.26 (s)	1.23 (s)
19	19.92	19.9	19	3H	1.22 (s)	1.20 (s)
20	120.66	120.7	-	-	-	-
21	141.18	141.2	21	1H	7.43 (s)	7.41 (s)
22	109.97	110.0	22	1H	6.37 (s)	6.35 (s)
23	143.11	143.0	23	1H	7.43 (d)	7.40 (d, J=1.3)
28	27.30	27.3	28	3H	1.11 (s)	1.09 (s
29	21.50	21.5	29	3H	1.12 (s)	1.09 (s)
30	18.68	18.7	30	3H	1.17 (s)	1.14 (s)
7-OH						2.65 (br, s)*

were comparable with those reported for 7-deacetoxy-7α-hydroxygedunin by Mitsui et al. (2006). The novel protocol reported here for the synthesis of 7-deacetoxy-7α-hydroxygedunin gave higher yield 88% and did not require column chromatographic purification compared with previously reported method that gave 43.4% yield (Akisanya et al., 1961).

Comparative antibacterial activities of 7-deacetoxy-7α-hydroxygedunin and 7-deacetoxy-7α-hydroxygedunin potassium salt

7-deacetoxy-7α-hydroxygedunin potassium salt had MIC of 1000 µg/ml against *K. pneumoniae,* 2000 µg/ml against *S. aureus*, and *E. coli*. However, *B. subtilis* was not susceptible to 7-deacetoxy-7α-hydroxygedunin potassium salt at 2000 µg/ml. On the other hand, 7-deacetoxy-7α-hydroxygedunin had MIC of 2000 µg/ml against *B. subtilis,* and *E. coli*; and 1000 µg/ml against *K. pneumoniae*. The standard drug Ciprotab had MIC of 48.8, 97.7, 390.6 and 390.6 ng/ml against *B. subtilis, S. aureus, K. pneumoniae and E. coli*, respectively (Tables 1 and 2). The characteristic structural difference between 7-deacetoxy-7α-hydroxygedunin potassium salt and 7-deacetoxy-7α-hydroxygedunin are essentially in the substituent at C-7 and the nature of lactone ring. The 7-deacetoxy-7α-hydroxygedunin potassium salt being a salt was more soluble in water compared to 7-deacetoxy-7α-hydroxygedunin which is more lipophilic.

However, the clinical significance of infections associated with the organisms investigated varies.

Table 2. Results of antibacterial MIC for 7-deacetoxy-7α-hydroxygedunin potassium salt and 7-deacetoxy-7α-hydroxygedunin.

Organism/compound	Bs	Sa	Kp	Ec
7-deacetoxy-7α-hydroxygedunin potassium salt	NA	2000 µg/ml	1000 µg/ml	2000 µg/ml
7-deacetoxy-7α-hydroxygedunin	2000 µg/ml	NA	1000 µg/ml	2000 µg/ml
Organism control	NA	NA	NA	NA
Ciprotab	48.8 ng/ml	97.7 ng/ml	390.6 ng/ml	390.6 ng/ml

NA, No activity observed; Bs, *Bacillus subtilis*; Sa, *Staphylococcus aureus*; Kp, *Klebsiella pneumonia*; Ec, *Escherichia coli*.

According to Kang et al. (2010), *S. aureus* is an important pathogen in patients with chronic liver diseases which is a significant factor associated with mortality along with old age immunosuppressive treatment, intubated state, pneumonia and concomitant bacteraemia. Bettiol et al. (1993) reported that *E. coli* Alkalescens-Dispar isolated from diarrheal samples were the potential pathogen responsible for diarrheagenic infection in Tasmania Australia. The significance of the observed MIC results is that 7-deacetoxy-7α-hydroxygedunin potassium salt and 7-deacetoxy-7α-hydroxygedunin could serve as leads in drug development in the treatment of infections caused by *S. aureus* and *E. coli*.

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