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ISOLATION AND INVESTIGATION OF ANTIMICROBIAL EFFECT OF 3,4,3′-TRI-*O*-METHYLFLAVELLAGIC ACID AND ITS GLUCOSIDE FROM *ANOGEISSUS LEOCARPUS*

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ABSTRACT. 3,4,3′-Tri-*O*-methylflavellagic acid and its glucoside (reported for the first time) were isolated from *Anogeissus leocarpus*. These compounds were analysed by GC-MS, IR, 1D and 2D-NMR, and also as acetates. Antimicrobial effect of the glucoside on *S. aureus, E. coli, Ps. aeruginosa* and *C. albicans* show that it possesses growth inhibitory effect at various concentrations.

INTRODUCTION

Anogeissus leocarpus belongs to the genus Combretaceae in which 16 species are distributed as trees, shrubs, and woody climbers, and partly as lianas in tropical and sub-tropical regions especially in Africa [1]. Plants in the *Anogeissus* genus have been used in herbal medicine in many places especially, Africa and India where the plants are widely distributed. For example, preparations from the leaves of *A. latifolia* are used in the treatment of diarrhoea and gonorrhoea in India and the extract is recorded as possessing demulcent and astringent properties [2]. *A. leocarpus*; the subject of the current study has numerous medicinal applications all over Africa.

The pre-1985 review on this plant by H.M. Burkill [3] mentioned various medicinal applications which include laxative, emulsifer, wounds and ulcer healing, for toothache, treatment of diarrhoea, syphilis chancres, stimulant and aphrodisiac, tanicide for horses and donkeys.

Previous phytochemical studies on some species of *Anogeissus* have yielded various classes of compounds such as sugars and their derivatives [4-6], acids [7-10], glycosides [11-13], flavones and flavonoidal glycosides [14], lignans [15] and tannins [16].

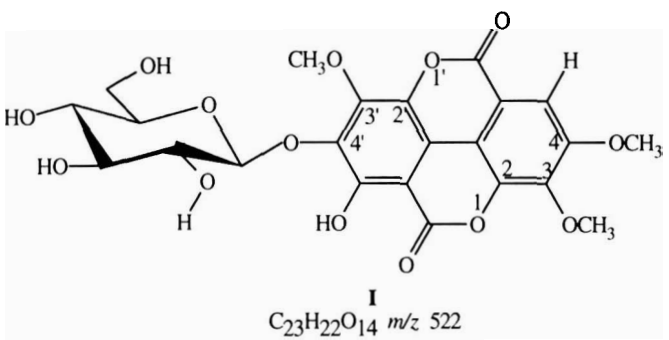
In our search for the active constituents of *Anogeissus leocarpus*, we hereby report the isolation, identification, and antimicrobial effects of both 3,4,3′-tri-*O*-methylflavellagic acid and its glucoside which were isolated from the plant.

RESULTS AND DISCUSSION

The solid which precipitated out of solution during extraction with ethanol was analysed and found to be 3,4,3′-tri-*O*-methylflavellagic acid-4′-*O*-glucoside, **I**. This compound was insoluble in cold and hot chloroform, ethanol, methanol and water, but it dissolved completely in DMSO. TLC in both n-BuOH:AcOH:water, 5:1:4, upper phase, (R_f 0.20), and

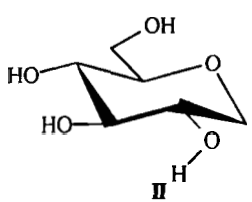
Forestal solvent [17], AcOH:conc. HCl:water, 10:1:3 (R_f 0.30), indicates that the compound contains only one component.

The UV spectrum in MeOH showed three absorption bands at λ_{max} 269 (ε 3,614), 364 (ε 2,309), and 374 nm (ε 2,309), indicating the extent of π-conjugation in the molecule. The IR spectrum (nujol mull), has peaks at 3250, 3200, 1740, and 1698 cm^{-1}, respectively, for free and hydrogen bonded -OH, and -C=O stretching vibrations of an aryl lactone. Early workers [18] have reported similar absorption frequencies. The ^1H NMR spectrum in DMSO-d_6 shows one aromatic proton as a singlet at δ 7.75, a broad doublet at δ 5.10 (*J* 7.3 Hz) for the H-1 proton of glucose, and 3 methyl singlets at δ 4.10, 4.02 and 3.82, respectively. It also has doublet at δ 3.68 (*J* 11.4 Hz) and multiplets at δ 3.45 for the two H-6 (the two H-6 resonate at slightly different positions due to a slightly different electronic environment), δ 3.38 for H-5, δ 3.34 for H-2 and H-3 and δ 3.20 for H-4. All these assignments were made from a ^1H-^1H-COSY spectrum. The DEPT ^{13}C NMR spectrum indicates the presence of 13 non-protonated carbons from the peaks at δ 161.05, 158.48, 152.71, 152.18, 147.34, 142.05, 141.60, 141.38, 134.24, 114.04, 112.68, 112.43, and 98.16. The 7-CH groups are at δ 113.44, 101.75 (CH, C-1 glucoside), 77.73, 76.93, 73.76, and 69.94, while the 3 methoxy carbons are at δ 62.20, 62.14 and 61.68. The C atom of the CH_2 group of C-6 of glucose absorb at δ 61.0. The EIMS measurement shows the aglygone peak as the base peak with *m/z* value of 360 and 100% intensity. This corresponds to the complete loss of the glucose unit. The peak at *m/z* 345 with 68% intensity is due to the loss of one methyl group from the aglycone. This fragmentation is shown in Scheme 1.



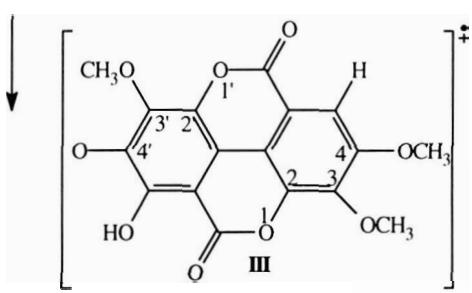
I

$C_{23}H_{22}O_{14}$ *m/z* 522



II

$C_6H_{10}O_5$ *m/z* 162



III

$_{17}H_{12}O_9$ *m/z* 360

Scheme 1

To further determine the structure and molecular weight of the sample, MS measurements were carried out using three methods viz: low resolution, EIMS and electrospray, (ES), and high resolution mass spectrum measurement (HRMS, using FAB, NOBA matrix). EI method

gave a very weak molecular ion peak for the peracetate (0.0065) at *m/z* 733, monoacetate aglycone peak (12%) at *m/z* 402, the aglycone base peak (78%) at *m/z* 360, and the (aglycone-CH_3) peak (42%) at *m/z* 345 among others. The ES spectrum shows peaks at *m/z* 391 (3%), 119 (19%), 101 (42%), 74 (38%), and 60 (100%), meaning that the base peak is that of acetic acid, CH_3COOH, an indication that this ionization method is inadequate for the sample in question. HRMS method shows *m/z* 755.1397 for $C_{33}H_{32}O_{19}Na$, which requires 755.1435 for M+Na meaning that the error is 5 ppm. It also has the expected molecular ion peak at *m/z* 733 (11%), aglycone peak at *m/z* 360 (11%), a peak at *m/z* 331 corresponding to a loss of $-C_2H_5$ group from the aglycone and a base peak at *m/z* 169 (100%).

It should be noted that the position of the -OH group(s) in of 3,3′,4′-tri-*O*-methylflavellagic acid was determined in literature by earlier workers [19-20] using shift reagents such as NaOAc, H_3BO_3, NaOEt, and $AlCl_3$, and the shift in λ_{max} measured to determine the nature of conjugation between the -OH and lactonic groups so as to deduce whether they are free or in close proximity. However, this procedure could not be carried out due to small sample size.

From the above, it can therefore be concluded that the sample is pure 3,4,3′-tri-*O*-methylflavellagic acid-4′-*O*-glucoside which was converted to the acetyl derivative 3,4,3′-tri-*O*-methylflavellagic acid-4′-*O*-glucoside pentaacetate.

The growth inhibitory effect of 3,4,3′-tri-*O*-methylflavellagic acid on microorganisms: *Staphylococcus aureus, Eschericia coli, Pseudomonas aeruginosa* and *Candida albicans* was determined. The result indicates that out of these organisms, the only organism that is highly susceptible to the compound is *Eschericia coli. Eschericia coli* is the microorganism commonly found in the alimentary canal of warm blooded animals [21]. At a concentration of 8.3×10^{-3} μg/cm^3, its growth was completely inhibited. The concentrations used are relatively small (8.3×10^{-5} - 8.3×10^{2} μg/cm^3), due to sample size, the compound may have inhibitory effect on these other organisms at higher concentrations.

To further confirm this identification the solid was derivatized by acetylation. After acetylation and first separation by flash column chromatography, four products were obtained, the fastest moving one is a single component analysed to be flavellagic acid diacetate, i.e. a derivative of flavellagic acid. ^{1}H-NMR spectra ran in $CDCl_3$ and C_6D_6 are consistent with flavellagic acid diacetate.

EXPERIMENTAL

The NMR spectra were recorded on a 400 MHz Bruker NMR Spectrophotometer model ADVANCE DPX400. UV and IR spectra were recorded using CE660 Multimude Computing UV Spectrophotometer 6000 Series and Perkin-Elmer FTIR Spectrophotometer 1600 Series, respectively. The GC-MS spectra were obtained from HP-GC 5890 + TRIO-1 Mass Spectrometer (VG Lab). The TLC and column chromatography were carried out on silica (0.2 mm) (Merck, Kieselgel 60F254) and silica gel (60-120 mesh), respectively. Microanalyses were carried out on a Perkin Elmer 240C Elemental Analyser.

Plant material. Fresh bark of *A. leocarpus* was collected at Grogoji village in Zaria Local Government area of Kaduna State. A voucher specimen (No. 167) is deposited in the herbarium of Botany Department of the Ahmadu Bello University, Zaria, Nigeria. The dried bark was powdered and used for extraction.

Extraction and isolation of 3,4,3′-tri-O-methylflavellagic acid and its -4′-O-glucoside. Powdered bark of *Anogeissus leocarpus* (200 g) was packed into a Sohxlet extractor and extracted using the solvents (in the order of use) petroleum spirit (40-60 °C), chloroform, ethanol, methanol and water. The weights of the extracts were 0.28% (petroleum spirit); 0.23% (chloroform); 6.09% (ethanol); 5.56% (methanol); and 9.0% (water).

Analysis of 3,4,3′-tri-O-methylflavellagic acid-4′-O-glucoside. M.p. 258-261°C (dec); anal., found C 52.86, H 5.01%; $C_{25}H_{28}O_{15}$ ($C_{23}H_{22}O_{14}.CH_3CH_2OH$) requires C 52.82, H 4.96%; IR ν_{max} (nujol mull) cm^{-1} 3520 (sh), 3200 (br), 1740 (sh), 1698 (sh); UV (MeOH) λ_{max} nm (ε) 269 (3,614), 364 (2,309), 374 (2,309); ^1H NMR (400 MHz, DMSO-d_6+ D_2O) δ 7.75 (1H, s), 5.10 (br d, J = 7.3, H-1), 4.10, 4.02, 3.82 (3 x 3H, 3 x s), 3.68 (1H, d, *J* 11.4, H-6), 3.45 (1H, m, H-6), 3.38 (1H, m, H-5), 3.34 (2H, m, H-2 and H-3), 3.20 (m, H-4). ^{13}C NMR (DMSO-d_6) δ 161.05, 158.48, 152.71, 152.18, 147.34, 142.05, 141.60, 141.38, 134.24, 114.04 (10 x C), 113.44 (CH), 112.68, 112.43 (2 x C), 101.75 (CH, C-1 glucoside), 98.16 (C), 77.73, 76.93, 73.76, 69.94 (4 x CH), 62.20, 62.14, 61.68 (3 x CH_3, -OCH_3); 61.0 (CH_2, C-6). EIMS *m/z* (rel. int.) 374 (2), 360 (100, found 360.0483, $C_{17}H_{12}O_9$ requires 360.0482), 345 (68), 317 (35), 299(16), 147 (23), 119 (35), 90 (45), 69 (75).

Acetylation of 3,4,3′-tri-O-methylflavellagic acid-4′-O-glucoside. Sample (50 mg) was acetylated to yield a product (40 mg, 80%), m.p. 237-240 °C (dec). IR ν_{max} (nujol mull) cm^{-1} 1745 (sh), 1218, 1045; UV ($CHCl_3$) λ_{max} nm (ε) 271 (14,348), 349 (11,713), 363 (12,592); ^1H NMR (400 MHz, C_6D_6) δ 7.98 (1H, s), 5.65 (1H, dd, J = 9.6 and 8.0, H-2), 5.52 (1H, t, J = 9.5, H-3), 5.24 (1H, t, J = 9.7, H-4), 4.88 (1H, d, J = 7.9, H-1), 4.25 (1H, dd, J = 12.3 and 1.6, H-6), 4.15 (1H, dd, J = 12.3 and 6.4, H-6), 3.90, 3.89, 3.77 (3 x 3H, 3 x s), 3.35 (1H, ddd, J = 10.0, 6.4 and 1.7, H-5), 2.32, 2.09, 1.90, 1.82, 1.78 (5 x 3H, 5 x s).

^1H NMR (400 MHz, $CDCl_3$) δ 7.94 (1H, s), 5.38 (2H, m), 5.17 (2H, m), 4.27 (3H, s), 4.25 (2H, m, 2H-6), 4.12 (3H, s), 4.02 (1H, m, H-5), 3.97 (3H, s), 2.60 (3H, s, $ArOC(=O)CH_3$), 2.20, 2.11, 2.08, 2.05 (4 x 3H, 4 x s); ^{13}C NMR (DEPT, C_6D_6) δ 170.18, 169.66, 168.97, 168.80, 168.64 (5 x C), 157.62, 154.92, 151.90, 147.56, 146.60, 142.94, 142.31, 141.88, 139.26 (9 x C), 114.12 , 113.16 (2 x C), 112.45 (CH), 106.23 (C), 99.47 (CH, C-1), 72.63, 72.39, 70.90, 68.17 (4 x CH), 61.66 (CH_2, C-6), 61.61, 61.58, 61.44 (3 x CH_3O), 20.41, 20.27, 20.10, 20.02, 19.93, 19.80 (6 x CH_3, $CH_3(C=O)O$). MS: EI *m/z* (rel. int.) 733 (0.006, M^+), 402 (12, monoacetylated aglycone), 360 (78, aglycone), 345 (42), 169 (34), 126 (38), 109 (59), 98 (83), 97 (100); electrospray *m/z* (rel. int.) 391 (3), 119 (19), 101 (42), 74 (38), 60 (100); FAB, NOBA matrix *m/z* 755 (25, found 755.1397, $C_{33}H_{32}O_{19}Na$ requires 755.1435, 5 ppm error, M+Na), 733 (11, M+H), 360 (11), 331 (81), 169 (100).

Determination of the effect of 3,4,3′-tri-O-methylflavellagic acid-4′-O-glucoside on micro-organisms. Sample (100 mg) was dissolved in 3 cm^3 of DMSO and then 9 cm^3 of normal saline solution was added. This gives a solution of concentration of 8.3 mg/cm^3. Serial dilution was made from this stock to have concentrations of 0.83 mg/cm^3, 0.083 mg/cm^3, 0.0083 mg/cm^3, 0.00083 mg/cm^3, 0.000083 mg/cm^3, 0.0000083 mg/cm^3, and 0.00000083 mg/cm^3. Each of these solutions was incorporated into culture medium using agar dilution technique to give various concentrations shown in the result table below.

Each test organism was inoculated into Trypticase Soy broth and allowed to grow overnight (about 18 h). Each plate Trypticase Soy agar containing the various concentrations was divided into 4 parts with a sterile inoculation loop. A loopful of organism was taken from each 18 h culture and striked on correspondingly labelled section of the plate. This was

repeated for each concentration. Control plate was also included which contains no extract. The growth on the test plates was compared with the growth of the organisms on the control plate. The results of the analysis together with the concentration and the observed effect are given in Table 1.

Table 1. Effect of 3,4,3′-tri-*O*-methylflavellagic acid-4′-O-glucoside on test organisms.

Conc (μg/cm^3)	Test organisms			
	Staphylococcus aureus	*Eschericia coli*	*Pseudomonas aeruginosa*	*Candida albicans*
830	++		++	++
	+++		++	++
	+++		++	++
	+++		++	++
0.083	+++		++	++
0.0083	+++		++	++
0.00083	++++	+	+++	+++
0.000083	++++	+	+++	+++
Control	++++	++++	++++	++++

Key: ++++ = abundant growth, +++ = less growth, ++ = few growth + = scanty growth, - = no growth.

Analysis of 3,4,3′-tri-O-methylflavellagic acid. Analysis: found C 54.62%, H 3.70%; $C_{17}H_{12}O_9$ requires C 56.44%, H 3.33%. IR ν_{max} (nujol mull) cm^{-1} 3302 (m, phenolic -OH), 1732 (sh, -C=O), 1695 (sh, -C=C-), 1613 (sh), 1462 (sh, $-CH_2$, $-CH_3$ stretch), 1378 (sh, $-CH_2$, $-CH_3$ stretch), 1232 , 1163 , 1084 (all weak, due to aromatic rocking modes). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.30 (15.5 mm, s), 3.94 (11.5 mm, s, 3H of $-OCH_3$), 4.10 (21 mm, s, 3H of $-OCH_3$), 4.23 (10.5 mm, 3H of - OCH_3), 7.54 (7 mm, 1H aromatic). EIMS *m/z* (rel. int.) 362 (3.6), 361 (19.5, M^++1), 360 (100, M^+), 345 (50), 330 (35), 317 (18), 68.5 (25).

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