Antitrypanosomal Activity of Glochidonol and Salacinin C from *Phyllanthus muellerianus*

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ABSTRACT:

Phyllanthus muellerianus is an important plant in Nigerian ethnomedicine. Several compounds were isolated from the hexane and methanol extracts of the stem and root bark of the plant using silica gel column chromatography and characterized by 1D and 2D NMR spectroscopic methods. The compounds were assayed for in vitro activity against Trypanosoma brucei brucei. The hexane and ethyl acetate extracts yielded friedelanone, physcion, glochidonol, and Salacinin C, while the methanol extract gave monoacetyl glycerol. Glochidonol (EC50 = 1.25 µg/ml) and Salacinin C (EC_{50} of 6.25 µg/ml) showed strong antitrypnosomal activity in vitro.

Keywords: Antitrypanosomal activity; Salacinin C, Friedelanone, Physcion, Glochidonol, Monoacetyl glycerol

INTRODUCTION

In addition to other diverse uses, plants have remained important sources of medicine throughout human history.[1] *Phyllanthus muellerianus* (Kuntze) Exell. is an African native woody climber or a little tree that has long branches with many small axillary shoots, alternate leaves, and unisexual greenish yellow or greenish white flowers.[2] Traditionally, the bark of the plant is an important source of a dye, while its wood is extensively used in making fishing lines, preparing baskets and for construction purposes. *P. muellerianus*, along with many other members of the genus, is widely used in ethnomedicine throughout tropical Africa. Its extracts are utilised as tonic for new mothers (Ghana and Nigeria), antidiarrhoeal (Tanzania), anti-emetic (Cameroon and

Nigeria), antipyretic (Nigeria), and aphrodisiac (Central African Republic). Parts of the plant have also been used in the treatment of eye infections, sore throat, paralysis (throughout West-Africa), dysmenorrhoea (Gabon), gonorrhoea (DR Congo), and constipation (Sierra Leone) amongst others.[3] *P. muellerianus* parts have been shown to contain compounds and constituents with biological activity. Bioactive compounds previously isolated from the plant include phthalates, lignans, cardiac glycosides, ellagitannins, flavonoids, phenylpropanoids and friedelin-type compounds.[4-6] The stem bark extract of the plant has been shown to possess wound healing and anti-tetanus activity.[7] The antimicrobial activity of the methanol extract of the plant was reported against *Clostridium sporogenes* and *Streptococcus pyogenes*. The aqueous leaf extract showed an increase in production of dermal fibroblasts and human keratinocytes.[5] Ethanolic extracts of *Phyllanthus muellerianus* leaves showed slight antiplasmodial activity against *Plasmodium falciparum* and considerable parasitic selectivity.[8] *Phyllanthus muellerianus* leaves extracted with methanol did not show antileishmanial activity.[9] Trypanosoma species cause important parasitic diseases in animals and humans. Human African Trypanosomiasis and African Animal Trypanosomiasis transmitted by tsetse flies affects millions of humans and animals with severe negative impacts on the African economy. Chemotherapy for trypanosomiasis is faced with the challenges of toxicity and resistance.[10] There is therefore a need for safer, cheaper and more effective trypanocides, especially if they can be sourced from locally available natural resources. This is the first report of antitrypanosomal activity for Salacinin C, and based on the results, the compound could serve as a lead toward the identification of more efficient antitrypanosomal drugs.

MATERIALS AND METHODS

General

The NMR spectra were obtained on JEOL (USA Inc) delta NMR spectrometer (Figure:2) (JNM-LA 400) and Ultra shield 600 MHz Bruker magnet with Avance II spectrometer. Residual solvent peaks were used as internal standard. *J*-modulated ^{1}H and ^{13}C were recorded at a relaxation delay (D1) of 2 seconds. Proton (^{1}H) and carbon (^{13}C) were recorded at 400 MHz. NMR (1D and 2D) methods used to determine the structure were Correlation spectroscopy (COSY), Heteronuclear single-quantum correlation

spectroscopy (HSQC) and Heteronuclear multiple-bond correlation spectroscopy (HMBC). Column chromatography was carried out using silica gel 60 (0.040-0.063 mm) (230-400 mesh ASTM). Thin-layer chromatography (TLC) was performed on pre-coated aluminium sheets with silica gel F250 (Merck, Germany).

Plant Material

The plant material, *Phyllanthus muellerianus* barks and roots was collected from plants growing freely, in Igwoke Uwokwu in Oju LGA of Benue state Nigeria. They were collected in July – September 2008. An herbarium sample was deposited at the University of Agriculture Makurdi plant herbarium.

Extraction and Isolation of Phytoconstituents

The bark and roots were completely air-dried, mixed, and ground into powder using a Fritsch grinder to obtain about 730 g of the powder. A Soxhlet apparatus was used to extract the powdered bark and roots successively using HPLC-grade hexane, ethyl acetate and methanol. Column chromatography (CC) using a glass column was used to purify the hexane and ethyl acetate extracts while Vacuum Liquid Chromatography (VLC) was used to purify the methanol extract. The column (CC) was packed wet with about 200 g of silica gel in hexane. The extracts, which were previously adsorbed on silica gel and dried, were loaded on top of the column. The column was eluted gradient-wise using increasing quantities of ethyl acetate in hexane and fractions were collected using glass vials. Similarly, VLC was carried out using an appropriate VLC glass column. All the fractions obtained from CC and VLC were examined by TLC and those with similar profiles were pooled and further examined by NMR.

Drug Sensitivity Using Blood Stream forms of Trypanosoma brucei brucei s427 lister strain

The plant extracts and isolated compounds were screened for activity against blood stream form of *Trypanosoma brucei brucei* (*T.b.brucei*) S427 using an Alamar blue™ assay as described previously.[11] Extracts and compounds for assay were prepared and stored as 10 mg/ml or 10 mM stock solutions in 100% DMSO. 100 µL of the extracts or compounds prepared in HMI-9 medium was placed in a respective well of 96-well plate. Initial screening was carried out at a single concentration of 20 µg/ml for extracts or 20 µM for pure compounds. The screening plate was arranged to include sterility control,

DMSO controls, and a concentration range of Suramin as the positive control. Trypanosomes were counted and prepared at a density of 3×10^4 cells/ml, 100 µL of this suspension was added to each well of the assay plate except well A1, which is meant for the sterility check. The assay plate was incubated at 37˚C and 5% CO_2 under a humidified atmosphere for 48 hours, after which 20 µL of Alamar blue dye was added to each well and the incubation continued for another 24 hours. Fluorescence was then determined using the Wallac Victor microplate reader at 530 nm excitation λ and 590 nm emission λ. The results were calculated as % of the DMSO control values. Minimum inhibitory concentration values (MICs) were determined in triplicates for samples that showed less than 10% of control values. About 200 µg/ml test solutions in HMI-9 medium were prepared and placed in column 2 of a 96-well plate. 100µl HMI-9 medium was pipetted into columns 1 and 3-11 and a 1:1 serial dilution was carried out from columns 2 to 11. A serial dilution of suramin in HMI-9 medium was prepared in column 12 to give a final concentration range of 0.008 to 1.0 µM. An inoculum of 100 µl of trypanosomes at a density of 3×10^4 cells/ml was added to each well except A1, and the procedure continued as described above. MIC values were determined as the concentration calculated to have <5% of control values.

RESULTS AND DISCUSSION

Isolated compounds

The following compounds were isolated from the plant material

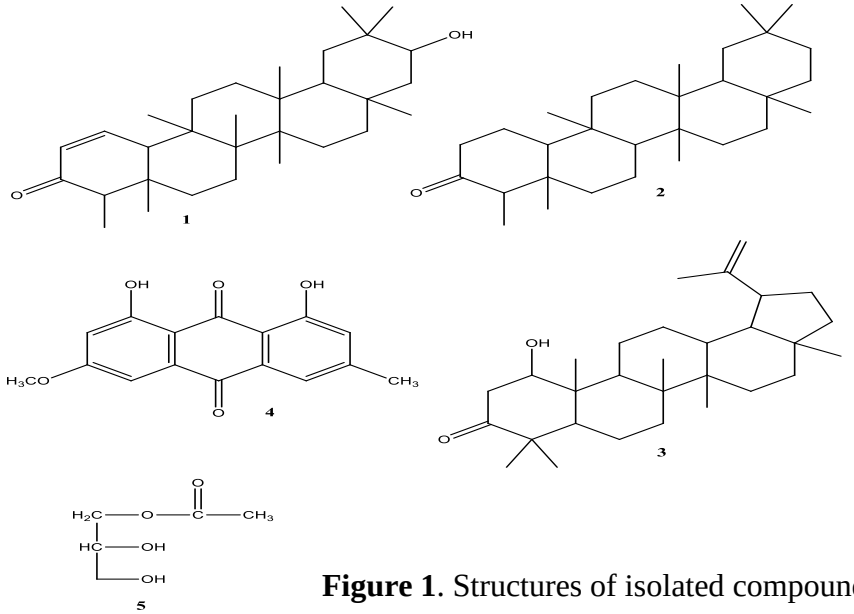

Figure 1. Structures of isolated compounds (**1-5**)

Characterization of fraction R75-85 as Salacinin C (1)

The ^{1}H and ^{13}C NMR spectra (Table 1) exhibited signals for seven quaternary methyls (δ_H 0.84, 0.97, 0.99, 1.05, 1.13, 1.16, and 1.26 ppm; δ_C 13.7, 17.4, 19.2, 20.1, 24.9, 31.8, and 33.1 ppm), one secondary methyl (δ_H 1.05, d, J = 4.9 Hz; δ_C 6.7 ppm), an sp^3 oxymethine (δ_H 3.76, dd, J = 12.2, 4.7 Hz; δ_C 74.2 ppm), two olefinic protons (δ_H 6.11, dd, J = 10.2, 2.9 Hz, and 6.99, d, J = 10.2 Hz; δ_C 130.2 and 148.9 ppm), and one carbonyl at δ_C 201.9. Based on the seven degrees of unsaturation, compound **1** must have a triterpene five ring system plus a carbonyl and a double bond. This is typical of a friedoolean-l-en-3-one skeleton. Since the conjugated ketone carbonyl is at C-3, the hydroxyl group must be substituted at another position in the compound. The structure was confirmed by examination of its 2D NMR spectra as follows: In the ^{1}H–^{1}H COSY spectrum of **1**, correlations were observed between H-1 and H-2 which were the olefinic protons and their cis-coupling is confirmed by their J values of 10.2 MHz. The correlations of a three-proton spin system comprising of H–6, H-7 and H-8 as well as couplings between H-11 and H-12, H-15 and H-16, H-21 and H-22 were also observed. In the HMBC spectrum of **1**, long-range correlations were observed from CH_3–29 (δ_H 1.05), CH_3–30 (δ_H 1.13), and Hß–22 (δ_H 1.76) to C–21 (δ_C 74.2). Thus, confirming the substitution of the hydroxyl group at C-21. The ß-orientation of H–21 was deduced from the cross-peaks of H–21 with CH_3–28 and CH_3–30 in the NOESY spectrum. Thus, the structure of **1** was elucidated as 21α-hydroxyfriedel-1-ene-3-one or Salacinin C (Fig. 1). The complete assignment of the proton and carbon chemical shifts for the compound is given in Table 1 and they compare excellently with literature reports[12]

Characterization of fraction R60-65 as Glochidonol (2)

The proton spectrum of the compound showed two vinyl protons at δ_H 4.70 (d, J = 2.0 Hz) and 4.59 (dd, J = 1.6, 2.4 Hz), an oxymethine proton at 3.92 (dd, J = 8.0, 3.6 Hz) and an allylic methyl at 1.70 ppm. It also had six other methyl groups between δ_H 0.82 and 1.08 ppm. The spectrum pattern was typical of a lupane-type triterpene. Two coupled geminal methylene protons at δ_H 3.02 (1H, dd, J = 14.4 and 8.0 Hz) and 2.25 (1H, dd, J = 14.4 and 3.6 Hz) ppm indicated a methylene group alpha to a carbonyl group. The compound showed 30 signals in its carbon-13 spectrum including one carbonyl at δ_C 215.9, one oxymethine at 79.9 and two olefinic carbons at 150.7 and 109.5 ppm. The

rest of the carbons were made up of seven methyl, nine methylene, five methine and six quaternary carbons. The compound was identified based on correlations in its 2D NMR and literature reports[13] as 1β-hydroxy-lup-20 (29)-en-3-one or glochidonol (**2**) (Fig. 1). The proton and carbon chemical shift assignments for the compound are given in Table 1.

The rest of the compounds were identified as physcion (**3**)[14], friedelanone (**4**)[15] and monoacetyl glycerol (**5**)[16]. (Fig. 1)

Position	**Compound 1**		**Compound 2**	
	^{13}C	**^{1}H**	**^{13}C**	**^{1}H**
1	130.2 (CH)	6.11 (dd, *J* = 10.2, 2.9 Hz)	79.9 (CH)	3.92 (dd, *J* = 8.0, 3.6 Hz)
2	148.9 (CH)	6.99 (d, *J* = 10.2 Hz)	45.1 (CH_2)	3.02 (dd, *J* = 14.4, 8.0 Hz) 2.25 (dd, *J* = 14.4, 3.6 Hz)
3	201.9 (C)	-	215.9 (C)	-
4	57.7 (CH)	2.29 (q, *J* = 4.9 Hz)	47.1 (C)	-
5	43.6 (C)	-	51.5 (CH)	1.37
6	39.8 (CH_2)	1.85 (m) 1.43 (m)	19.6 (CH_2)	1.5-1.65
7	17.7 (CH_2)	1.60 (m) H–7α; 0.83 (m)	32.9 (CH_2)	1.4-1.55
8	50.7 (CH)	1.51 (br d, *J* = 7.9 Hz)	41.2 (C)	-
9	36.6 (C)	-	50.7 (CH)	1.54
10	62.2 (CH)	2.32 (d, *J* = 2.9 Hz)	43.0 (C)	-
11	36.0 (CH_2)	1.59	23.0 (CH_2)	1.87, 1.38
12	30.2 (CH_2)	1.49 (m), 1.36 (m)	25.2 (CH_2)	1.16, 1.72
13	39.1 (C)	-	38.1 (CH)	1.70
14	38.8 (C)	-	42.9 (C)	-

15	29.9 (CH_2)	1.45 (m)	27.5 (CH_2)	1.71, 1.05
16	36.0 (CH_2)	1.59 (m)	35.5 (CH_2)	1.41, 1.51
17	32.5 (C)	-	43.0 (C)	-
18	44.3 (CH)	1.61 (br d, *J* = 8.7 Hz)	48.3 (CH)	1.41
19	34.3 (CH_2)	1.69 (m)	48.1 (CH)	2.40
20	36.0 (C)	-	150.7 (C)	-
21	74.2 (CH)	3.76 (dd, *J* = 12.2, 4.7 Hz)	30.0 (CH_2)	1.94, 1.65
22	47.0 (CH_2)	1.76 (dd, *J* = 12.7, 12.2 Hz), 1.30 (dd, *J* = 12.7, 4.7 Hz)	40.0 (CH_2)	1.41, 1.22
23	6.7 (CH_3)	1.05 (3 H, d, *J* = 4.9Hz)	19.9 (CH_3)	1.06 (s)
24	13.7 (CH_3)	0.84 (3H, s)	27.9 (CH_3)	1.08 (s)
25	17.4 (CH_3)	0.97 (3H, s)	11.9 (CH_3)	0.86 (s)
26	20.1 (CH_3)	0.99 (3H, s)	16.0 (CH_3)	1.08 (s)
27	19.2 (CH_3)	1.16 (3 H, s)	14.5 (CH_3)	1.00 (s)
28	33.1 (CH_3)	1.26 (3 H, s)	18.1 (CH_3)	0.82 (s)
29	24.9 (CH_3)	1.05 (3 H, s)	109.5 (CH_2)	4.70 (d, *J* = 2.0 Hz) 4.59 (dd, *J* = 1.6, 2.4 Hz)
30	31.8 (CH_3)	1.13 (3 H, s)	19.3 (CH_3)	1.70 (s)

Table 1. ^{1}H and ^{13}C-NMR data for compounds **1** and **2**

Antitrypanosomal activity screening

Salacinin C and some compounds and fractions obtained showed good activity against *T. brucei brucei* (Table 2).

Particularly, fraction R15-19 which contains triglycerides; R20-25 made up of triglyceride and fats; R26-49 physcion; R60-65 glochidonol; R75-82, a mixture of triterpenes; and R94-105, a mixture of benzaldehydes, were active. In addition, R30:70 HE, R50:50HE and R50:50 EM, and some fractions obtained from vacuum liquid chromatography were also active.

Fraction	Compound	% of control	MIC (µg/ml)
R9-14	**4**	104.4	-
R15-19	Mixture of triglycerides	-0.1	12.5
R20-25	Mixture of triglycerides	-1.8	12.5
R26-49	**3**	-0.4	12.5
R52-57	Sitosterol + Stigmasterol	106.4	-
R60-65	**2**	3.8	1.56
R75-85	**1**	0.8	6.25
R94-105	Mixture of aromatic aldehydes	-1.1	6.25
R30:70 HE	Fatty substances	-14.3	20.0
R100E	**5**	597.5	-

Table 2. Effect of Salacinin C from *Phyllanthus muellerianus* and other isolated compounds and fractions on *Trypansonsoma brucei brucei* s427 lister strain

CONCLUSION

Several compounds including salacinin C, friedelanone, physcion, monoacetyl glycerol, and glochidonol were obtained from *Phyllanthus muellerianus* extracts. Only three of these compounds demonstrated significant activity against *Trypanosoma brucei brucei.*

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