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Weak Membrane Stabilizing Activity of Sydowinin B and Pitholide B

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Authors' contributions

This work was carried out in collaboration between both authors. The study was carried out by author GOA while spectra analysis and manuscript preparation were carried out by the two authors. Both authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Background: There is a dire need for anti-inflammatory agents presently to solve many health challenges. Extracts of endophytic fungi have been implicated a lot in this area. The aim of the study was to test isolated compounds from the extract of *Pestalotiopsis clavispora* residing in *Phoenix reclinata*.
Methods: Fungal isolation from *Phoenix reclinata*, cultivation, extraction isolation of compounds, characterisation and membrane stabilisation assay were carried out by standard methods.
Results and Discussion: From the extract of *P. clavispora*, was isolated three compounds which are 7,8-dihydroxanthenone-8-carboxylic acid methyl ester,sydowinin B and pitholide B. The fungus was from the mangrove plant *Phoenix reclinata* Jacq. (Arecaceae).
Sydowinin B and pitholide B exhibited a very weak membrane stabilizing activity of 4.5 and 2% respectively, at 100 µg/mL and 5% at 200 µg/mL for the two compounds.
Conclusion: The compounds have not been isolated before from this fungus and they exhibited a very weak membrane stabilizing activity.

Keywords: Membrane; fungus; inflammatory; red blood cells.

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1. INTRODUCTION

A number of models have been described and used to evaluate potential anti-inflammatory drugs, some of which include platelet aggregation, cotton pellet granulation in animals and erythrocyte membrane stabilization [1]. Membrane stabilizing evaluation of erythrocytes membrane shown by some agents is a vital *in vitro* evaluation method for antiinflammatory action [2]. Hypotonicity-induced haemolysis inhibition has been utilised as a measure of the anti-inflammatory activity of various extracts, fractions and isolated compounds from medicinal plants [3]. The plant, *Phoenix reclinata*, commonly known as Date palm, is widely distributed throughout the mangrove of sub-Saharan Africa [4] and it is utilised folklorically for problems associated with erection and male infertility [5]. Extracts of several endophytic fungi have exhibited excellent biological activities. The essence of the work is to test the effect of isolated compounds from *Pestalotiopsis clavispora* on inflammation.

2. MATERIALS AND METHODS

2.1 Materials

Sephadex LH-20, Silica gel, heparinized syringe, methanol (hplc grade), hexane, ethylacetate.

2.2 Methods

2.2.1 Plant collection

Collection of a few leaf samples of *Phoenix reclinata* was done at Onne, Rivers State, Nigeria. Dr. Omokafe A. Ugbogu of the Faculty of Agriculture, University of PortHarcourt did the identification. The voucher specimen (NDUP 200) of the authenticated sample was kept and stored at the Herbarium of the Department of Pharmacognosy & Herbal Medicine, Niger Delta University, Wilberforce Island, Nigeria.

2.2.2 Endophytic fungal isolation and its identification

A non-infected leaf of *P. reclinata* was gently washed in sterilised water without allowing the tissues to squeeze and then disinfected with ethyl alcohol [70% ($^{v}/_{v}$)] for approximately three minutes and ultimately re-rinsed in sterilised water. Complete disinfection was justified by the application of the sample on malt agar. The sliced leaf samples were then applied to agar incorporated with antibiotics as described earlier by Kjer and co-workers [6]. The plates were thereafter left at 21 - 22°C to allow hyphae growth. Some of the hyphae were subsequently subcultured into fresh agar until pure colonies were established. The colonies were identified using established molecular techniques via DNA extraction, PCR amplication of ITS-1 region and sequencing [6]. The resulting strain coded LNG-S2 was thereafter banked at the Institute of Pharmaceutical Biologie and Biotechnologie, Heinrich-Heine University, Duesseldorf, Germany.

2.2.3 Cultivation and extraction of fungus

To the one hundred grams of commercially available rice in a one-litre flask, one hundred and ten millilitres of distilled water was added and subjected to autoclave at 121°C, 2 bar for a third of an hour. After cooling, ten pieces of the fully grown mycelia were gently added to the top of the rice medium in sterile conditions. It was left for a month at room temperature for full growth. The growth was then abruptly terminated using ethyl acetate (about 500ml) which at the same time acted as the solvent of extraction. It was left in this solvent for 11-12 hours after which complete dryness of the extract was achieved *in vacuo* resulting in a 2.51g of the extract.

2.2.4 Chromatographic purification

The LNG-S2 extract (2.51g) was dispersed in 20 ml of water and then partitioned into *normal*-hexane (70 ml x 2) and methanol/water [(9:1) (70 ml x 2)] and this afforded 1.32, 1.11 and 0.08 g of hexane (LNGS-2'), methanol (LNG-S2 and aqueous (LNGS-2'' fractions, respectively. Vacuum liquid chromatography (VLC) of LNGS-2 (1.3 g) was carried out on silica for TLC. The solvent systems for the elution process were hexane: ethyl acetate in the ratio: of 10:0, 9:1…5:5…. up to 2:8 and this ended up with nine VLC fractions. These were eventually bulked and pooled together into four by TLC (n-hexane: ethyl acetate 8/2 and 7/3 and observed in UV light at ʎ 236 and 254 nm. Using HPLC profile and TLC, about 0.53g of the second bulked fraction which was eluted in hexane: ethyl acetate (3:2, 1:1 & 2:3) was taken for further separation on Sephadex LH-20 (DCM / MeOH; 1/1) and this gave 7 subfractions using HPLC for this process. Fractions 1 and 2 were ultimately cleaned on a semi-preparative HPLC (MeOH/0.1% formic acid in water); 0 sec., 10% MeOH; 300 sec., 10% MeOH; 2100 sec., 100% MeOH; 2700 sec.,

100% MeOH) to obtain pure compounds coded LNGS2-E1 (1.0 mg) and E2 (5.10 mg). About 200 mg of the third Sephadex fraction was obtained pure and coded LNGS2-E3. Structural elucidation was carried out using NMR (AVANCE DMX 600) and mass spectroscopic (LC-MS Agilent 1100 series) methods.

2.2.5 Membrane stabilization activity

Evaluation of the anti-inflammatory effects of isolated compounds was carried out by *in vitro* membrane stabilization according to the method described by Awe *et al.*, (2009) [7,8]. The activity could not be carried out for compound 1 due to low yield. Briefly, 5 mL of fresh whole blood was collected into a heparinized syringe, mixed with an equal volume of Alsever's solution (dextrose 2%, sodium citrate 0.8%, citric acid 0.05%, sodium chloride 0.42% and distilled water 100 mL).

2.2.6 Hypotonic solution induced haemolysis

Each compound was dissolved in 5 mL distilled water (hypotonic solution) in graded doses (25, 50, 100, 200 µg/mL). An equal volume (1 mL) of the hypotonic solution containing the compound and the blood were separately transferred in triplicate into centrifuge tubes. This was repeated for the isotonic solution containing the compound and distilled water and 100 mg indomethacin served as control. The tubes were gently and slightly shaken for the content to mix properly and incubation was carried out for half an hour at 98.6^{o}F. This was subjected to centrifugation at 1300 *gravity* for 180 sec. Evaluation of the absorbance of the haemoglobin of the supernatant was performed by using a spectrophotometer (560 nm) by taking the produced haemolysis of distilled water to 100%. The inhibition (%) of the haemolysis was estimated as:

% haemolysis inhibition = 1- [AL_2-AL_1/AL_3-AL_1] X100

Where,

AL_1 = Value of absorbance of the compound in an isotonic solution
AL_2 = Value of absorbance of the test compound in a hypotonic solution
AL_3 = Value of absorbance of indomethacin in a hypotonic solution

3. RESULTS

The spectra data from the proton and Carbon – 13 nmr are presented in Tables 1 and 2 while the membrane stability effect is presented in Fig. 1.

Table 1. NMR spectra data of LNGS2-E1 and E2 in 600 MHZ, CD_3OD

Position	LNGS2-E1	LNGS2-E2	
	^{1}H -NMR δ (ppm)	^{1}H -NMR δ (ppm)	^{13}C –NMR δ (ppm)
1			163.4
2	6.76 (1H, s)	7.39 (1H, d, J = 9.11 Hz)	110.9
3			151.7
4	6.99 (1 H, s)	7.53 (1H, d, J = 9.12 Hz,)	106.5
4'			157.1
5	6.34, (1H, dd)	6.75 (1H, s, H)	122.2
6	6.47 (1H, dd)	6.99 (1H, s)	140.6
7	5.01 (1H, d)		66.5
8	4.15 (1H, d)		46
8'			110.9
9			183.1
9'			110.9
10'			158.0
11	4.65 (2H, s)	4.68 (2H, s)	66.5
12			171.9
13	3.69 (3H, s)	3.97 (3H, s,)	54.5

Table 2. NMR spectra data of LNGS2-E3 in 600 MHZ, CD_3OD

Position	LNGS2-E3	
	^{1}H -NMR δ (ppm)	^{13}C –NMR δ (ppm)
1	8.03 s	155.1
2		
3		166.5
4	7.90 s	107.9
4’		
5		147.3
6		108.6
7		166.7
8		119.8
8’		
9		173
9’		
10’		
11		85
12	4.52 s	69.9
13		122.2
14		18.6
15	9.79 s	187.9
16	1.38 s	24
17		200.9
18	2.84 (m, 2H)	43.7
19	1.63 (m, 2H)	23.8
20	1.32 (m, 8H, H-20-23)	30
21		30.4
22		33.0
23		22.2
24	0.90 (t, 3H)	14

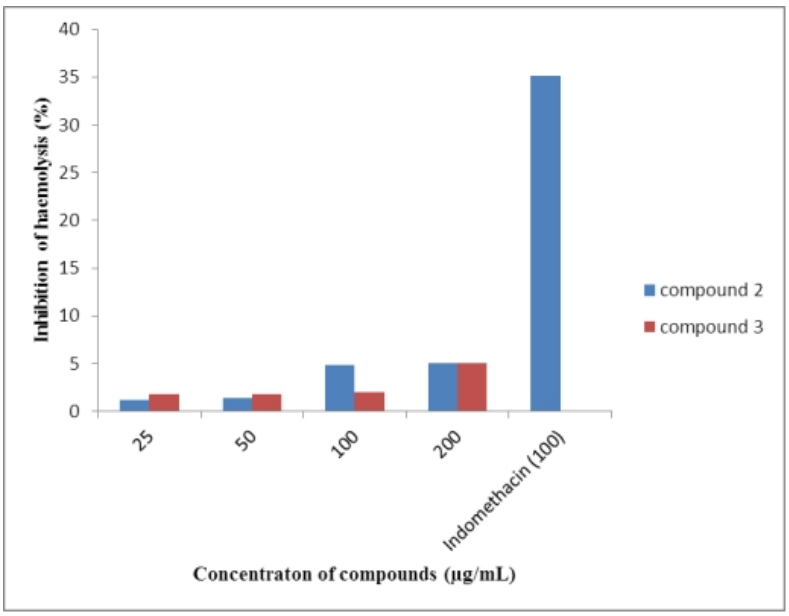
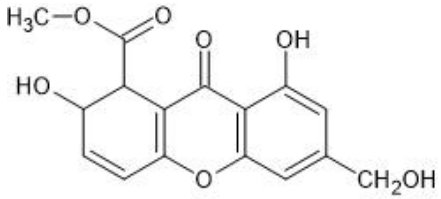


Fig. 1. Membrane stabilising activity of LNGS2-E1 and E2

4. DISCUSSION

4.1 Structural Elucidation

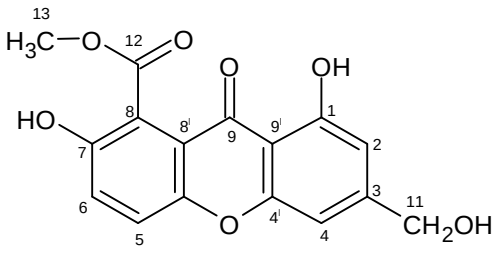
The first compound; LNG-S2-E1 (Fig. 2) was powdery in its physical nature and the colour was yellow, The ^{1}H NMR (600 MHZ, CD_3OD) spectrum data are as indicated in Table 1, it was characterised as 7,8-dihydroxanthenone-8-carboxylic acid methyl ester by comparison an earlier study [9].



LNGS-2-EI

Fig. 2. Chemical structure of LNG-S2-E1

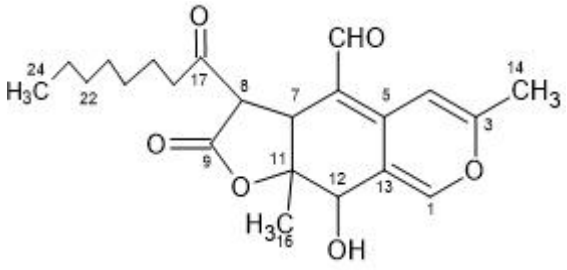
The second compound; LNG-S2-E2 (Fig. 3) was needle-like and yellow. It showed a peak at a retention time; of 22.647 (235 nm). The molecular weight of 316 g/mol was deduced for $C_{16}H_{12}O_7$ as shown by the LC-MS containing quasi-molecular ion peaks at mass/charge of 317.1 and 315.2 which indicated $[M+H]^+$ and $[M-H]^-$, respectively. The proton and carbon -13 nmr at 600 Megahertz indicated the peaks shown in Table 1. LNG-S2-E2 was characterised to be as Sydowinin B when compared to the literature [10].



LNGS-2-E2

Fig. 3. Chemical structure of LNG-S2-E2

LNG-S2-E3 (Fig. 4) is oily in nature and red in colour. It showed UV absorbance at λ_{max} (MeOH) 414.8 nm. As shown by the LC-MS, molecular weight and formula were respectively deduced as 386g/mol and $C_{22}H_{26}O_6$. Quasi-molecular ion peaks at mass/charge 387.2 and 385.3 ($[M+H]^+$ and $[M-H]^-$), respectively shown at both positive and negative modes of 386g/mol. Also, The mass/charge peak of 794.9 indicated $[2M+Na]^+$. From the ^{13}C spectrum, three CH_3, six CH_2, four CH and 9 quaternary carbons which amounted to $C_{22}H_{25}$ were observed. ^{1}H NMR (600 MHZ, CD_3OD) δ (ppm) showed a singlet each with a proton at 8.03, 7.90, 4.52 and 9.79 ppm and a singlet with three protons each of two different positions. Also, two meta protons each of 2.84 and 1.63 were assigned for positions 18 and 19 while others are two protons which are meta each at positions 20 t0 23 and 3 proton peak at position 24 (Table 2). The data extracted from the ^{13}C NMR (600 MHZ, CD_3OD) are as also indicated in Table 2. This compound was therefore identified as Pitholide B by comparing it with literature data [11].



LNGS-2-E3

Fig. 4. Chemical structure of LNG-S2-E3

4.2 Membrane Stabilizing Activity

LNG-S2-2 and LNG-S2-3 exhibited a low protective action against red blood cells and lysosome lysis which was influenced by hypotonic solution in rats. The latter is a clear indication of stabilizing action of the membrane. Inflammation may come with age and diseases such as diabetes and cancer among several others [12]. Test for membrane stabilization of red blood corpuscles is gaining popularity in screening for potential anti-inflammatory /agents. Exposure of these blood cells to a medium which is hypotonic, haemolysis results. Any compounds or agents which can maintain the integrity of the membrane will be suitable anti-inflammatory drugs [7,13,14]. The slight cell membrane stabilizing action exhibited by these compounds is an indication that one of the pathways is mediated via lysosomal membrane stabilization.

5. CONCLUSION

Sydowinin B and pitholide B showed very weak protection against hypotonic solution-induced

erythrocyte and lysosome lysis. The weak activity is probably due to de-replication of compounds.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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