Original Research Article

Weak membrane stabilizing activity of Syndowinin B and pitholide B

ABSTRACT

Introduction: There is a dire need for anti-inflammatory agents presently to solve many health challenges. Extracts of endophytic fungi have been proved implicated in a lot in this area.
Aim: To test isolated compounds from the extract of *Pestalotiopsis clavispora*,

Material and Method: Isolation of compounds, characterisation and membrane stabilisation assay were carried out by standard methods.
Results: From the extract of *Pestalotiopsis clavispora*, an endophytic was isolated three compounds which are 7,8-dihydroxanthenone-8-carboxylic acid methyl ester, syndowinin B and pitholide B. The fungus was from the mangrove plant *Phoenix reclinata*Jacq. (Arecaceae).
Discussion: Syndowinin B and pitholide B exhibited 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 exhibited very weak membrane stabilizing activity.

Keywords: membrane, fungus, inflammatory, red blood cells

INTRODUCTION

A number of models has been described and used to evaluate potential anti-inflammatory drugs, some of which include platelet aggregation, cotton pellet granulation in animals and erythrocyte membranes 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 extract, 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

of associated with erection and male infertility [5]. Extracts of several endophytic fungus 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 Plant collection

Collection of a few leaves sample 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 Bayelsa.

2.2 ENDOPHYTIC FUNGAL SOLATION AND ITS IDENTIFICATION

A none 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 application of the sample on malt agar. The sliced leaf samples were then and applied to agar incorporated with antibiotics as described earlier [6]. Theplateswere thereafter left at 21 - 22°C to allow hyphae growth. Some of the hypha were subsequently sub cultured into fresh agar until pure colonies are established. The colonies were identified using established molecular technique [6].The resulting strain coded LNG-S2 was thereafter banked at the the Institute of Pharmaceutical Biologie and Biotechnologie, Heinrich-HeineUniversity, Duesseldorf, Germany.

2.3 CULTIVATION AND EXTRACTION OF FUNGUS

To the one hundred gram of commercially available rice in a one litre flask, one hundred and ten millilitre of distilled water was added and subject to autoclave at 121°C, 2 bar for one third

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of a minute. After cooling, a piece of the fully grown mycelia was gently added on the top of the rice medium in sterile conditions. It was left for a monthly at room temperature for full growth. The growth was then abruptly terminated using ethylacetate (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 extract.

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2.4 CHROMATOGRAPHIC PURIFICATION

The LNG-S2extract (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: 10:0, 9:1…5:5…. up to 2:8 and this ended up with nine VLC fractions, These were eventually bulked to which were pooled together into four by TLC(n-hexane : ethyl acetate 8/2 and 7/3 and observed in UV light and 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 sub fractions using HPLC for this process. Fractions 1 and 2 were ultimately cleaned on 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.

2.5 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]. 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 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).

2.6 HYPOTONIC SOLUTION INDUCED HAEMOLYSIS

Each compound was dissolved in 5 mL distilled water (hypotonic solution) in graded doses (25, 50, 100, 200 µg/mL). 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 isotonic solution containing the compound and distilled water and 100 mg indomethacin served as controls. 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 spectrophotometer (560 nm) by taking the produced haemolysis of distilled water to be 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 compound in isotonic solution

AL_2 – value of absorbance of test compound in hypotonic solution

AL_3 - value of absorbance of indomethacin in hypotonic solution

3. RESULTS

The spectra data from the proton and Carbon – 13 nmr are as presented in Tables 1 and 2 while the membrane stability effect is presented in Figure 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-E3in 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

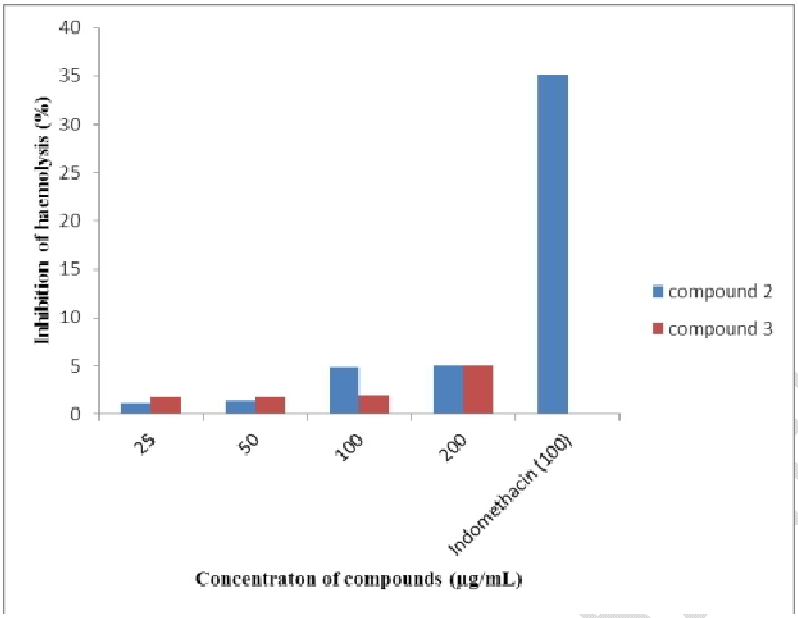
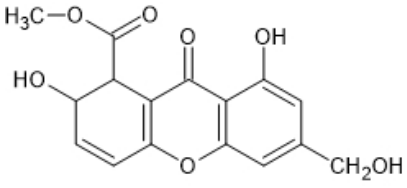


Figure 1: Membrane stabilising activity of LNGS2-E1 and E2

4. DISCUSSION

4.1 Structural elucidation

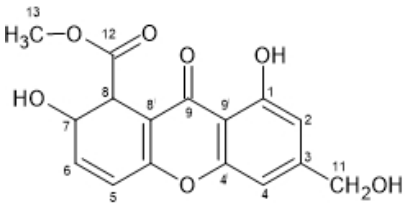
The first compound; LNG-S2-E1 (Figure 2) was powdery in its physical nature and the colour was yellow, The ^{1}H NMR (600 MHZ, CD_3OD) spectrum data are as indicated in Table 1, it was characterised as 7,8-dihydroxanthenone-8-carboxylic acid methyl ester by comparison an earlier study [9].



LNGS-2-EI

Figure 2 Chemical structure of LNG-S2-E1

The second compound; LNG-S2-E2 (Figure 3) was needle like and yellow in colour. It showed a peak at retention time; 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 Syndowinin B when compared to the literature [10].

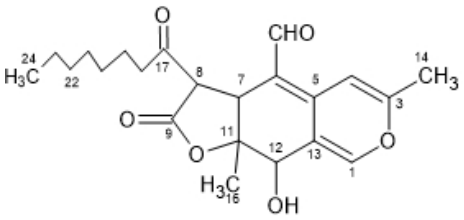


LNGS-2-E2

Figure 3Chemical structure of LNG-S2-E2

LNG-S2-E3 (Figure 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, a molecular weight and formula were respectively deduced as 386g/mol and $C_{22}H_{26}O_{6,}$ respectively. 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. ^1H 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 Bby comparing with literature data [11].



LNGS-2-E3

Figure 4Chemical 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 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 a medium which is hypotonic, haemolysis results. Any compounds or agents which can maintain the integrity of the membrane will be a suitable anti-inflammatory drug [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.

4. CONCLUSION

Syndowinin B and pitholide B showed very weak protection against hypotonic solution-induced erythrocyte and lysosome lysis.

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