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Isolation of wound healing compounds from *Heliotropium indicum*

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ABSTRACT

Heliotropium indicum (Boraginaceae) is used in the traditional Ivory Coast pharmacopeia to treat asthma. In the present study, wound healing effect of *n*- butanol fractions was evaluated in H292- cells. Fractions which possessed better wound healing activity were fractionated on Sephadex LH20 column chromatography. Two compounds have been isolated which were responsible for this wound healing effect. Their structures were established as Pestalamide B (**1**) and Glycinamide,N-(1-oxooctadecyl)glycyl-L-alanylglycyl-L-histidyl (**2**) on the basis of spectral analysis. Both compound **1** and **2** presented wound healing effect compared with the control (P<0.05).

Keywords: *Heliotropium indicum*, Boraginaceae, Isolation, Wound healing, H292-Cells,

INTRODUCTION

Extracts of several plants were used for their wound healing properties (Diwan *et al*., 1982; Udupa *et al*., 1989; Suguna *et al*., 1996; Saha *et al*., 1997; Sunilkumar et al., 1998; Rasik *et al*., 1999; Mukherjee and Suresh, 2000; Park and Chun, 2001; Nagappa and Cheriyan, 2001). *Heliotrpium indicim* is an herb grown throughout Africa. This herb was used in Ivorian Pharmacopeia for it antipyretic; antiasthmatic effects (Adjanohoun *et al*., 1983). The plant is reported to be highly valued in the folklore medicine and is believed to be useful in treating malaria, abdominal pain, fever, dermatitis, venereal diseases, insect bites, menstrual disorder, urticaria, and sore throat (Duttagupta and Dutta, 1977). The plant decoction is considered as diuretic and remedy for the treatment of kidney stone (Quisumbing, 1951; Berhault, 1974). The leaf paste is applied externally to cure rheumatism and skin infections (Nagarajuand and Rao, 1990; Barrett, 1994). The various tribes in India use the leaf paste over fresh cuts and wounds and claim for its promising activity (Kumar *et al.,* 2007). A number of natural products from *H. indicum* such as volatile oil, Indicine-N-oxide, esters and terpenes have shown potent wound healing, antitumor and antileukemic activities (Machan *et al.,* 2006; Yasukawa *et al*., 2002; Kupchan *et al*., 1976). Wound healing process involves several steps, which are coagulation, inflammation, formation of granulation tissue, matrix formation, remodeling of connective tissue, collagenization and acquisition of wound strength. During the formation of new tissue, endothelial cells proliferate and form new blood vessels. Previous study demonstrated *in vitro* and *in vivo* wound healing effect of whole and fractions of extracts from *H. indicum* (Srinivas *et al.,* 2000, Dodehe *et al* 2011a). The aim of this research was to isolate compounds responsible for this effect.

MATERIEL AND METHODS

Plant material

The aerial part (stem and leaves) of *Heliotropium indicum* (Boraginaceae) was collected in Korhogo, Ivory Coast during the month of November 2009 and authenticated by the Department of Botany, University of Cocody-Abidjan, Ivory Coast. The voucher specimen N° 6693 was kept in a herbarium.

Chemical analysis

General experimental

Column chromatography was carried out with Diaion HP-20 (Mitsubishi Chemical, Japan) and C18-ODS (Merck, Germany). Preparative-HPLC was performed using a Tosoh HPLC instrument on a TSK ODS-80Ts gel column (250 × 10 mm, 5 µm). NMR spectra were recorded on a Varian Inova 400. The ESIMS were detected on an Esquire 3000 plus ion trap mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA). LC-MS were conducted using an Agilent LC-MSD ion trap mass spectrometer (Bruker Daltronics, Bremen, Germany) equipped with a 1100 series HPLC and a Tosoh ODS-80Ts (150 mm × 4.6 mm, 5 µm) column. The UV detection was set at 270 nm and the chromatographic separation was carried out using a gradient elution of solvent A: methanol and solvent B: 5‰ formic acid in water at a flow rate of 0.8 mL/min as follows: 0-30 min, 15–35% A; 31-45 min, 35% A; 46-55 min, 35-15% A.

Extraction and isolation

The aerial part of the plant were cut into small pieces, dried in the shade and powdered. Following the harvesting of plant material, it was dried in the open air at room temperature until they attained constant weight and powdered in a mortar. The powder (100g) was extracted with 2L of distilled water for 2x24 h on a hot plate. The mixture was filtered over a cheese cloth; cotton wool and Wathman filter paper N°1, respectively. The aqueous extract was concentrated over a water bath to the desired consistency (100 mL). The aqueous extract was therefore extracted with ethyl acetate (3x100 mL). The ethyl acetate extract was concentrated under vacuum (1.3 g) and re-extracted with *n*-butanol (3x90 mL). This *n*-butanol extract was concentrated under vacuum (2.5 g).

Only the *n*-butanol extract showed the best activity (Dodehe *et al.,* 2011b) and it was fractionated on Sephadex LH20 column. Elution was performed with MeOH-H_2O (60:40, v/v). Twenty (20) fractions were obtained. Only fractions six (6) to fifteen (15) were retained and put together (1.1g) according to their activity and their chromatography profile. It was future analyzed on HPLC to verify it purity (Fig.1) and fractionated on Sephadex LH-20 column using MeOH-H_2O (70:30, v/v) as elution solvent. Two fractions (1 and 2) were obtained according to their activity and their chromatography profile under UV-light (254 and 365 µm). They were future purified by recrystallization method to yield compound **1** (55 mg) and compound **2** (120 mg) respectively.

*Pestalamide B (**1**)*

A colorless amorphous; ^{1}H NMR (400MHz, CD_3OD), δ 8.20 (1H, s, H-2), 5.89 (1H, s, H-5), 3.90 (2H, s, H-7), 7.06 (2H, *dd, J=8.4, 2.5 Hz*, H-9 and H-13), 7.26 (2H, *dd, J=8.4, 7.8Hz*, H-10 and H-12), 7.12 (1H, m, H-11), 2.29 (1H, *dd, J=18.1, 8.2Hz*, H-17α), 2.60 (1H, *J=18.1, 5.6Hz*, H-17β), 2.82 (1H, m, H-18), 1.19 (3H, d, *J=6.7Hz*, H-20); ^{13}C NMR (100Mz, CD_3OD), δ 156.9 (C-2), 119.0 (C-3), 182.0 (C-4), 110.2 (C-5), 160.8 (C-6), 41.0 (C-7), 137.2 (C-8), 129.1 (C-9 and C-13), 128.7 (C-10 and C-12), 125.1 (C-11), 165.0 (C-14), 173.3 (C-16), 41.5 (C-17), 37.5 (C-18), 178.2 (C-19), 17.0 (C-20); LC-ESIMS: ions $[M+H]^+$ m/z:343.12966 and $[M+Na]^+$ m/z 365.12081, molecular formula $C_{18}H_{18}N_2O_5$.

*Glycinamide,N-(1-oxooctadecyl)glycyl-L-alanylglycyl-L-histidyl (**2**)*

Yellow-orange amorphous; ^{1}H NMR (400MHz, CD_3OD), δ 2.18 (2H, *t, J=7.2Hz*, -$NCOCH_2$, H-2), 1.57 (2H, *m*, H-3), 1.29 (26H, *br, s*, 13CH_2, H-4 to H16), 1.33 (2H, *m*, H-17), 0.96 (3H; *t, J=3.5 Hz*, CH_3, H-18), 8.0 (5H, *br s*, NH), 4.09 (6H, br s, H-20, H-26 and H-32), 4.71 (1H, *q, J=5.2Hz*, H-23), 6.0 (2H, br s, NH_2), 1.48 (3H, *d, J=5.2Hz*, H-35), 2.87 (1H, *dd, J=6.2 and 3.6Hz*, H-36α), 3.12 (1H, *dd, J=6.2 and 3.6Hz*, H-36β), 6.80 (1H, *d; J=7.3Hz,* H-37), 7.50 (1H, *s*, H-39), 1.47 (1H, *m,* H-41); ^{13}C NMR (100MHz, CD_3OD): δ 175.3 (C-1), 35.8 (C-2), 25.7 (C-3), 28.7 (C-4), 29.7 (C-4 to C-15), 34.9 (C-16), 22.8 (C-17), 14.1 (C-18), 43.1 (C-20), 170.8 (C-21 and C-27), 48.3 (C-23), 172.1 (C-24, C-30), 45.7 (C-32), 169.9 (C-33), 17.8 (C-35), 33.4 (C-36), 164.0 (C-37), 160.5 (C-39), 45.1 (C-41); LC-ESIMS: ions $[M+C_2H_5NH_3]^+$ m/z: 708.51207 and $[M+H]^+$ m/z:663.44803, molecular formula $C_{33}H_{58}N_8O_6$.

Tissue culture

The cultivation of cells let's get in a few days a well-differentiated epithelium like *in vivo*. These models purely apprehend the mechanisms of epithelial repair and differentiation after a chemical (Buisson *et al*., 1996) or mechanical attack (Lechapt-Zalcman *et al.,* 2006). H292 human lung cells were maintained in RPMI-1640 medium (Newland and Richter, 2008). This medium contained 10% foetal bovine serum (FBS), and was supplemented with 2 mM glutamine, 50 U/mL penicillin and 50 µg/ml streptomycin. The cells were cultured, at 37°C, 5% CO_2 in a humidified incubator. After a 12 h period, fresh serum-free medium was added.

Wound healing assays

The scratch assay was performed on cells to study the effect of *n*-butanol fraction from *H. Indicum* on cell migration. Cells were scraped in a straight line to create a "scratch" with a p200 pipet tip and washed once with 1 mL of the growth medium (RPMI). Four groups of dishes were created with three in each group. Group 1 served as control with FBS (0.3 %) and Group 2 was used as reference with the *Transforming Growth Factor* β (TGF β). Group 3 to Group 4 received fraction 1 and fraction 2 from *H. indicum*. Wound healing effect ach drug was observed at 300 µg/ml. At time t_0, areas were measured. For every 2 h (t_i) the same scratched region was observed until the scratch completely

close. Areas of scratch were measured with the logiciel "image J". Percentage of wound was calculated using the equation: Percentage of wound= (Area of initial wound at time t_0 – Area of wound at time t_i/Area of initial wound at time t_0) X 100 (Dodehe *et al.,* 2011b).

Statistical analysis

Data obtained were presented as mean s ± standard error of mean (SEM.). Each concentration was tested three times. The differences between the data obtained from 'test' dishes groups and the data obtained from untreated dishes groups, were subjected to one-way analysis of variance (ANOVA; 95% confidence interval), and followed by Dunnett's test. Necessary time for 50 % of *in vitro* wound healing effect (T_{50}) was calculated. Values with $p < 0.05$ compared with the control group were considered as being significantly different.

RESULTS AND DISCUSSIONS

The *n*-butanol extract prepared from the leaves and stems of *H. indicum* was subjected to a series of column chromatography using Sephadex LH-20. Compounds **1** and **2** were isolated. Identifications were based on HPLC, LC-ESIMS and NMR 1D (^{1}H, ^{13}C) and 2D (^{1}H-^{1}H COSY, HMBC and HSQC) spectroscopic data.

Pestalamide B (**1**) was identified as follow: In the HPLC spectrum (Fig.1) peak of **1** was observed at the retention time (R_T) 5.493 minutes. The LC-ESIMS spectrum gave the peak of the ion $[M+H]^+$ at m/z:343.12966 and that of $[M+Na]^+$ is observed at m/z 365.12081. It was deducted the molecular weight 342.12184 g/mol with the molecular formula $C_{18}H_{18}N_2O_5$. The ^{1}H NMR spectroscopic data of **1** showed signals at 8.20 ppm (1H, s) and 5.89ppm (1H, s) what result from the ring of pyridin-4(1*H*)-one. Aromatic protons gave signals at 7.06 ppm (*dd, J=8.4, 2.5 Hz)*, 7.26 ppm (*dd, J=8.4, 7.8Hz*) and 7.12 ppm (m), this showed a monosubstitued aryl ring. The ^{1}H-^{1}H COSY NMR confirmed this observation with the sequence H_9-H_{10}-H_{11} or H_{13}-H_{12}-H_{11}. The ^{13}C NMR spectrum confirms the presence of aryl ring with the peaks at 129.1, 128.7 and 125.1 ppm. A ketone α,β-unsaturated gave the peak at 182.0 ppm (-C=C-CO-C=C-). The HMBC correlation shows that this ketone resulted from the pyridin-4(1*H*)-one. The amide system gave the peaks at 165.0 and 173.3 ppm (-CO-NH-CO-) and the carboxylic acid group gave the one at 178.2ppm (-COOH). The HMBC spectrum showed sequences which leaded to fragments C_2-C_3-C_4[C_{14}], C_4-C_5-C_6-C_7-C_8 and C_{16}-C_{17}-C_{18}-C_2[C_{19}]-. Comparing our spectroscopic data with those reported by Gang et al.; compound **1** was identified as Pestalamide B; a compound previously isolated from *Pestalotiopsis theae* (Amphisphaeriaceae) (Gang *et al.,* 2008).

Glycinamide, N-(1-oxooctadecyl)glycyl-L-alanylglycyl-L-histidyl (**2**) was isolated as an amorphous, yellow-orange. In the HPLC spectrum (Fig.1), peak of **2** was observed at the retention time (R_T) 12,392 minutes. The development of this peak by LC-ESISMS method gave, the ions $[M+C_2H_5NH_3]^+$ m/z 708.51207 and $[M+H]^+$ m/z:663.44803. So, it was deducted the molecular weight

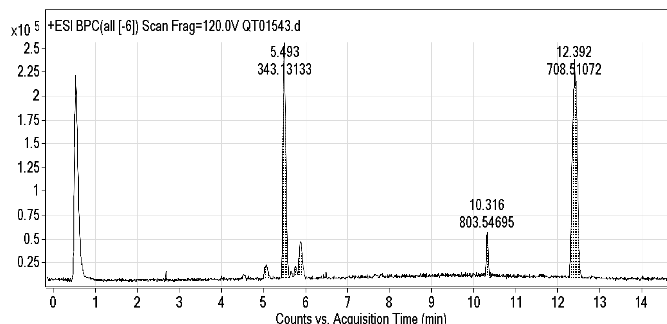

Fig 1: HPLC of the *n*-butanol extract from *Heliotropium indicum.*

M=662.44729 g/mol with the molecular formula $C_{33}H_{58}N_8O_6$. The ^{1}H NMR spectroscopic data of **2** showed a signal at 8.0 ppm corresponding to that of a proton carried by a nitrogen; this requires the presence of an amide (HN-C=O). The signals at 7.44 ppm (H-39, s) and 6.80 ppm (H-37, *s*) respectively indicated the presence of a *1H-imidazole* ring. Chemical shifts between 4.92 and 4.09 ppm result from the protons of amide groups (HN-C=O); probably eight (8) amide groups in it structure. This could justify the presence of 8 nitrogens in the molecular formula. The intense peak at 1.29 ppm requires the presence of a long carbonyl chain n(CH_2). The methyl groups (CH_3) gave signals at 0.95 ppm. The presence of amide groups was confirmed by the ^{13}C NMR spectroscopic data, that shown N-C=O signals at 193.0, 172.1, 170.8 and 169.9 ppm. Each peaks at 172.1 and 170.8 ppm counted for two carbonyl groups (C=O). Three peaks at 135.5, 133.5 and 119.8 ppm indicated the presence of double bonds (C=C), probably those of the *1H-imidazole*. An intense peak at 29.7 ppm confirmed the presence of the long carbonyl chain n(CH_2). The DEPT135 showed the presence of two methyl groups at 17.8 and 14.1 ppm. The HMBC correlation leaded to the sequence C_1-C_2-C_3-C_4, C_{18}-C_{17}-C_{16}, C1-C_{20}-C_{21}, C_{41}-C_{23}-C_{24}, C_{30}-C_{29}-C_{35}-C_{36}, C_{33}-C_{32}-C_{30} and C_{36}-C_{37}-C_{39}. All these spectroscopic data leaded to the formal identification of compound **2**. This known compound was already decrypted in the literature (Huo *et al., 2001*; Orbulescu *et al.,* 2001). Structure of compounds **1** and **2** were reported in Figure 2.

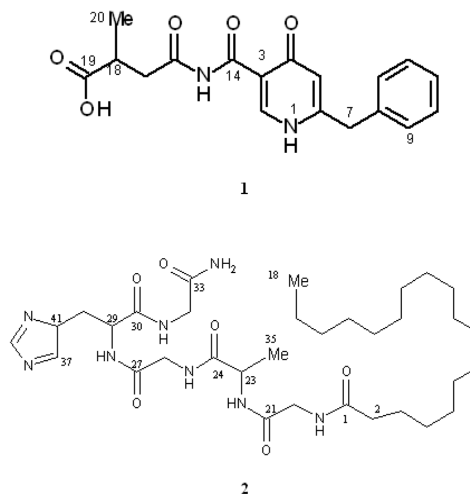

Fig 2: Isolated compounds from *H. indicum*: Pestalamide B (**1**) and Glycinamide, N-(1-oxooctadecyl)glycyl-L-alanylglycyl-L-histidyl **(2).**

Present bio-guided study of *H. indicum* has leaded to the isolation and identification of two alkaloids: Pestalamide B (**1**) and Glycinamide, N-(1-oxooctadecyl)glycyl-L-alanylglycyl-L-histidyl (**2**). Our results are in accordance with those of literature, which indicated that *Heliotropium* genus is a reach source of alkaloid (Pandey *et al.,* 1996; Pandey *et al.,* 1982; Hoque *et al.,* 1976) and most of the alkaloids isolated from *H. indicum* have pyrrolizidine structure (Newland and Richter, 1988). All compounds isolated in our study were for the first time in *H. indicum*. We can explain it by the very high polarity of our extract.

Compounds **1** and **2** were test for their *in vitro* wound healing effect. *In vitro* complete wound healing of the compounds were studied by counting the number of days and also by measuring the percentage of wound (Figure 3).

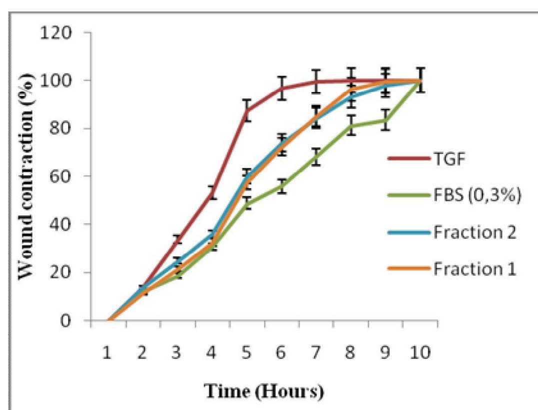

Fig 3: Wound healing effect of compounds **1** and **2**. TGF=Tumor Growth Factor was used as reference; Fetal Bovine Serum (FBS) at 0.3% (poor medium) was used as control. Each point represented mean percentage of wound area ±S.E. This figure showed wound healing effect of *Heliotropium indicum*, compared to the control ($p<0.05$) at 300 µg/ml. Data were shown as mean percentage of wound area ± SEM for 3 separate experiments.

The wound contracted progressively when it was treated with the compounds (**1**; **2**) and TGFβ ($P<0.05$). T_{50} for the control (FBS; 0.3%) was 28.5 hours (1 hour-2 hours; CI 95%) and the T_{50} of TGFβ was 24 hours (0.2 hours-0.6 hours; CI 95%). The T_{50} of compounds **1** and **2** were respectively 25.12 hours (*0.8 hour-1 hour*; CI 95%) and 24.75 hours (*0.7 hour-1 hour*; CI 95%). Values were summarized in Table 1. The results indicated compounds **1** and **2** induce wound effect compared to the control ($P<0.05$).

Table 1. Comparison of T_{50}.

Fractions	T_{50} (hours)	*Confidence Interval (CI) at 95 %*
FBS(0.3%)	28.5	*1 hour-2 hours*
TGFβ	24	*0.2 hour-0.6 hour*
Compound **1**	**25.12**	***0.8 hour-1 hour***
Compound **2**	**24.75**	***0.7 hour-1 hour***

T_{50} = Necessary time to have 50 % of in vitro wound healing effect. Fetal Bovine Serum (FBS) was used as a control and TGF represented the reference. Compounds 1 and 2 showed a wound healing effect compared to the control ($p < 0.05$).

Wound healing or repair is a natural process of regenerating dermal and epidermal tissue, and may be categorized into three phases, viz, inflammation, proliferation and remodeling phase (Koff *et al.,* 2006). In the inflammation phase, various growth factors such as tumour necrosis factor (TNF), interleukins (IL) are released to initiate the proliferation phase. The latter is characterized by angiogenesis, collagen deposition, granular tissue, epithelialization and wound contraction (Darbelley *et al.,* 1986). Intervention in any one of these phases by drugs would eventually lead to either promotion or depression of collagenation, wound contraction and epithelialization. Epithelialization involves that stabilization of lysosomal membranes, inhibition of cellular migration and inhibition of fibroblast contraction are responsible for their anti-healing effects.

CONCLUSION

The bio-guided investigation of the *n*-butanol crude extract from *H. indicum* leaded to the isolation and identification of two alkaloids: Pestalamide B and Glycinamide, N-(1-oxooctadecyl)glycyl-L-alanylglycyl-L-histidyl. These compounds were isolated for the first time from *H. indicum* and presented an excellent wound healing activity. Both compound had wound healing effect compared to the control (FBS 0.3%) and reference (TGFβ).

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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