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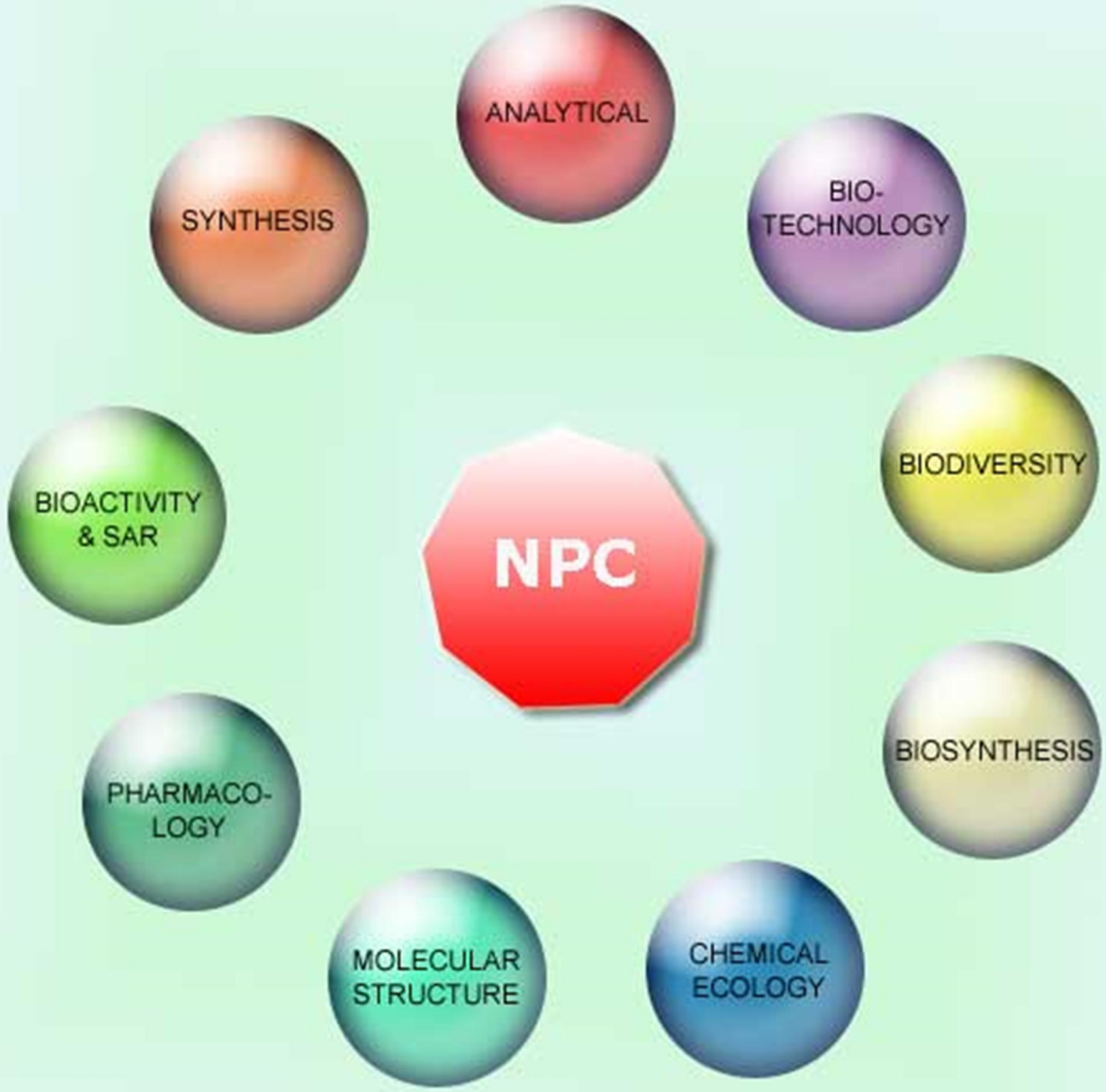

Volume 8. Issue 9. Pages 1195-1352. 2013
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

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NPC Natural Product Communications

2013
Vol. 8
No. 9
1213 - 1216

Isolation of Cycloeucalenol from *Boophone disticha* and Evaluation of its Cytotoxicity

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Received: November 21st, 2012; Accepted: June 6th, 2013

Boophone disticha (Amaryllidaceae) is widely used in traditional medicine in southern Africa. Several alkaloids, volatile oils and fatty acids have been isolated from the plant. However, there has been no literature report of a triterpene from *B. disticha*. Cycloeucalenol, a cycloartane triterpene, together with its regio-isomer, was isolated from the ethyl acetate extract of the bulbs using column chromatography and preparative thin layer chromatography. Structural elucidation was carried out using 1D and 2D NMR and mass spectroscopy. The MTT and neutral red assays were used to assess the cytotoxicity of the compound in human neuroblastoma (SH-SY5Y) cells. The compound was obtained as a mixture of two regio-isomers, which were separated for the first time by chromatographic optimization. Integration of the ^{1}H NMR spectrum showed that cycloeucalenol and its regio-isomer were present in a ratio of 1.04:1. A dose-dependent decrease in cell viability was observed using both cytotoxicity assays. IC_{50} values of 173.0 ± 5.1 μM and 223.0 ± 6.4 μM were obtained for the MTT and neutral red assays, respectively, indicative of the low toxicity of the compound. This work describes for the first time, the presence of triterpene compounds from the genus *Boophone*.

Keywords: Amaryllidaceae, *Boophone disticha*, Cycloeucalenol, Cytotoxicity, SH-SY5Y cells, Regio-isomer.

Boophone disticha (L.f.) Herb, a member of the Amaryllidaceae family, is an attractive, bulbous plant with a thick covering of dry scales [1]. The large, round heads occur on short stems so that they appear to grow directly from the bulb, almost at ground level. The flowers vary from shades of pink to red and are sweetly scented [2]. The pedicels (flower stalks) elongate after flowering to form a large seed-head. This breaks off at the top of the scape (stalk) and tumbles across the veld dispersing the seed. The greyish green leaves are erect, arranged in a conspicuous fan and are usually produced after flowering [2]. *B. disticha* is used traditionally to treat several diseases. Fresh scales are applied to burns and used to treat rashes and skin disorders including eczema. It is also used to relieve rheumatic pains, arthritic swelling, sprains, muscular strains, painful wounds, eye conditions, headaches, anxiety, the pain of abrasions and inflammatory conditions [3,4]. Bulb decoctions are administered either orally or as enemas to adults suffering from headaches, abdominal pain, weakness, sharp chest pains and persistent bladder pains [3]. The bulb is also used in the treatment of varicose ulcers and for the relief of urticaria, as well as a treatment for cancer [3].

The Amaryllidaceae alkaloids, a group of isoquinoline alkaloids are found in various *Boophone* species [3]. Alkaloids isolated to date include crinine, buphanisine, buphanamine, distichamine, buphacetine, crinamidine, lycorine, nerbowdine, undulatine, 3-*O*-acetylnerbowdine, buphanidrine and 6-hydroxycrinamine [5,6]. Buphanidrine, buphanamine and distichamine have been reported to have affinity to the serotonin transporter indicating their potential in treatment of depression and anxiety [7,8]. Also, 6-hydroxycrinamine has been shown to contain acetylcholinesterase inhibitory activity [6]. Several other compounds have been isolated from the plant and these include; a volatile oil containing furfuraldehyde, acetovanillone, chelidonic acid, copper, laevulose, petatriacontane, ipuranol and a mixture of free and combined fatty acids [3,9]. However, there has been no literature report of the detection of a triterpene from *B. disticha*. This paper describes the isolation and structural elucidation of a cycloartane triterpene from *B. disticha*. Toxicity of the isolated compound was determined using both the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) and neutral red uptake assays. In addition, as the compound was obtained as a mixture of two regio-isomers, the separation of the regio-isomers was achieved by chromatographic optimization.

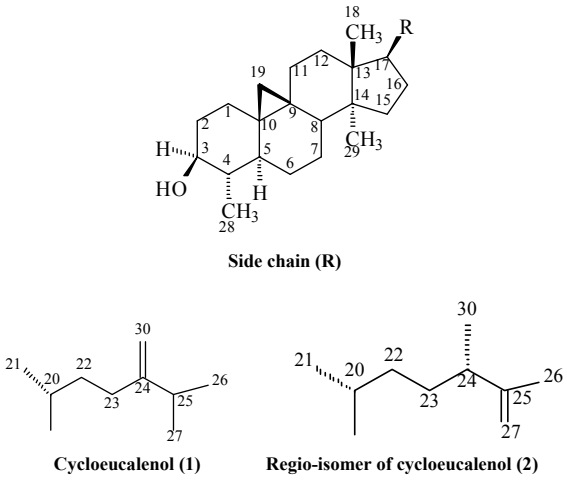

Figure 1: Structure of cycloeucalenol and its regio-isomer.

The triterpene was isolated from the ethyl acetate extracts of the bulbs of *B. disticha* as white crystals. MS data showed the pseudo

molecular ion $[M + H]^+$ peak as the base peak at *m/z* 427 which corresponds to the molecular formula, $C_{30}H_{50}O$ (MW = 426.3942 Da; iFit = 0; DBE = 6). The compound was observed to be non-polar and was dissolved in deuterated chloroform for NMR analysis (^1H, ^{13}C and 2D experiments). The signals obtained from both the ^1H and ^{13}C NMR spectra were complex suggesting that the isolated compound was a mixture of two regio-isomers. Analyses of both the NMR and MS data revealed that the structure of the isolated compound was cycloeucalenol (**1**), together with its regio-isomer (**2**) (Figure 1). The NMR data obtained was compared with that of the published data on cycloeucalenol [10,11], and our extensive literature search revealed that cycloeucalenol and its regio-isomer have not previously been isolated from any species of *Boophone*. However, this class of compound, the cycloartanes, including cycloeucalenol, have previously been reported from *Ammocharis coranica*, a member of the Amaryllidaceae family [12]. The first literature report of a cycloartane from this family was from the plant *Crinum asiaticum* var. *japonicum* [13].

The ^1H NMR spectra of cycloeucalenol and its regio-isomer are very similar, with the only difference observed in the position of the double bond on the side chain. The methyl protons of the regio-isomer (**2**) (Figure 1), Me-28 and Me-21, appeared as broad singlets (0.95 and 0.86); Me-26 appeared as a multiplet (δ_H 1.64), while Me-29 was observed as a singlet (δ_H 0.88). A hextet was observed at δ_H 2.22 (J = 7.0 Hz), while an olefinic proton, which appeared as a doublet, was observed at δ_H 1.00 (J = 6.6 Hz). The ^1H NMR data compares well with that of Akihisa *et al.* [10]. The ^{13}C NMR spectra of cycloeucalenol and its regio-isomer are very similar for C-1 to C-21, with the only difference observed in the side chain from C-22, because of the difference in position of the double bond. C-25 is an olefinic quaternary carbon at δ_C 150.5, while C-27 is an exomethylene carbon at δ_C 109.6.

Cycloeucalenol and its regio-isomer co-chromatographed. To date there has been no literature report in which the separation of these regio-isomers was accomplished. This study is the first to separate these isomers into two distinct compounds, as evident from the chromatographic profile. Integration of the ^1H NMR spectrum showed that cycloeucalenol and its regio-isomer are present in a ratio of 1.04:1.

The continuous use and growing demand for herbal therapies have invigorated the quest for validating the efficacy and safety or toxic implications of medicinal plants. This is very important, as it helps in developing safe and cheap alternative medicines. One of the fundamental *in vitro* toxicological assays performed is the direct assessment of the effects of a plant extract or compound on the viability of a cell line. Data obtained in these assays are very useful in selecting the most promising candidate for further development and obtaining data for future studies [14]. The human neuroblastoma (SH-SY5Y) cell line, which is widely used in experimental neurological studies, analysis of neuronal differentiation, metabolism and function related to neurodegenerative and neuroadaptive processes, neurotoxicity and neuroprotection [15], was selected to assess the cytotoxicity of cycloeucalenol and its regio-isomer. The MTT and neutral red uptake assays were selected to determine cell viability. Both assays were run in parallel in order to improve the reliability of the cytotoxicity data thereby providing a more comprehensive picture of the potential cellular toxicity through different mechanisms.

Cytotoxicity tests were carried out to assess the effect of cycloeucalenol and its regio-isomer on the viability of the cells. A dose-dependent effect on cell viability was observed and results obtained from both cytotoxicity assays were comparable (Figure 2). IC_{50} values of 173.0 ± 5.1 μM and 223.0 ± 6.4 μM were obtained for the MTT and neutral red assays, respectively. Cycloeucalenol and its regio-isomer were observed to have high IC_{50} values for both assays, which is indicative of their low toxicity. Two cycloartane triterpenes; 25-*O*-acetylcimigenol-3-*O*-β-D-glucopyranosyl (1″→2′)-β-D-xylopyranoside and 25-*O*-acetyl-cimigenol-3-*O*-β-D-galactopyranoside showed low toxicity when tested against mouse hepatocytes, with IC_{50} values >100 μM [16]. This result supports the findings of the present study.

Cycloeucalenol has been reported to show anti-inflammatory, cardiotonic and spasmolytic effects [17,18], and its low toxicity indicates that it could be studied further as a potential lead in developing drugs useful in treating inflammation and with cardioprotective properties.

In conclusion, we have described the isolation of cycloeucalenol, a cycloartane triterpene, together with its regio-isomer, from the bulbs of *Boophone disticha*. The separation of both regio-isomers into two distinct compounds is also reported for the first time. The low toxicity of cycloeucalenol and its regio-isomer make it a suitable agent for further testing for pharmacological activity.

Experimental

General experimental procedures: NMR spectroscopy was performed using a 600 MHz Varian NMR spectrometer. Structural characterizations were carried out using a combination of 1D (^1H, ^{13}C) and various 2D experiments. The 2D experiments carried out included DEPT, COSY, HSQC and HMBC. Chemical shifts are reported in units of δ (ppm) and coupling constants (*J*) are expressed in Hz. UV-VIS detection was achieved on a WATERS PDA scanning from 200 – 600 nm. All chemicals for UPLC-MS were of ultra-pure LC-MS grade and purchased from Fluka (Steinheim, Germany), while ultra-pure solvents were purchased from Honeywell (Burdick & Jackson, Muskegon, USA). Ultra-pure water was generated from a Millipore Elix 5 RO system and Millipore Advantage A10 Milli-Q system (Millipore SAS, Molsheim, France). Silica gel 60 (0.063-0.2 mm) was used for CC, while pre-coated glass plates (Merck, SIL G-25 UV_{254}, 20 cm x 20 cm) were used for TLC and preparative TLC. Compounds on the TLC plates were detected under UV light at short wave (250 nm) and long wave (365 nm) lengths, and by spraying with vanillin-H_2SO_4 reagent. MTT and neutral red dye, purchased from Sigma were used for the cytotoxicity assays.

Plant material: Bulbs of *Boophone disticha* (L.f.) Herb. (Amaryllidaceae) were a gift from the South African National Biodiversity Institute, Pretoria.

Extraction and isolation of cycloeucalenol and its regio-isomer: Plant material was cut into small pieces and air-dried at room temperature. Dried material was ground to a fine powder using an Ika Analytical Mill (Staufen, Germany), and stored at ambient temperature in the dark till use. Powdered plant material (250 g) was extracted with 2.5 L of ethyl acetate for 24 h while shaking. The extracts were filtered, concentrated using a rotary vacuum evaporator and further dried under reduced pressure. The ethyl acetate extract (1.4 g) was subjected to silica gel CC (65 g; particle size 0.063 - 0.2 mm).

The separation and purification was carried out using a stepwise gradient mixture of *n*-hexane: ethyl acetate starting from 100:0 until 0:100 as eluent to give 70 fractions. Fractions were collected every

5 min at a rate of 1 mL/min. The fractions were pooled together based on the similarity in their R_f values on a TLC plate to give 4 sub-fractions. Sub-fraction 2, which contained cycloeucalenol, was further purified by CC. This sub-fraction was subjected to further silica gel column chromatographic purification and subsequently eluted using a stepwise gradient mixture of *n*-hexane: ethyl acetate, starting from 90:10 until 0:100, to give another set of 18 fractions. These fractions were pooled together based on the similarity in their R_f values on a TLC plate. Cycloeucalenol and its regio-isomer (0.3 g) were obtained as white crystals. These were further analyzed using UPLC-QTOF (mass spectrometric determination) and NMR spectroscopy (1D and 2D experiments). The separation of the 2 regio-isomers into 2 distinct compounds was evident from the chromatographic profile (data not shown).

Instrumental: A Waters UPLC coupled in tandem to a Waters photodiode array (PDA) detector and a SYNAPT G1 HDMS mass spectrometer was used to generate accurate mass data. Chromatographic separation of the purified sample utilized a Waters HSS C18 column (150 mm x 2.1 mm, 1.8 µm) with temperature controlled at 60ºC. A binary solvent mixture was used consisting of water (Eluent A) containing 10 mM formic acid (natural pH of 2.3) and methanol (Eluent B). The initial conditions were 40% A at a flow rate of 0.4 mL/min, which was maintained for 1 min, followed by a linear gradient to 5% A at 12 min. The conditions were kept constant for 3 min and then changed to the initial conditions. The runtime was 20 min and the injection volume was 5 µL. The PDA detector was scanned between 200 and 500 nm (1.2 nm resolution), which collected 20 spectra per second.

The SYNAPT G1 mass spectrometer was used in V-optics and operated in electrospray ionization mode to enable detection of terpenes. Leucine enkephalin (50 pg/mL) was used as reference calibrant to obtain typical mass accuracies between 1 and 3 mDa. The mass spectrometer was operated in positive mode with a capillary voltage of 3.0 kV, the sampling cone at 25 V and the extraction cone at 4 V. The scan time was 0.1 sec covering the 100 to 1000 Da mass range. The source temperature was 120ºC and the desolvation temperature was set at 400ºC. Nitrogen gas was used as the nebulization gas at a flow rate of 800 L/h. The software used to control the hyphenated system and for data manipulation was MassLynx 4.1 (SCN 704).

Cells and cell culture: Human neuroblastoma (SH-SY5Y) cells (ATCC CRL-2266) were used for the cytotoxicity studies. Cells were cultured in Ham's F-12 supplemented with 2% heat-inactivated fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37°C in a humidified incubator at 95% air and 5% CO_2. For use in the assay, the cells were trypsin-treated for 10 min, decanted from culture flasks and centrifuged (200 *g*, 10 min). The pellet was re-suspended in 1 mL Ham's F-12 medium supplemented with fetal calf serum, and enumerated by staining with trypan blue. The cells were diluted to a concentration of 1×10^5 cells/well in Ham's F-12 medium and 100 µL of the cell suspension plated into each of the wells of a 96-well microtiter plate. Ham's F-12 medium (80 µL) was added and plates were then incubated for 1 h at 37°C in a humidified incubator with 95% air and 5% CO_2 to allow for cellular re-attachment.

MTT assay: The MTT assay as described by Mossmann [19] was used to measure cell viability. The principle of the assay is based on generation of formazan (a blue product) in the mitochondria of active cells, which is measured by photometric techniques [20]. The compound was dissolved in 0.3%, v/v, DMSO in distilled water. The vehicle was used as control.

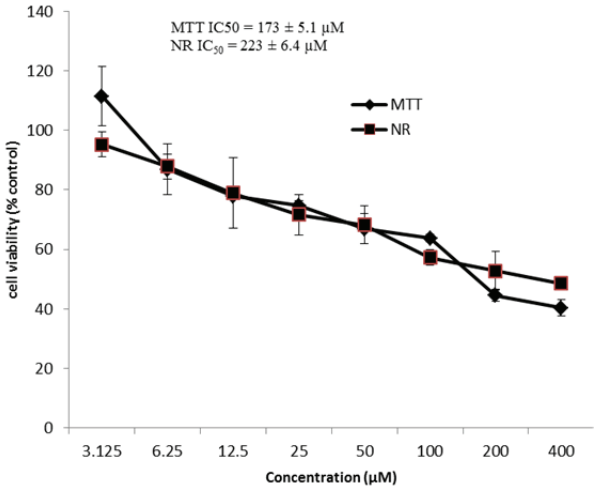

Figure 2: Effect of cycloeucalenol and its regio-isomer on the viability of SH-SY5Y cell lines as determined by the MTT and neutral red uptake assays after 72 h of incubation.

The cells were plated into 96-well culture plates, as described above, and treated with various concentrations of the compound ranging from 3.125 µM to 400 µM for 72 h. Thereafter, 20 µL of MTT solution (5 mg/mL) was added to the wells and further incubated for 3 h. A solution (50 µL) containing 30%, w/v, *N,N*-dimethylformamide and 20% sodium dodecyl sulfate in water was then added to breach the cells and dissolve the formazan crystals. The plates were incubated overnight at 37°C, after which absorbance was measured at 570-630 nm using a microtiter plate reader (Labsystems Multiscan EX type 355). Wells without cells were used as blanks and were subtracted as background from each sample. Cytotoxicity results are expressed as the percentage cell survival compared with the untreated control using a dose response curve and extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage versus extract concentration.

Neutral red assay: The neutral red uptake assay, as described by Borenfreund and Puerner [21], was also used to assess cell viability. This method is based on the determination of the accumulation of the neutral red dye in the lysosomes of viable, uninjured cells. The compound was dissolved in 0.3%, v/v, DMSO in distilled water. The vehicle was used as control. The cells were plated into 96-well culture plates, as described above, and treated with various concentrations of the compound ranging from 3.125 µM to 400 µM for 72 h. Thereafter, 150 µL of neutral red dye (100 µg/mL) dissolved in serum free medium (pH 6.4) was added to the culture medium for 3 h at 37°C. Cells were washed with Phosphate Buffered Saline (PBS), and 150 µL of elution medium ($EtOH/AcCOOH/H_2O$, 50%/1%/49%) was added, followed by gentle shaking for 60 min, so that complete dissolution could be achieved. Absorbance was recorded at 540-630 nm using a microtiter plate reader (Labsystems Multiscan EX type 355). Cytotoxicity results are expressed as the percentage cell survival compared with the untreated control using a dose response curve and extract concentration providing 50% inhibition (IC_{50}) of cell death was calculated from the graph.

Statistical analysis: Tests were carried out where possible at least in triplicate and on 3 different occasions. The results are reported as mean ± standard deviation (S.D.). Standard curves were generated and calculation of the 50% inhibitory concentration (IC_{50}) values was made using GraphPad Prism Version 4.00 for Windows

(GraphPad Software Inc.). Cytotoxicity results are expressed as the percentage cell survival compared with the untreated control using a dose response curve. Data obtained from mass spectroscopy were analyzed using MassLynx 4.1 (SCN 704) software.

Acknowledgements - We are grateful to the National Research Foundation (NRF) of South Africa for funding the Waters UPLC Synapt HDMS GI system as a joint venture between CSIR Biosciences and University of Johannesburg Biochemistry Department.

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