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Cytotoxicity of latex of *Synadenium compactum* var. *rubrum* S. Carter.

By Aynalem Redae (B. Pharm)

October, 2023

Addis Ababa

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Cytotoxicity of latex of *Synadenium compactum* var. *rubrum* S. Carter

A thesis Submitted to The Department of Pharmaceutical Chemistry and Pharmacognosy as partial fulfillment of the requirements for the degree of Master of Science in Pharmacognosy

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Under the supervision of Mariamawit Yonathan Yeshak (PhD)

Abstract

Cytotoxicity of latex of *Synadenium compactum* var. *rubrum* S. Carter

Aynalem Redae

Addis Ababa University, 2023

Cancer has become the second leading cause of death globally. While it was once predominately a disease affecting economically developed countries, it is now impacting developing nations more accounting for over half of all new cases worldwide. This has significant social and economic implications for countries like Ethiopia. One of the major drawbacks in treating cancer is the prevalence of drug resistance, along with the unbearable side effects and high costs associated with current treatments. Therefore, there is an urgent need to discover new, safe and effective drugs to combat this advancing disease. Natural products have proven to be a valuable source for the development of cancer drugs, as they make up over half of the currently used drugs. Traditional medicine attends to almost 80% of population's primary health needs in Ethiopia. In this research, traditionally claimed cytotoxic latex and its fractions from *Synadenium compactum* var. *rubrum* S. Carter, a member of the Euphorbiaceae family, were evaluated for their cytotoxic activity. The latex and its ethyl acetate fraction showed potent cytotoxicity in an MTT assay against the ovarian cancer cell line A2780, with GI_{50} values as low as 1.98 ± 0.45 µl/ml and 0.911± 0.296 µl/ml, respectively. Additionally, the sub-fractions of the ethyl acetate fraction showed potent activity against histiocytic human lymphoma cell line U-937 in a Fluorometric microculture cytotoxicity assay (FMCA), with an SI value as low as 8% at a dose of 100 µl/ml. Through bioassay guided fractionation and preparative HPLC, a previously

reported lathyrane diterpenoid known as 2-methylbutanoate tetraacetate synadenol was isolated from the most active ethyl acetate sub fraction. The structural elucidation of the compound was achieved using ^{1}H NMR, ^{13}C NMR, HMQC, HMBC and HRESIMS spectroscopic techniques. The finding of this study strongly supports the antitumor traditional claim of the latex of *S. compactum.*

Keywords: *Synadenium compactum*, Cytotoxicity, lathyrane diterpenoid, 2-methylbutanoate tetraacetate synadenol

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Addis Ababa University

School of Graduate Studies

Department of pharmaceutical chemistry and pharmacognosy

This is to certify that the thesis prepared by Mr. Aynalem Redae, entitled: "**Cytotoxicity of latex of *Synadenium compactum* var. *rubrum* S. Carter** " and sub-mitted in partial fulfillment of the requirements for the Degree of Master of Science (Pharmacognosy) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the Examining Committee:

External examiner_____ Signature_____ Date_____

Internal examiner_____ Signature_____ Date_____

Dr. Mariamawit Yonathan Yeshak (PhD) (Advisor) Signature_____ Date_____

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Chair of Department or Graduate Program Coordinator

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List of Abbreviations

CC	Column Chromatography
COSY	Correlation Spectroscopy
FMCA	Fluorometric microculture cytotoxicity assay
GI_{50}	Half-maximal growth inhibitory concentration
HMBC	Heteronuclear Multiple Bond Correlation
HRESIMS	High-resolution Electrospray Ionization Mass Spectrometry
HSQC	Heteronuclear Single Quantum Coherence
Hx	Hexane
IC_{50}	Half-maximal inhibitory concentration
NMR	Nuclear Magnetic Resonance Spectroscopy
Q-TOF	Quadrupole-time-of-flight
RP HPLC	Reversed phase High-performance Liquid Chromatography
SI	survival index
TLC	Thin Layer Chromatography

1 Introduction

1.1 Definition and epidemiology of cancer

The world health organization (WHO) defines the term cancer as the uncontrolled growth and spread of cells, which can affect any part of the body. It can invade surrounding tissues and can metastasize to distant sites (WHO, 2021). And, according to the national cancer institute of America (NCI), cancer is a term for diseases in which abnormal cells divide without control and can invade nearby tissues spreading through blood and lymph system (NCI, 2021).

Cancer is the second leading cause of death globally and was responsible for close to 10 million deaths in 2020. According to WHO, one in six deaths globally is caused by cancer. The number of new cases is expected to rise by about 70% over the next 2 decades (IARC cancer report, 2020). As to the total annual economic cost of cancer, in 2010 cancer was estimated at approximately US$ 1.16 trillion (WHO fact sheet, 2020). Over the years, the burden has shifted to less developed countries, which currently account for about 57% of new cancer cases and 65% of cancer deaths worldwide. Liver and stomach cancer among males and cervical cancer among females are the leading causes of death in these countries (Torre *et al*., 2015). The GLOBOCAN (2020) data estimates 51,865 deaths due to cancer and 77,352 new cases in Ethiopia in the year 2020. The data indicates women to be affected more with breast, cervical and colorectal cancer taking 20.9%, 9.6% and 7.8% respectively. The data also indicated colorectal, prostate and leukemia cancer 11.7%, 10.2%, 9.6% respectively, are among the highest incidences in male (Global cancer observatory, IARC cancer report, GLOBOCAN 2020). Further data is shown in Table 1.

Table 1: Summary of cancer statistics for Ethiopia (taken from the global cancer observatory website https://gco.iarc.fr/today/data/factsheets/populations/231-ethiopia-fact-sheets.pdf)

	Males	Females	Both sexes
Population	57 516 835	57 446 748	114 963 583
Number of new cancer cases	26 754	50 598	77 352
Age-standardized incidence rate (World)	77.9	134.2	106.7
Risk of developing cancer before the age of 75 years (%)	8.2	13.5	11.0
Number of cancer deaths	18 895	32 970	51 865
Age-standardized mortality rate (World)	58.0	91.7	75.3
Risk of dying from cancer before the age of 75 years (%)	6.2	9.8	8.1
5-year prevalent cases	43 705	87 153	130 858
Top 5 most frequent cancers excluding non-melanoma skin cancer (ranked by cases)	Colorectum Prostate Leukaemia Non-Hodgkin lymphoma Liver	Breast Cervix uteri Colorectum Ovary Thyroid	Breast Cervix uteri Colorectum Leukaemia Non-Hodgkin lymphoma

1.2 Etiology of cancer

Causes of cancer were previously perceived to be more of an environmental factor, in the 1980s studies showed chronic infections in additional to environmental exposure are possibly the causes to cancer. Oxidative damage, diet, tobacco and inflammation due to chronic infection play a major role compared to occupation hazards which are to be the less important contributors (Betteridge, 2000; Blackadar, 2016). Study done by Danaei *et al*. (2005), depicted the impact of income with the contrast of lifestyle and environment as possible causes of cancer. The study showed smoking, alcohol use, and low fruit and vegetable intake were the leading risk factors for death from cancer worldwide and in low-and-middle-income countries. In high-income countries, smoking, alcohol use, and overweight and obesity were the most important causes of cancer. Treatment modalities of cancer include surgery, radiotherapy and chemotherapy,

depending on the specific case of the patient these modalities are administered alone or as a combination (Hosseinzade *et al.,* 2017).

1.3 Statement of the problem

Cancer in the past decades has become the second leading cause of disease globally (Danaei *et al*., 2005; WHO, 2020). Apart from the rise of number of new cases, the treatment for cancer is often ineffective, has unbearable side effects and expensive for patients (Prakash *et al*., 2013). The emergence of drug resistance to chemotherapeutic agents is another factor that limits the efficacy of treatment (Kibria *et al*., 2014). Search for sources of newer and effective drugs is a task that gives no time.

One such source of newer and effective drugs are natural products (Demain and Vaishnav, 2011). Of the 185 anti-cancer agents approved since 1940 and available for use, over 64.9% are of natural origin or can be traced to a natural product (Cragg *et al*., 2009; Newman and Cragg, 2020). The search for newer drugs with less down sides obtained from natural product is gaining ground (Kinghorn *et al*., 2009; Newman and Cragg, 2020). In Ethiopian traditional medicine where 80% of the population attendance to, different plants are used to treat ailments including cancer (Wondimu *et al.,* 2007; Yineger *et al.,* 2008). In this thesis work, in a verbal communication with a local traditional healer, we were informed that a white milky exudate from a red leafed plant is used to treat patients with topical tumors by directly applying the exudate on the tumor. The latex from this red leafed plant later identified as *Synadenium compactum* var. *rubrum S.* Carter (Euphorbiaceae) was evaluated for cytotoxic activity.

1.4 Literature review

The Euphorbiaceae is a family of succulent plants containing over 50 tribes, 300 genera and 7500 species described worldwide (Costa *et al*., 2012; Vasas and Hohmann, 2014). It is known for its many toxic species containing cytotoxic agents, potent irritants and co-carcinogens: the phorbol esters and diterpenoid polyosl being the well-known esters (Olivier *et al*., 1992). The family is predominantly found in Africa and the Americas, mainly in tropical or arid habitats (Costa *et al*., 2012). The genus *Synadenium* is one of the small genera, containing 19 species, belonging to this family Euphorbiaceae.

1.5 The Genus *Synadenium*

The genus *Synadenium* is a small genus in the family Euphorbiaceae, and is indigenous to east Africa (Kinghorn, 1980; Costa *et al*., 2012). Species belonging to this genus are widely used in folk medicine to treat several diseases like cancer, peptic ulcers and other health problems (Costa *et al*., 2012). A red-leaved form of *Synadenium* species have long been used for its ornamental value especially in Kenya, as a foliage plant (Carter susan, 1987).

1.5.1 Phytochemistry of the genus *Synadenium*

Plants in this genus have been reported to contain different phytochemicals. *Synadenium grantii* (synonyms: *Euphorbia umbellata* and *Synadenium umbellatum*) a well-studied species of the genus, reportedly contains euphol **(1)**, citrostadienol **(2)** from its latex, lanosterol **(3)** along with pentacyclic terpenes friedelin **(4)** and 3β- friedelinol **(5)** from the bark (Figure 1) (Oliveira *et al*., 2013; Munhoz *et al*., 2014). The phorbol esters 4-deoxyphorbol-13-(phenylacetate)-12-tiglate **(6)** which was reported to be a skin irritant, a polyacetyl phorbol ester synagrantol A **(7)** and 4-

deoxyphorbol ester synagrantol B **(8)** were isolated from chloroform extract of the leaves *S. grantii* (Bagavathi *et al*., 1987; Costa *et al*., 2012; Hassan *et al*., 2012). Campos *et al.* (2010) isolated the phorbol diterpene ester 3,4,12,13-tetraacetylphorbol-20-phenylacetate **(9)** from the chloroform fraction of the stem whereas Li *et al.* (2022) isolated a non-carcinogen lathyrane type diterpene ingol 7,8,12-triacetate 3-phenylacetate **(10)** along with β-sitosterol **(11)** from the leaves of *S. grantii.*

Anthocyanin glycosides that contain the monosaccharide apiose, cyanidin 3-O-(2”-(5’’’-(E-p-coumaroyl)-β-apiofuranosyl)-β-xylopyranoside)-5-O-β-glucopyranoside **(12)**, cyanidin 3-O-(2’’-(5’’’-(Ecaffeoyl)-β-apiofuranosyl)-β-xylopyranoside) **(13)** from the leaf extracts of *S. grantii* have also been reported by Andersen *et al*. (2010). Another species *S. glaucescens* has also been reported to contain the triterpenoids euphol and β-sitosterol (Nyigo *et al*., 2016). Souza *et al.* (2005) isolated a lectin from the latex of *S. carinatum* that binds to D-galactose.

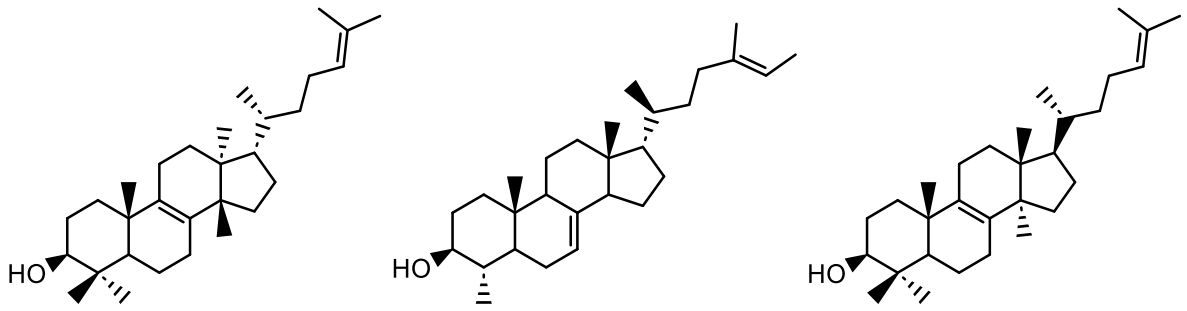

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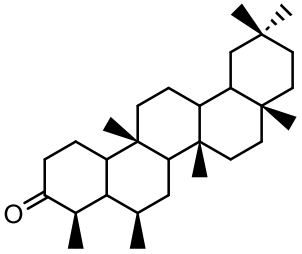
Figure 1: Chemical structures of compounds reported from the genus *Synadenium*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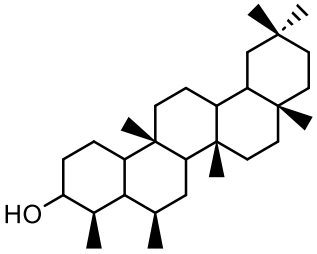
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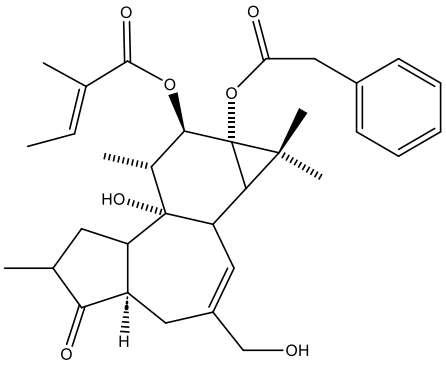
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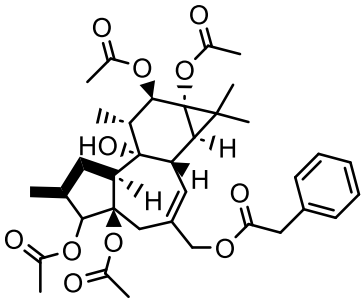
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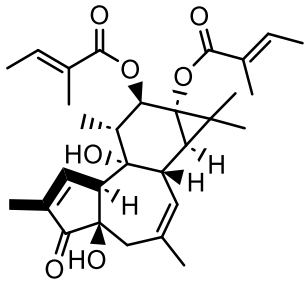
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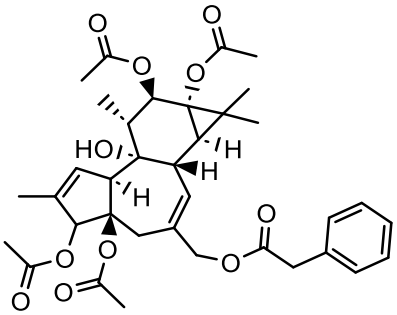
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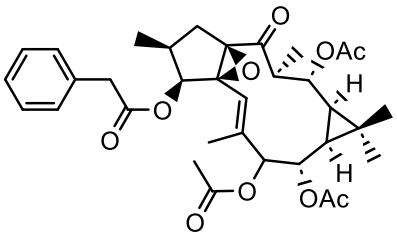
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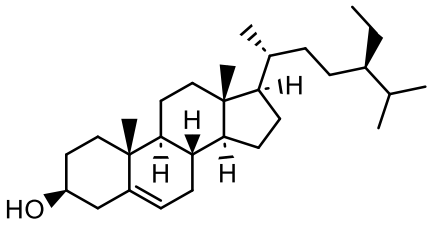
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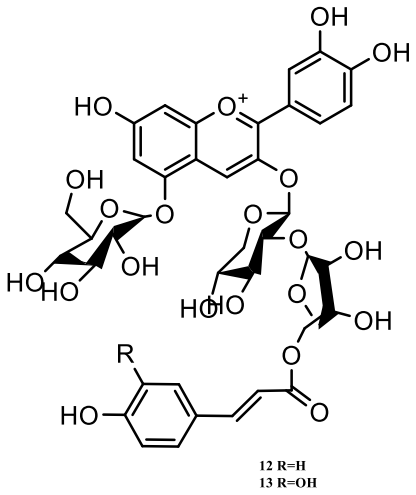


Figure 2: Chemical structures of compounds reported from the genus *Synadenium* (continued)

1.5.2 Biological activity of the genus *Synadenium*

Latex and different extracts of *S. grantii*, have shown to have antitumor, antioxidant, antiulcer, and skin irritant activities (Kinghorn, 1980; Hassan *et al*., 2012; Oliveira *et al*., 2013; Munhoz *et al*., 2014). Moreover, extracts of *S. umbellatum* extracts showed antinociceptive and anti-inflammatory activities (Borges *et al*., 2013).

Crude extract of aerial parts of *S. umbellatum* showed cytotoxicity at IC_{50} of 0.08 mg/ml on K-562 cell lines. The same extract showed an *in vivo* tumor suppression activity of 36% at a dose of 25 mg/kg (Nogueira *et al*., 2008). The latex and fractions of *S. grantii* decreased cell viability of melanoma cell line, dichloromethane fraction exhibited cytotoxicity with IC_{50} value of 30 µg/ml in a time-dependent manner and arrested cell cycle at S-G2/M phase. The latex on the other hand showed a 40% reduction in the volume of tumors of mice with melanomas (Oliveira *et al*., 2013).

The hexane fraction of the latex of *S. umbellatum* arrested the cell cycle of lukemia cell (Jurkat cell line) in the G0/G1 phase in a dose and time dependent cytotoxicity effect with IC_{50} value of 1.87 µg/mL (Luz *et al.*, 2016).

Antitumor activity of the chloroform extract of stem of *S. grantii* showed cytotoxicity against different human tumor cell lines, GI_{50} values as low as 0.37 µg/ml on lung cancer cell NCI-H460 (Campos *et al*., 2010). In the same study, the phorbol diterpene ester 3,4,12,13-tetraacetylphorbol-20-phenylacetate **(9)** (Figure 1) showed lesser effect as compared to extract with total growth inhibition (TGI) value of 24.1 µg/ml.

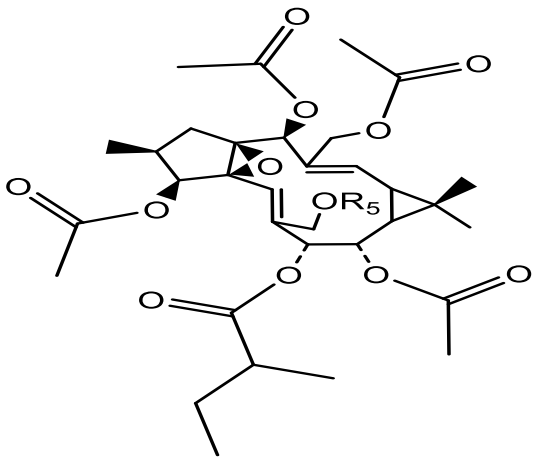
A lathyrane diterpenoid ingol 7,8,12-triacetate 3-phenylacetate **(10)** (Figure 1) isolated from the leaves of *S. grantii* exhibited a Growth Inhibition Percent (GIP) against leukemia (SR) and renal cancer (CAKI-1) 33% and 21% respectively, at the concentration of 10 µM. Phosphatidylinositol 3-kinases (PI3Kα) a tumor promoter, was inhibited by the same compound almost comparable to that of the standard molecule GDC-0326 (docking scores of ingol 7,8,12-triacetate 3-phenylacetate −9.62 kcal/mol and GDC-0326 −9.03 kcal/mol) (Li *et al.*, 2022).

1.5.3 *Synadenium compactum* var. rubrum S. Carter

Synadenium compactum var. rubrum S. Carter (Euphorbiaceae) also known as *Euphorbia bicompacta* Bruyns is a many branched, small tree native to East Africa. It is commonly referred to as African milk bush due the white milk like exudate. *S. compactum* has two variants, *S. compactum var. rubrum* which has purplish-red leaves and *S. compactum* var. *compactum* has dark green leaves and a flaking silvery colored bark, both are cultivated as an ornamental plant in many parts of the world (Olivier *et al*., 1992).

Figure 3: Photograph of *S. compactum var. rubrum* located in garden in Tikur Anbesa specialized hospital Addis Ababa, Ethiopia.

Ethnoveterinary studies showed that the latex of *S. compactum* is used to treat ectoparasitism, anaplasmosis, weakening of bones, and theileriosis in rural Kenya (Njoroge and Bussmanni, 2006). Olivier *et al.* (1992) isolated two lathyrane diterpenoids (synadenol derivatives) namely 2-methylbutanoyk tetraacetyl synadenol **(14)** and 2-methylbutanoate pentaacetate synadenol **(15)** (Figure 3) from the latex of *S. compactum*.



14 R_5 = H **15** R_5 = Ac

Figure 4: Chemical structure of 7-(2-methylbutanoyl)-3,8,14,20-tetraacetyl synadenol (**14**) and 7-(2-methylbutanoyl)-3,8,14,17,20- pentaacetyl (**15**) synadenol.

These compounds are structurally characterized by the acetylation at various sites and also inclusion of 2-methylbutyrate function at carbon seven of the molecule. The latex of *S. compactum var. rubrum* used locally for antitumor activity and lacks published studies on its cytotoxicity, this study is aimed at testing the latex and its constituents for cytotoxicity.

2 Objectives of the study

2.1 General objective

- To evaluate cytotoxic activity of the latex of *Synadenium compactum var. rubrum* S. Carter (Euphorbiaceae).

2.2 Specific objectives`

- To test latex for cytotoxicity on selected human cancer cell lines.
- To test different fractions of the latex for cytotoxicity.
- To isolate major compounds using bioassay guided technique from *S. compactum* and on selected human cancer cell lines.
- Structural elucidation of isolated compounds

3 Materials and Methods

3.1 Materials

3.1.1 Plant Latex

The latex *Synadenium compactum* var. *rubrum* S. Carter was collected in March 2017, from a plant growing in the compound of Ethiopian National Archive and Library Agency, Addis Ababa, Ethiopia. Identification of the plant material was done by Mr. Melaku Wondafrash, Senior Botanist at the National Herbarium, Addis Ababa University.

3.1.2 Chemicals

Chloroform, water, ethyl acetate and hexane for extraction, partition and fractionation on open column chromatography (CC) were either purchased from Sigma-Aldrich (Sigma-Aldrich Co., MO, USA) or Thermo Fisher Scientific (Thermo Fisher Sci-entific Inc., Waltham, MA, USA) and all were of analytical grade. HPLC grade methanol, acetonitrile and water purchased from CARLO ERBA reagents S.A.S. Silica gel (SilicaFlash®P60, 230–400 mesh; SiliCycle Inc., Quebec, Canada) were used for open CC. Analytical TLC was performed using pre-coated silica gel 60 F254 plates (Merck KGaA, Darmstadt, Germany). The deuterated NMR solvents CD_3OD was either purchased from Sigma-Aldrich or Cambridge Isotope Laboratories, Inc (Tewksbury, MA, USA).

3.1.3 Instruments

Lyophlilizer instrument (Alpha 1-2LD plus Christ Co. Ltd., Germany). Organic solvents from fractions and compounds were removed using Heidolph rotary evaporators (Heidolph Instruments GmbH & Co.KG, Germany).

Preparative HPLC was performed on Shimadzu SPD-M10AVP with Shimadzu LC-10AD pump, using Phenomenex Kinetex XB-C18 (150x21.2mm) column. Fractions were collected via Pharmacia LKB fraction collector. Analytical HPLC was performed with a Shimadzu UFLC HPLC (Shimadzu Corporation, Tokyo, Japan) equipped with Shimadzu SPD-M20A diode Diode Array Detector, Sil-20 ACHT Autosampler and LC-20Ad pump with degasser and a semi-preparative C18 column (Kintex C18 HPLC Column 10 x 250 mm). ^{1}H and ^{13}C, HSQC, HMBC, COSY NMR spectra were recorded at room temperature on a Bruker $500H_z$ NMR instrument (Bruker, Billerica, MA, USA). HRESIMS were measured on a Q-TOF mass spectrometer (Waters Corp., Milford, MA, USA) in the positive-ion mode with Na being used for mass calibration.

3.1.4 Cancer cell lines

Ovarian cancer cells (A2780) were obtained from the cell bank at the Centre for Drug Discovery and Development, University of South Australia. The cell lines were maintained following ATCC recommendations either in RPMI-1640 (Roswell Park Memorial Institute), DMEM (Dlbecco's Modified Eagle's Medium) or MEM (Minimum Essential Media) with 10% fetal bovine serum. All cell lines were cultured at 37°C in a humidified incubator in the presence of 5% CO_2.

3.2 Methods

3.2.1 Collection of latex

The latex was collected by making small puncture wounds to the stem of the plant. Approximately 15 ml of latex was collected and freeze dried to give 2g of white pellet.

3.2.2 TLC analysis of latex and Solvent-solvent fractionation

Analytical TLC was used to assess for possible constituents of the crude latex. In doing so, portion of the latex was dissolved in methanol and applied on a normal phase analytical TLC with 4:1 ratio of chloroform and methanol as a solvent system. Chromatograms were visualized using a UV cabinet at a wavelength of 254 and 366 nm.

Then after, the latex (1g) was partitioned into chloroform and water using a separatory funnel. The chloroform fraction was dried using a rotavapor and water fraction was dried using a lyophilizer.

3.2.3 Fractionation of the chloroform partition using Normal phase Column chromatography

The chloroform fraction was subjected to normal phase open column chromatography. The following gradients of mobile phase were used 100% hexane, hexane and ethyl acetate 1:1, 100% ethyl acetate, 100% methanol. Elutes were dried using a rotavapor and further evaluated for spots using analytical TLC at 254nm and 366nm.

3.2.4 Isolation of compound by preparative RP-HPLC

The ethyl acetate eluate of the column chromatography was selected for the isolation of a pure compound using a preparative HPLC on the bases of its considerable cytotoxicity activity. Utilizing a C18 column and a gradient mobile phase. Sample was adsorbed on C18 powder and packed in a loading device. A gradient mobile phase system was utilized with acetonitrile (ACN)-water with 0.05% of trifluoroacetic acid (TFA) as a buffer. Elution started with 25% organic solvent (acetonitrile) to 95% organic over a time period of 65 min with flow rate of 9 ml/min. Samples were collected every one minute. The purity of the fractions was analyzed by performing H^1 NMR for every other fraction collected. Since there were no pure compounds in the 65 fractions collected the fractions were regrouped into six groups, each containing ten consecutive fractions. The regrouped fractions were coded as F1-F6. Collected samples were dried using vacuum concentrator. The bioassay guided fractionation of the latex and isolation of AY07'11 is depicted through a flow chart on Figure 4.

A compound was isolated using HPLC with a semi-preparative column and DAD (Diode array detector) as a detector. Isocratic eluent of 60% ACN (0.05% formic acid) and 40% water (0.05% formic acid) was utilized at a flow rate of 4 ml/min for a duration of 30 min. The sample collector was set to collect starting from the tenth up to the twentieth minute with an interval of 30 sec. Collected samples were dried using vacuum concentrator. Each fraction was subjected to NMR analysis to check for purity, a fraction collected at sixteenth minute was pure enough and was subjected for further analysis.

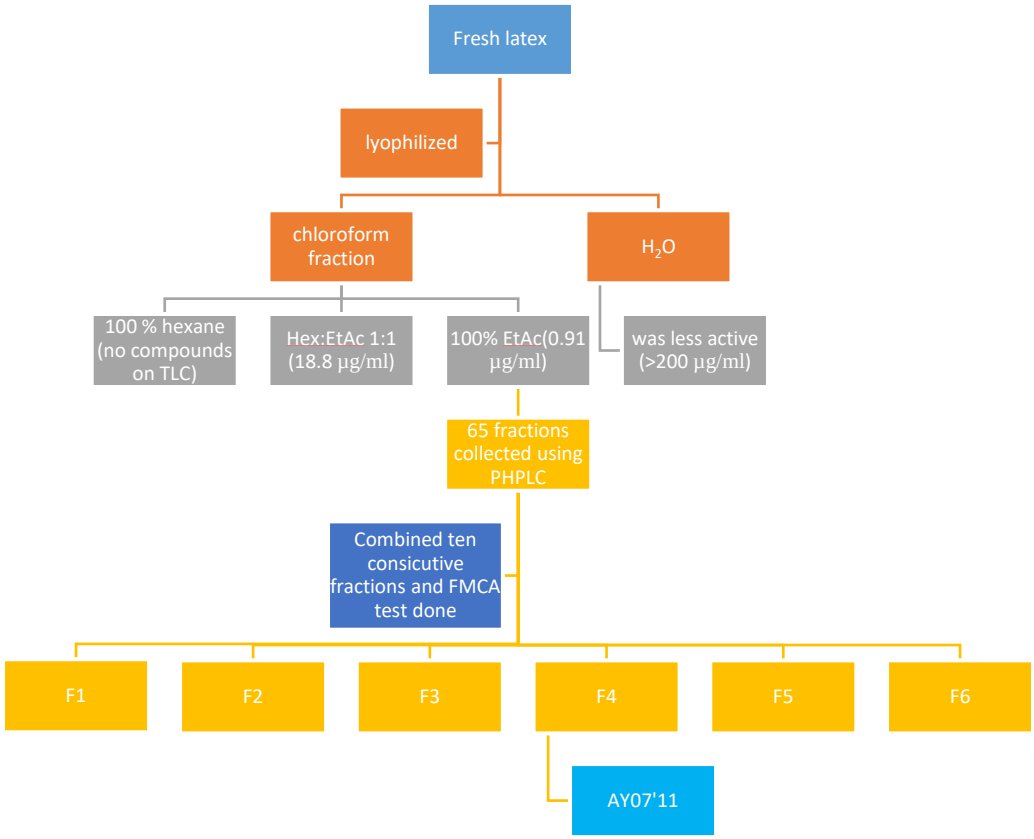


Figure 5: Flow chart of fractionation of the latex and isolation of compound AY07'11

3.2.5 Spectroscopic techniques

High resolution mass spectra were recorded using a Waters XEVO QTof mass spectrometer (Milford, USA), and ionization of all samples was carried out using ESI. ^{1}H and ^{13}C NMR spectra were obtained at 500 and 125 MHz, respectively. The 2D NMR analyses include correlated spectroscopy ^{1}H-^{1}H COSY, heteronuclear multiple bond correlation (HMBC), and heteronuclear single quantum coherence (HSQC). Chemical shifts are reported in units of ***d*** (ppm) and coupling constants (*J*) are expressed in Hz. Multiplicity of ^{1}H NMR signals is reported as s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m =

multiplate and br=broad. All spectroscopic techniques were carried out at the Department of Pharmaceutical Biosciences, Uppsala University, Uppsala Sweden.

3.2.6 Cytotoxicity

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay performed on ovarian cancer cell lines (A2780) was used as a preliminary guide for the fractionation of the latex. The assay was performed as reported by Wang *et al*. (2004). Approximately 1×10^5 cells/well were seeded into 96-well plates and incubated overnight at 37 °C. Test samples were dissolved in dimethyl sulfoxide (DMSO), and diluted (3x serial dilution) in a 100 µl of cell medium, added to seeded cells, and incubated for 72h at 37 °C. Tests were done in triplicate. MTT stock solution of 5 mg/mL was prepared in cell medium, the resulting solution was filtered and sterilized. Medium was removed from cells followed by a wash with 200 µl/well phosphate buffered saline (PBS). MTT solution was then added at 20 µl/well and incubated in the dark at 37 °C for 4 h. MTT solution was removed and cells were again washed with 200 µl of PBS. MTT dye was solubilized with 200 µl/well of DMSO with agitation. Absorbance was read at 540 using an EnVision multi-label plate reader (PerkinElmer, Beaconsfield, Buckinghamshire, UK). The above experiment was conducted at University of South Australia, Australia.

The cytotoxicity assay that was used as a guide for the isolation of an active compound was performed at the Department of Pharmaceutical Biosciences, Uppsala University, Uppsala Sweden. Regrouped fractions (F1-F6) of the EtOAc fraction, were evaluated for cytotoxic activity using the fluorometric microculture cytotoxicity assay (FMCA) (Lindhagen *et al*., 2008), which is based on monitoring of fluorescence arising from fluorescein that is produced as a result

of fluorescein diacetate (FDA) hydrolysis by cells with intact cell membranes. The histiocytic human lymphoma cell line U-937 GTB suspended in growth medium was dispensed into the test sample containing microtiter plates.

Test sample was added at of concentration of 100 μg/ml to each well that was seeded with 200 μl of cell suspension, containing approximately 20,000 cells, to give a total volume of 200 μl per well. The plates were then incubated for 72 h at 37 °C in 5% CO_2 atmosphere. The plates were then centrifuged at 1000 rpm for 5 min at 37 °C, the medium was removed by aspiration, and the cells were washed with phosphate buffered saline (PBS) 80.0 mg of NaCl, 2.0 mg of KCl, 14.4 mg of Na_2HPO_4 and 2.4 mg of KH_2PO_4 in 10.0 mL of distilled water. FDA (10 mg FDA dissolved in 1 mL 100% DMSO, w/v) was added to preheated (37 °C) Q2-buffer (40 mL of 125 mM NaCl, 10 mL of 25 mM Hepes added up to 400 mL with MQ-H_2O, pH 7.4). A portion of this solution (100 μl) was then added to each well and incubated for 40 min at 37°C. Fluorescence at 538 nm in each well was measured (Thermoscientific Varioskan Flash) with excitation at 485 nm.

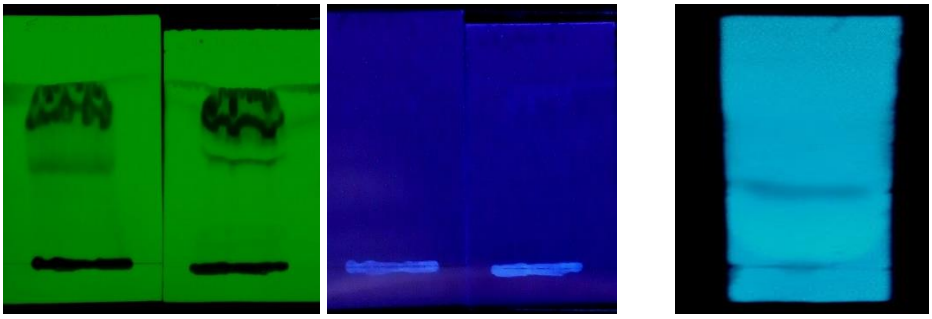
The fluorescence in each well is proportional to the number of living cells and the cytotoxic activity is thus inversely proportional to fluorescence intensity and expressed in terms of Survival index (SI). Survival index is calculated as fluorescence in the experimental wells, articulated as a percentage of the control wells after the fluorescence of the blank wells is subtracted. The experiment was done in duplicates and reported as mean ±SEM of the two independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) to compare the means of two different groups, followed by Tukey's post hoc test. All statistical analysis were conducted at significance level of $p < 0.05$.

4 Results and Discussion

4.1 Chromatographic analysis

4.1.1 TLC analysis

Lyophilized latex pellets were dissolved in methanol and were analyzed for constituents using a normal phase analytical TLC with a solvent system $CHCl_3$: MeOH (4:1). TLC showed two bands with a higher R_f value and a single band at the baseline (Figure 5). Accordingly, 1g of the latex was partitioned with $CHCl_3$ and H_2O. The H_2O fraction was lyophilized to a fluffy white powder. Reversed phase TLC of the lyophilized H_2O fraction showed a single band (at 254nm) with mobile phase of H_2O: MeOH (2:1). Thus, it was considered to be a single compound and was labeled as AYSC1.



(A) (B) (C)

Figure 6: Thin layer chromatogram of crude latex *Synadenium compactum* using $CHCl_3$: MeOH (4:1) solvent system, visualized under 254 nm (A) and 366 (B). Thin layer chromatogram of water fraction of the latex (AYSC1) using solvent system H_2O: MeOH (2:1) visualized under UV light at 366nm.

The $CHCl_3$ fraction was run through column chromatography using eluents 100% Hx, 1:1 Hx:EtOAc, 100% EtOAc and finally the column was washed with 100% MeOH. By performing TLC on the eluates, 1:1 Hx:EtOAc, 100% EtOAc along with the latex and water fraction were subjected to cytotoxicity test. The Hx and MeOH eluates did not show any spot in both 254 and 366 nm wave lengths thus were not further investigated.

4.1.2 RP HPLC analysis and isolation of compound

Based on the results obtained from the MTT, 65 fractions using a preparative HPLC were collected with the intent to isolate a pure compound from the active fraction (EtOAc fraction). While analyzing every other fraction of the 65 fractions collected using a ^1H NMR, the spectrum showed overlapping of peaks at different regions indicating that the samples were not pure enough. Accordingly, it was advantageous to re-combine ten consecutive fractions based on a comparable retention time and NMR data to form six groupings.

Using a semi preparative HPLC for isolation of compounds, fraction F4 had a major peak and a cluster of other peaks near both sides of the major peak while using a gradient elution (Figure 6). Isocratic elution was selected as an optimal method to try and single out the major peak from other minor peaks nearby. In doing so, 60% ACN with the addition of acid buffer was used as an eluent for the isolation of the major compound in the fraction. A colorless compound with a retention time of 16 min was obtained and was coded to be AY07’11.

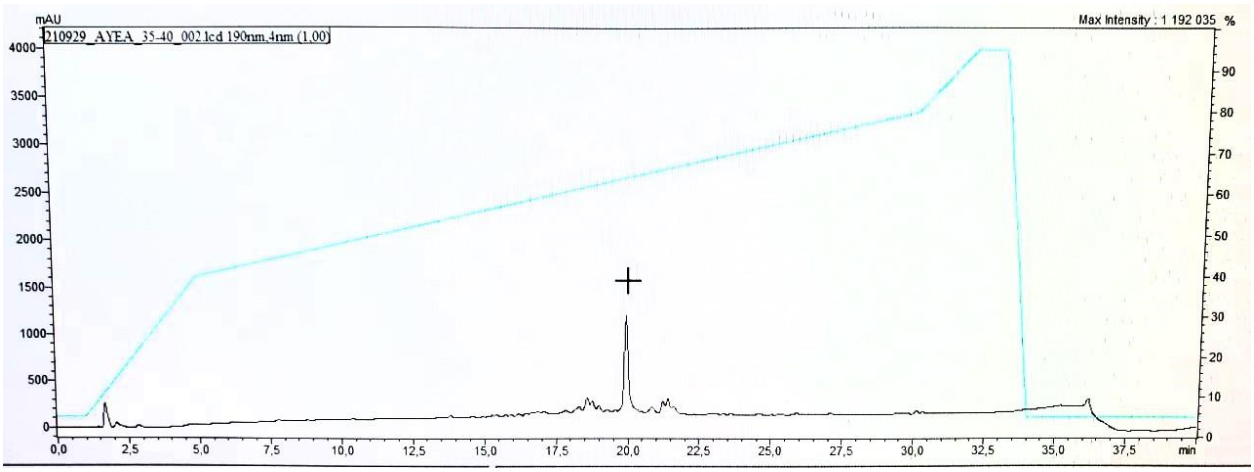


Figure 7: HPLC analysis of fraction F4 (gradient elution of 50% ACN to 80% ACN over a period of 40 minutes on a reversed phase C18 column)

4.2 Cytotoxicity activity of the latex and its fractions

MTT cytotoxicity assay using A2780 ovarian cancer, the EtOAc elute showed promising result with GI_{50} of 0.911± 0.296 µg/ml, latex 1.98 ± 0.45 µg/ml. Hx:EtOAc fraction showed almost 20 times less potent activity as compared EtOAc (GI_{50} =18.88 ± 0.296 µg/ml). In contrast, the water fraction AYSC1 was not active as the GI_{50} value was >200 µg/ml.

The latex, EtOAc fraction and the six fractions of the EtOAc fraction obtained from preparative HPLC yet again were subjected to FMCA cytotoxicity assay against histiocytic human lymphoma cell line U-937. In the experiment, the latex and F4 showed remarkable cytotoxicity by reducing the survival of the cancer cells to 2.5 and 8 percent at a dose of 100 µg/ml, respectively (Figure 7). Both MTT and FMCA bioassay result showed that the latex is active against different cell lines, which may imply the broad spectrum of its activity.

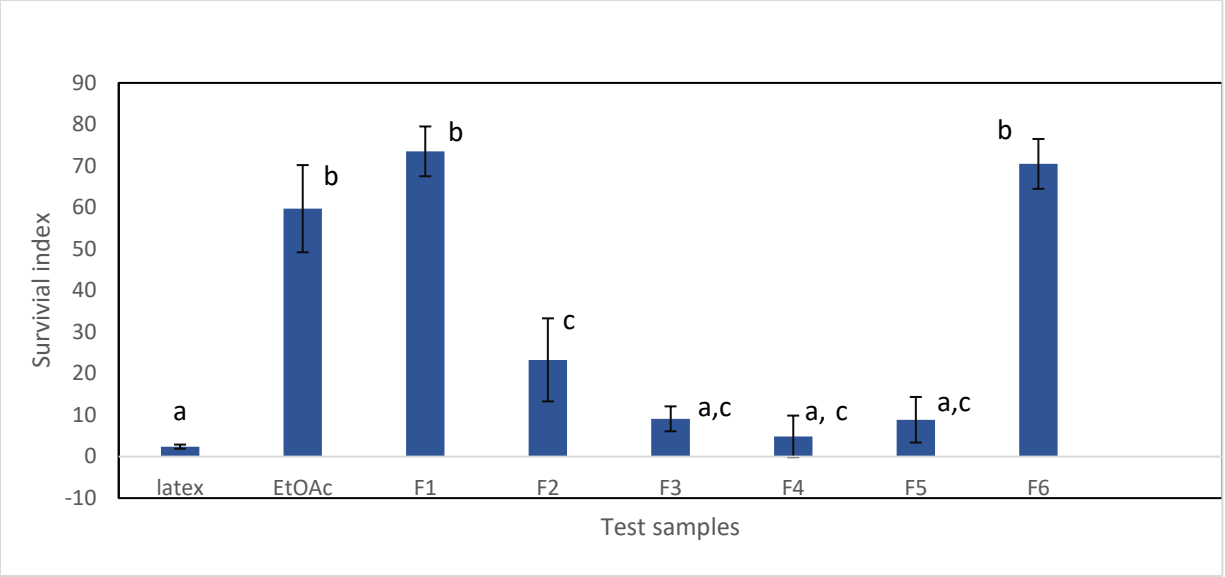


Figure 8: FMCA cytotoxicity assay of latex, EtOAc fraction and HPLC fractions of the EtOAc, survival index reported as mean ±SEM of duplicate tests against histiocytic human lymphoma cell line U-937. **Note:** Different letters show statistically significant difference between samples ($p < 0.05$)

4.3 Structural elucidation of compound AY 07'11

Mass spectroscopic analysis of AY07'11 presented a sodiated pseudomolecular ion peak at $[M+Na]^+$ *m/z* 658.2861. From the information gathered from ^{13}C NMR and HRESIMS results the molecular formula was calculated to be $C_{33}H_{46}O_{12}$ (exact molecular mass of 634.30 Da). Distinct fragmentation patterns such as deacetylation (with 60 mass units of each acetyl group) were observed; resulting in peaks at 575, 515, 455, 395 and also at 473 to 413 to 353 to 293 Figure 8. The deduction of 2-methylbutyrate (102 mass units) can also be seen with mass unit debit of 575 to 473 and 395 to 293. This finding aligns to that of the discoveries of Olivier *et al.* (1992) and to the molecule 7-(2-methylbutanoyl)-3,8,14,20-tetraacetyl reported by the author.

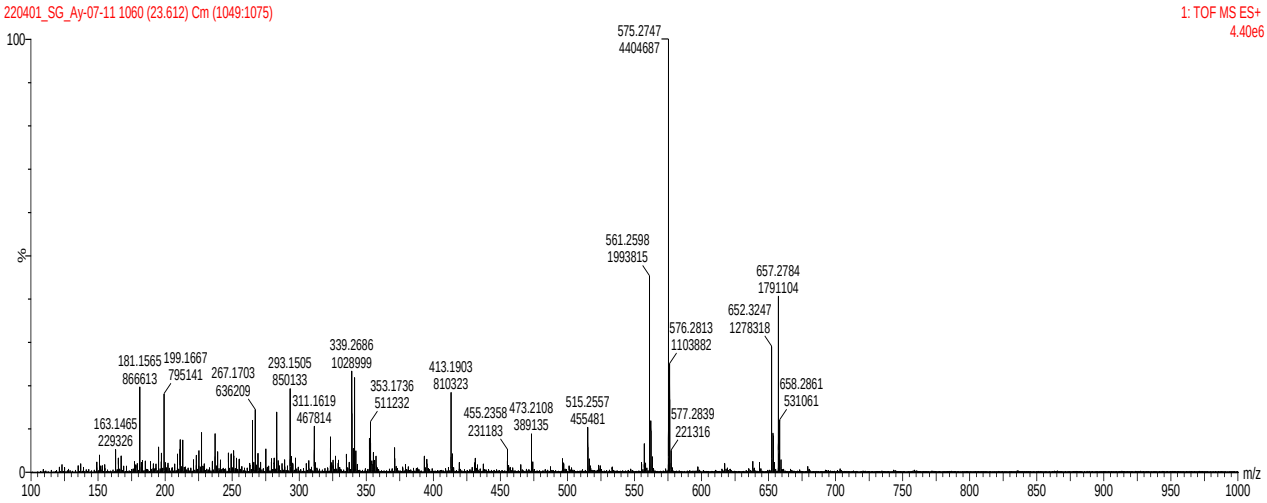


Figure 9: HR ESI MS spectrum of compound AY 07' 11 isolated from the EtOAc fraction of the latex of *S. compactum.*

The ^{1}H NMR data of AY07'11 (Figure 9) showed four singlet signals from the methyl protons of the acetyl functions at 2.02,2.08,2.13,2.15 ppm, which indicate the presence of 4 acetyl functional groups. A sharp singlet at 3.37 ppm indicates a hydroxyl proton that is a product of a hydrolysis of an acetyl function at position 17 as discussed by Olivier *et al*. (1992). Two olefinic proton suggesting the presence of unsaturation are observed, integration of a doublet signal at 3.36 ppm suggests an overlapping proton, later to be explained in 2D experiments. A sextet signal at 2.53 ppm that corresponds to a CH proton that is coupled by CH_2 and CH_3 protons which in the presence of a 2-methylbutyrate functional group as reported by Olivier *et al.* (1992).

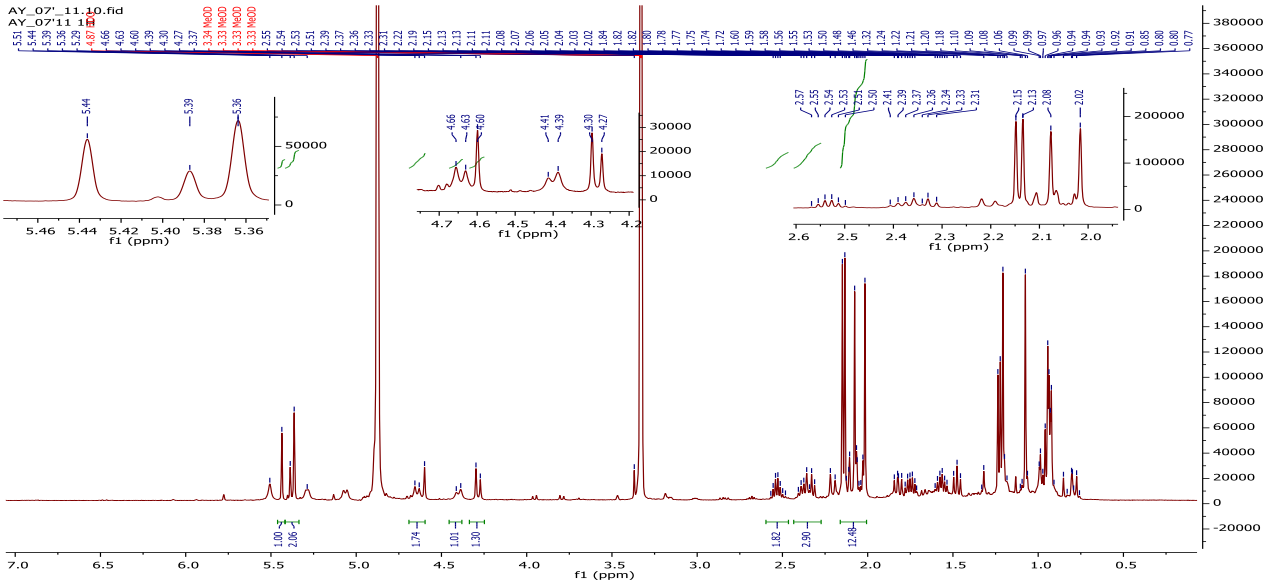


Figure 10: ^{1}H NMR spectrum of compound AY07'11 (CD_3OD)

The data gathered from ^{13}C NMR spectrum of compound AY07'11 (Figure 10) showed olefinic carbon signals at 120.9, 124.5, 131.28, 137.9 ppm. Furthermore, five carbonyl carbons are observed, three signals from the acetate functional groups in the molecule (169.6, 170.5, 170.8) and the one other signal originating from the 2-methylbutharate functional group (175.15 ppm).

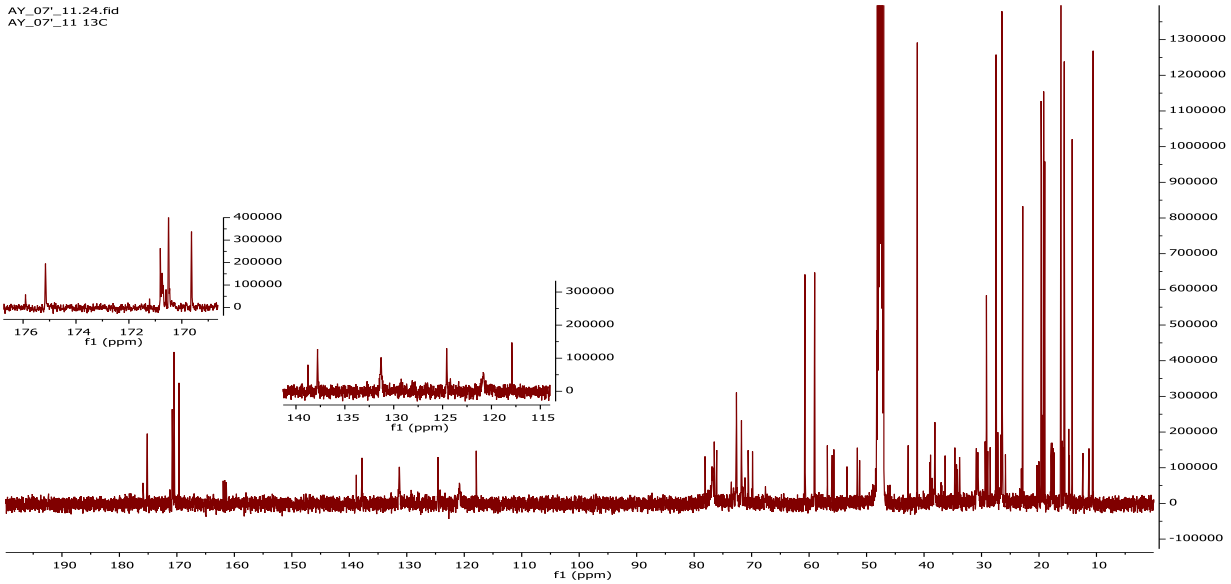


Figure 11: ^{13}C NMR spectrum of compound AY07'11 (CD_3OD)

In the 2D NMR experiments of compound AY07'11, HSQC spectrum (Appendix 1) shows the presence of four methylene group and also, a more important information obtained is that an overlapped doublet olefinic proton signal in the ^{1}H NMR spectrum actually belong to two olefinic protons, one as a singlet the other as a doublet. HMBC spectrum (Appendix 2) among other information clearly shows the difference in chemical environment of the two quaternary olefinic carbons in the molecule. In the interpretation current HMBC spectrum, the chemical shifts of the two quaternary olefinic carbons differ than that of the work of Olivier *et al.* (1992). The HMBC correlation of compound AY07'11 is shown in Figure 11. Table 2 depicts the chemical shifts obtained from the ^{1}H NMR, ^{13}C NMR and HMBC correlations.

Table 2: ^{1}H, ^{13}C NMR chemical shifts and HMBC correlations of compound AY 07'11

Assignment	**H^{1} NMR**		**C^{13} NMR**		
	AY07'11	Olivier *et al.* (1992)	AY07'11	Olivier *et al.* (1992)	HMBC
1	2.21; 2.33	2.23m	38.1	38.6	H-2, H-3
2	2.37m	2.37m	29.1	29.2	H-3,H-1w
3	5.14 br d	5.14br d	76.3	76.3	-
4	-	-	78.0	77.2	H-3
5	5.36s	5.25m	120.9	120.5	H-7a
6	-	-	137.9	124.0	H-5, H-7a
7	5.44br s	5.40br s	71.8	71.4	H-5,H-9
8	5.06br d	5.07br d	72.8	72.8	H-9,H-12
9	1.48 dd	1.39dd	30.9	30.9	H-8, H-12,H-18
10	-	-	22.9	23.3	H-9,H-11,H-12H-18,H-19
11	1.82 dd	1.63dd	26.4	26.3	H-18,H-19
12	5.36 d	5.44d	131.3	132.2	H-14,H-20
13	-	-	124.5	120.5	H-11, H-12,H-14,H-20b,
14	5.44s	5.47s	71.7	71.5	H-12
15	-	-	78.1	77.8	H-1
16	0.93 d	0.92d	16.2	17.1	H-1,H-2,H-4
17 OH	3.37s	3.23br d	-	-	-
17a	4.29 d	4.02dd	59.2	60.1	H-5
17b	4.40 br d	4.55br d	-	-	-
18	1.21 s	1.19s	27.5	28.7	H-9,H-11, H-19
19	1.08 s	1.06s	14.2	15.1	H-9,H-11,H-18
20a	4.64d	4.64d	60.7	61.2	H-12,H-14
20b	4.88d	4.72d	-	-	-
2'	2.53 sextet	2.49 sextet	41.4	41.2	H-3b, H-4', H-5'
3'a	1.56 m	1.52 m	26.4	26.7	-
3'b	1.75 m	1.71 m	26.4	26.7	H-2', H-4', H-5'
4'	0.94 t	0.9 t	10.5	11.4	H-2'H-3', H-5'w
5'	1.23 d	1.21 d	15.7	16.5	H-2', H-3'
4x $\underline{CH_3}$-CO	2.02,2.08,2.13,2.15 s	2.02, 2.07, 2.12, 2.15 s	18.9,19.1,19.5,19.6	20.4, 20.8, 20.9, 21.2	-
4xCH_3-$\underline{C}$O	-	-	169.6,170.5,170.8	169.4,170.3,170.6 ,174.9	-
methylbutanoyl carbonyl	-	-	175.15	-	H-3', H-5'

Note: solvents used to solubilize sample are $CDCl_3$ for Olivier *et al.* (1992) and present study CD_3OD.

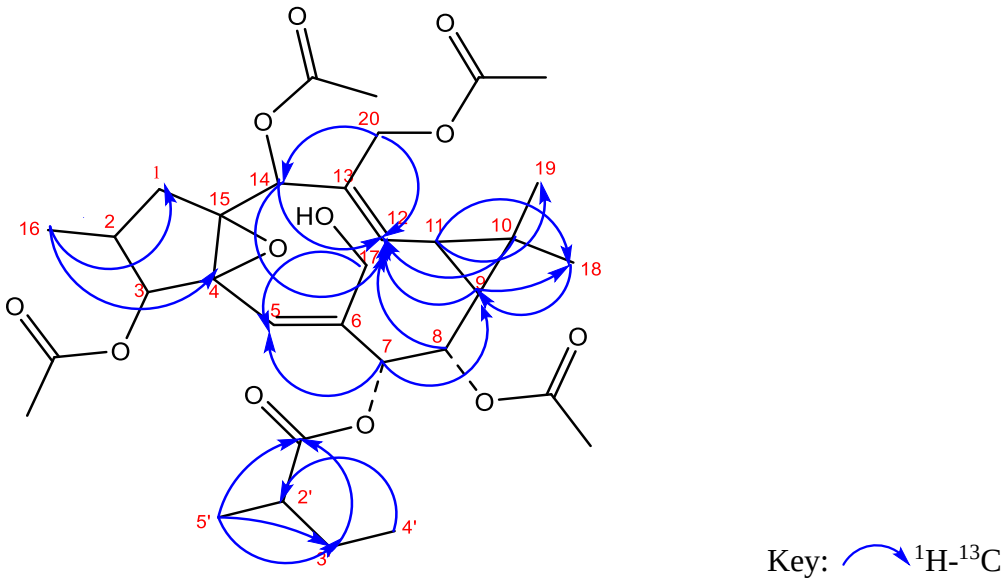


Figure 12: HMBC correlations of compound AY07'11

Form the data gathered and discussed above the chemical structure of the AY07'11 is confirmed to be as 7-(2-methylbutanoyl)-3,8,14,20-tetraacetyl synadenol (Figure 12) previously reported from the same plant by Olivier *et al.* (1992).

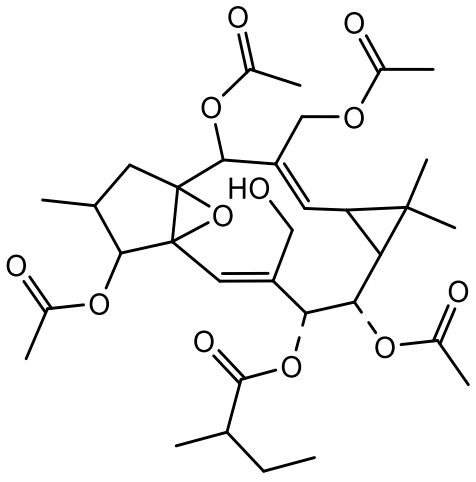


Figure 13: Chemical structure of AY07'11 (7-(2-methylbutanoyl)-3,8,14,20-tetraacetyl synadenol)

Lathyrane diterpenes have a 5-11-3 fused tricyclic basic skeleton which are polyacetylated (Vasas and Hohmann, 2014). These polycyclic diterpenes are synthesized by intra molecular cyclization of geranylgeranyl pyrophosphate (GGPP) to its precursor casbane by the enzyme casbene synthase (Figure 13). Further intra molecular cyclization of Lathyrane skeleton leads to synthesis of different classes of compounds like jatropholane, premyrsinane, tigliane (phorbols) (Dueber *et al*., 2007; Duran-Pena *et al.*, 2014). The ring strain present in the gem-dimethylcyclopropane moiety is important for its activity (Duran-Pena *et al.*, 2014).

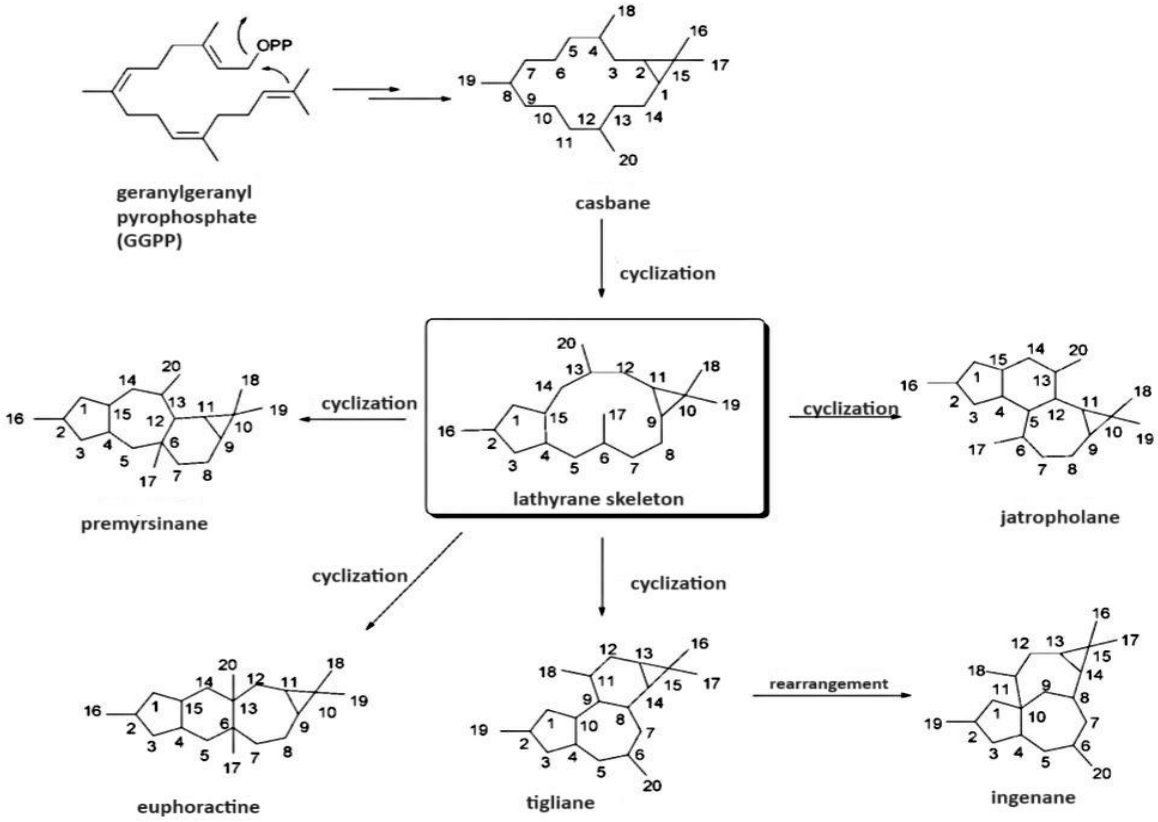


Figure 14: Biosynthetic pathways of lathyrane diterpenes and other casbane derivatives.

This compound belongs to the lathyrane diterpenoids that are gaining popularity due to their potent cytotoxic effects with an added value of lacking in carcinogenicity (Appendino *et al.,* 2001; Olivier *et al.,*1992). These type of diterpenoids have reportedly shown to have potent cytotoxicity and as apoptosis inducers against different cell lines (Duarte *et al.,* 2007; Li *et al.,* 2022; Pusztai *et al*., 2007; Wang *et al*., 2018).

Compounds of this class of diterpenoids are reported to inhibit cellular P-glycoprotein type efflux pumps that expel chemotherapeutic drugs from within cancer cells resulting in resistance to chemotherapeutic agents (Appendino *et al.,* 2001; Li *et al.,* 2022; Yao *et al.,* 2020). The presence of unsaturation at position C-5 in the lathyrane diterpene skeleton is an important factor to inhibit P-glycoprotein (Jiao *et al*., 2009). The combination of cytotoxicity and efflux pump inhibition effects of this class compounds, advocates promising results in further investigating the compound isolated in this study.

5 Conclusion and Recommendation

The latex of *S. compactum* and its ethyl acetate fraction showed promising cytotoxicity activity against ovarian cancer cell line A2780 and histiocytic human lymphoma cell line U-937. In this study, via bioassay guided technique and using preparative HPLC a previously reported lathyrane diterpenoid 2-methylbutanoyl tetraacetate syn`adenol (AY07'11) has been isolated and its structure elucidated. The finding of this study supports the antitumoral traditional claim of the latex of *S. compactum.*

Based on the findings and limitations of this study, further recommendations are stated as follows.

- Isolate other corresponding compounds from fraction F4
- Isolate and test compounds from fractions F3 and F5, which have comparable activity to that of F4.
- Latex of *S. compactum* showed noticeable activity in both cytotoxicity assays, thus testing the latex on different cell lines could lead to other active compounds with in the latex.
- Test AY07'11 on different cancer cell lines and study its selectivity profile
- As shown in the result and discussion section, lathyrane diterpenoids similar to AY07'11 reportedly inhibit P-glycoprotein type efflux pump, thus performing in silico molecular docking of AY07'11.

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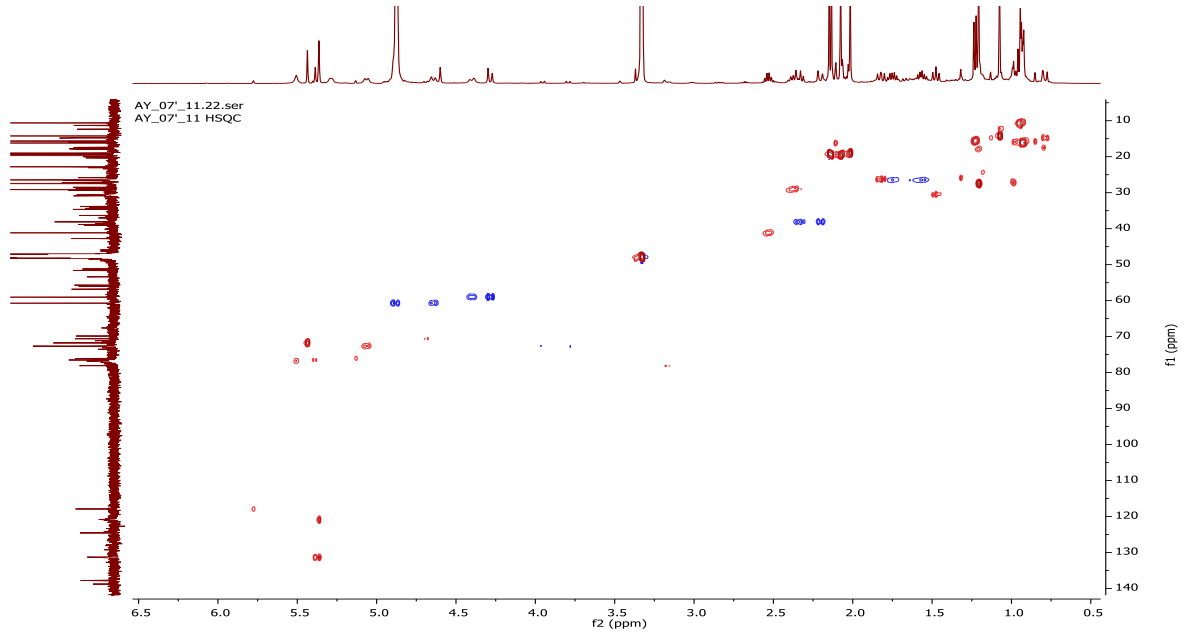
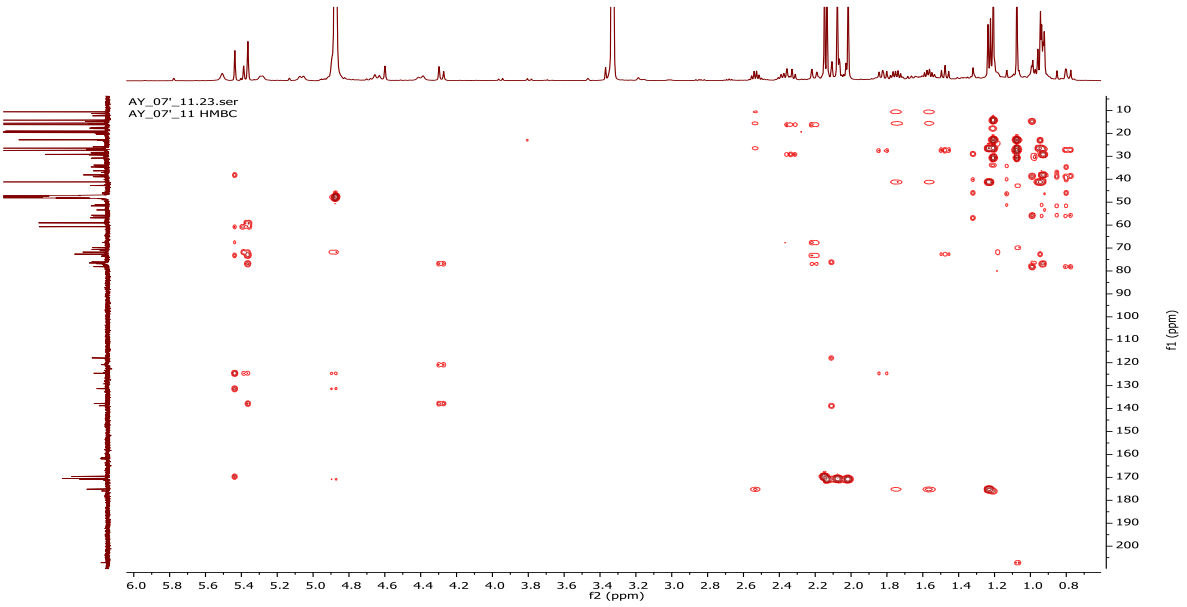
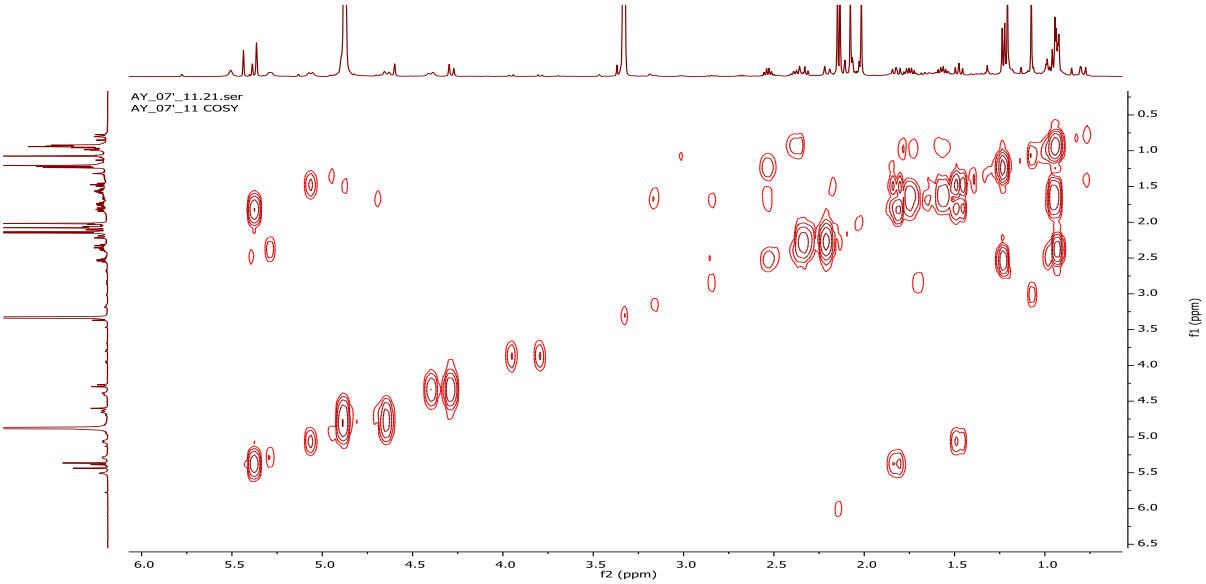
Appendices



Appendix 1: HSQC correlation spectrum of compound AY07'11



Appendix 2: HMBC correlation spectrum of compound AY07'11



Appendix 3:^{1}H ^{1}H COSY correlation spectrum of compound AY07'11

Cytotoxicity of latex of *Synadenium compactum* var. *rubrum* S. Carter.

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Abstract

Cancer has become the second leading cause of death globally. One of the major drawbacks in treating cancer is the prevalence of drug resistance, along with the unbearable side effects and high costs associated with current treatments. Natural products have proven to be a valuable source for the development of cancer treatments, as they make up over half of the drugs currently used in treatment. In this research, latex and its constituents from *Synadenium compactum var. rubrum* S. Carter, a member of the Euphorbiaceae family, were evaluated for their cytotoxic activity. The latex and its ethyl acetate fraction showed potent cytotoxicity against the ovarian cancer cell line A2780, with GI_{50} values as low as 1.98 ± 0.45 µl/ml and 0.911± 0.296 µl/ml, respectively. The sub-fractions of the ethyl acetate fraction showed potent activity against histiocytic human lymphoma cell line U-937, with an SI value as low as 8% at a dose of 100 µl/ml. Through bioassay guided fractionation using Fluorometric microculture cytotoxicity assay (FMCA) and preparative HPLC, a previously reported lathyrane diterpenoid known as 2-methylbutanoate tetraacetate synadenol was isolated from the most active ethyl acetate sub fraction. The structural elucidation of the compound was achieved using 1H, 13C, HMQC, HMBC and HRESIMS spectroscopic techniques. The finding of this study strongly supports the antitumor traditional claim of the latex of *S. compactum.*

Key words: *Synadenium compactum* var. *rubrum* S. Carter., Cytotoxicity, FMCA assay, lathyrane diterpenoid

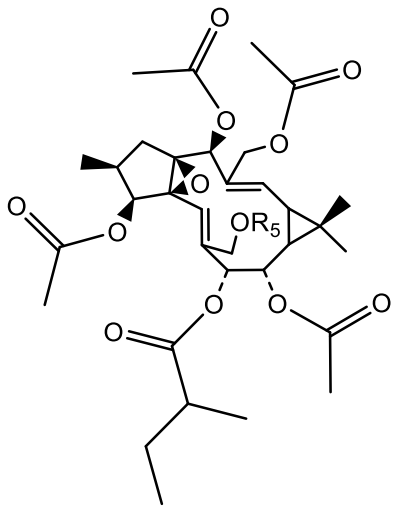
Introduction

The Euphorbiaceae is a family of succulent plants containing over 50 tribes, 300 genera and 7500 species described worldwide (Costa *et al*., 2012; Vasas and Hohmann, 2014). It is known for its many toxic species containing cytotoxic agents, potent irritants and co-carcinogens: the phorbol esters and diterpenoid polyosl being the well-known esters (Olivier *et al*., 1992). The family is predominantly found in Africa and the Americas, mainly in tropical or arid habitats (Costa *et al*., 2012).

The genus *Synadenium* is one of the small genera, containing 19 species, belonging to this family Euphorbiaceae and is indigenous to east Africa (Kinghorn, 1980; Costa *et al*., 2012). Species belonging to this genus are widely used in folk medicine to treat several diseases like cancer, peptic ulcers and other health problems (Costa *et al*., 2012). Plants in this genus reportedly contains phytochemicals like euphol, citrostadienol, lanosterol, pentacyclic terpenes friedelin, 3β-friedelinol (Oliveira *et al*., 2013; Munhoz *et al*., 2014). The phorbol esters 4-deoxyphorbol-13-(phenylacetate)-12-tiglate a known skin irritant, a polyacetyl phorbol ester synagrantol A and 4-deoxyphorbol ester synagrantol B were isolated from chloroform extract of the leaves *S. grantii* (Bagavathi *et al*., 1987; Costa *et al*., 2012; Hassan *et al*., 2012).

Synadenium compactum var. *rubrum* S. Carter (Euphorbiaceae) also known as Euphorbia bicompacta Bruyns is a many branched, small tree native to East Africa. It is commonly referred to as African milk bush due the white milk like exudate and it is cultivated as an ornamental plant in many parts of the world (Olivier *et al*., 1992).

The latex of *S. compactum* is used to treat ectoparasitism, anaplasmosis, weakening of bones, and theileriosis in rural Kenya (Njoroge and Bussmanni, 2006). Olivier *et al*. (1992) S. compactum isolated two lathyrane diterpenoids (synadenol derivatives) namely 2-methylbutanoate tetraacetate synadenol (**1**) and 2-methylbutanoate pentaacetate synadenol (**2**) (**Figure 1**)



1 R_5 = H **2** R_5 = Ac

Figure 1: Chemical structure of 7-(2-methylbutanoyl)-3,8,14,20-tetraacetyl and 7-(2-methylbutanoyl)-3,8,14,17,20-pentaacetyl esters of synadenol respectively.

The latex of S. compactum var. rubrum used locally for antitumor activity and lacks published studies on its cytotoxicity, this study is aimed at testing the latex and constituents for cytotoxicity.

Results and discussion

RP HPLC analysis and isolation of compound

Based on the results obtained from the MTT, 65 fractions using a preparative HPLC were collected with the intent to isolate a pure compound from the active fraction (EtOAc fraction). While analyzing every other fraction of the 65 fractions collected using a ^{1}H NMR, the spectrum showed overlapping of peaks at different regions indicating that the samples were not pure enough. Accordingly, it was advantageous to re-combine ten consecutive fractions based on a comparable retention time and NMR data to form six groupings.

Using a semi preparative HPLC for isolation of compounds, fraction F4 had a major peak and a cluster of other peaks near both sides of the major peak while using a gradient elution. Isocratic elution was selected as an optimal method to try and single out the major peak from other minor peaks nearby. In doing so, 60% ACN with the addition of acid buffer was used as an eluent for the isolation of the major compound in the fraction. A colorless compound 1 was obtained at a retention time of sixteen minutes.

Cytotoxicity activity of the latex and its fractions

MTT cytotoxicity assay using A2780 ovarian cancer, the EtOAc elute showed promising result with GI^{50} of 0.911± 0.296 µl/ml., latex 1.98 ± 0.45 µl/ml. Hx:EtOAc fraction showed almost 20 times less potent activity as compared EtOAc (GI_{50} 18.88 ± 0.296 µl/ml). In contrast the water fraction AYSC1 was not active as the GI_{50} was >200 µl/ml.

The latex, EtOAc fraction and the six fractions of the EtOAc fraction obtained from preparative HPLC yet again were subjected to FMCA cytotoxicity assay against histiocytic human lymphoma cell line U-937. In the experiment, the latex and F4 showed remarkable cytotoxicity by the reducing the survival of the cancer cells to 2.5 and 8 percent at a dose of 100µg/ml, respectively (Figure 2).

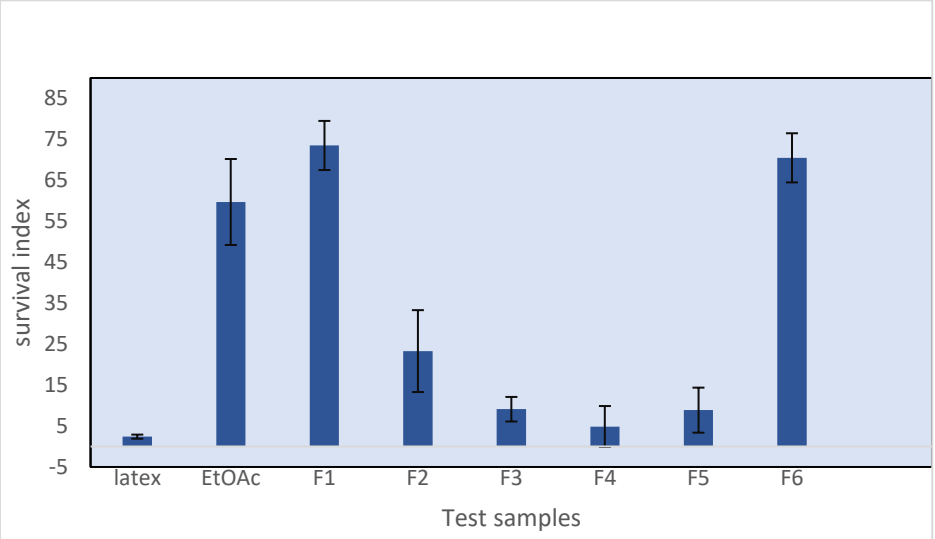


Figure 2: FMCA cytotoxicity assay of latex, EtOAc fraction and HPLC fractions of the EtOAc, survival index reported as mean ±SD of duplicate tests against histiocytic human lymphoma cell line U-937.

Structural elucidation

Mass spectroscopic analysis of compound 1 presented a sodiated pseudomolecular ion peak at [M+Na] + m/z 658.2861. From the information gathered off of 13C NMR and HRESIMS results the molecular formula was calculated to be C33H¬¬46O12 (exact molecular mass of 634.30 Da). Distinct fragmentation patterns such as deacetylation (with 60 mass units of each acetyl group)

were observed; resulting in peaks at 575,515, 455, 395 and also at 473 to 413 to 353 to 293 Figure 8. The deduction of 2-methylbutyrate (102 mass units) can also be seen with mass unit debit of 575 to 473 and 395 to 293. This finding aligns to that of the discoveries of Olivier *et al*. (1992) and to the molecule 7-(2-methylbutanoyl)-3,8,14,20-tetraacetyl reported by the author.

The 1H NMR data of AY07'11 (Figure 9) showed four singlet signals from the methyl protons of the acetyl functions at 2.02,2.08,2.13,2.15 ppm, which indicate the presence of 4 acetyl functional groups. A sharp singlet at 3.37 ppm indicates a hydroxyl proton that is a product of a hydrolysis of an acetyl function at position 17 as discussed by Olivier *et al*. (1992). Two olefinic proton suggesting the presence of unsaturation are observed, integration of a doublet signal at 3.36 ppm suggests an overlapping proton, later to be explained in 2D experiments. A sextet signal at 2.53 ppm that corresponds to a CH proton that is coupled by CH_2 and CH_3 protons which in the presence of a 2-methylbutyrate functional group as reported by Olivier *et al*. (1992).

The data gathered from 13C NMR spectrum of compound 1 (Figure 10) showed olefinic carbon signals at 120.9, 124.5, 131.28, 137.9 ppm. Furthermore, five carbonyl carbons are observed, three signals from the acetate functional groups in the molecule (169.6, 170.5, 170.8) and the one other signal originating from the 2-methylbutharate functional group (175.15 ppm). Form the data gathered and discussed above the chemical structure of the compound 1 is confirmed to be as 7-(2-methylbutanoyl)-3,8,14,20-tetraacetyl synadenol (Figure 3) previously reported to be isolated from the same plant by Olivier *et al*. (1992).

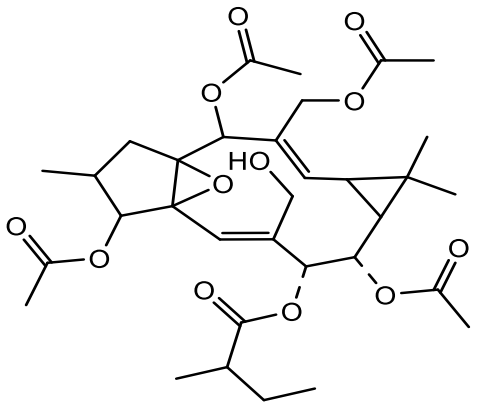


Figure 3: Chemical structure of compound 1 (7-(2-methylbutanoyl)-3,8,14,20-tetraacetyl synadenol)

This compound belongs to the lathyrane diterpenoids that are gaining popularity due to their potent cytotoxic effects with an added value of lacking in carcinogenicity (Appendino et al., 2001; Olivier et al.,1992). Lathyrane diterpenes have a 5-11-3 fused tricyclic basic skeleton which are polyacetylated (Vasas and Hohmann, 2014). These type of diterpenoids have reportedly shown to poses potent cytotoxic activity and as apoptosis inducers against different cell lines (Duarte et al., 2007; Li et al., 2022; Pusztai et al., 2007; Wang et al., 2018).

Compounds of this class of diterpenoids have been also reported to inhibit cellular P-glycoprotein type efflux pumps that expel chemotherapeutic drugs from within cancer cells resulting in resistance to chemotherapeutic agents (Appendino et al., 2001; Li et al., 2022). The presence of unsaturation at position C-5 in the lathyrane diterpene skeleton is an important factor to inhibit P-glycoprotein (Jiao et al., 2009) The combination of cytotoxicity and efflux pump inhibition effects of this class compounds, advocates promising results in further investigating the compound isolated in this study.

Conclusion

The latex of S. compactum and its ethyl acetate fraction S. compactum have shown promising cytotoxicity activity against ovarian cancer cell line A2780 and histiocytic human lymphoma cell line U-937. In this study, via bioassay guided techniques and using preparative HPLC a previously reported lathyrane diterpenoid 2-methylbutanoate tetraacetate synadenol has been isolated from the most active fraction and its structure was elucidated.

Experimental

Collection of latex

The latex was collected by making small puncture wounds to the stem of the plant. Approximately 15 ml of latex was collected and freeze dried to give 2g of the latex as a white pellet form.

Fractionation of the latex and isolation of compound 1

Dried latex was partitioned using water and chloroform. The chloroform partition was further fractionated using an open column chromatography. The following gradients of mobile phase were used 100% hexane, hexane and ethyl acetate 1:1, 100% ethyl acetate, 100 methanol. Elutes were dried using a rotavapor and further evaluated for spots using analytical TLC read at 254nm and 366nm. The ethyl acetate elute of the column chromatography was selected for the isolation of a pure compound using a preparative HPLC on the bases of its considerable cytotoxicity activity. Utilizing a C18 column and a gradient mobile phase. Sample was adsorbed on C18 powder and packed in a loading device. A gradient mobile phase system was utilized with acetonitrile (ACN)-water with 0.05% of trifluoroacetic acid (TFA) as a buffer. Elution started with 25% organic solvent (acetonitrile) to 95% organic over a time period of 65 min. with flow

rate of 9ml/min. Samples were collected every one minute. fractions were regrouped into six groups, each containing ten consecutive fractions. The regrouped fractions were coded as F1-F6.

Compound was isolated using HPLC with a semi-preparative column and DAD (Diode array detector) as a detector. Isocratic eluent of 60% ACN (0.05% formic acid) and 40% water (0.05% formic acid) was utilized at a flow rate of 4 ml/min for a duration of 30 min. fraction collected at sixteenth minute was pure enough and was subjected for further analysis.

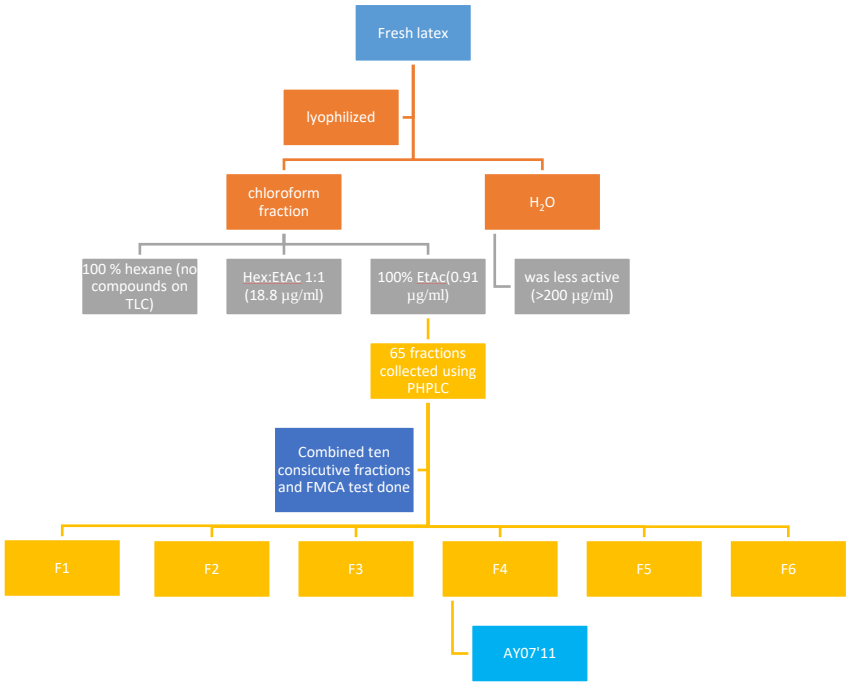


Figure 15: Flow chart of fractionation of the latex and isolation of compound 1

Cytotoxicity

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay performed on ovarian cancer cell lines (A2780) was used as a preliminary guide for the fractionation of the latex. The assay was performed as reported by (Wang et al., 2004). Approximately 1×105 cells/well were seeded into 96-well plates and incubated overnight at 37 °C. Test samples were dissolved in dimethyl sulfoxide (DMSO), and diluted (3x serial dilution) in a 100 µl of cell

medium, added to seeded cells, and incubated for 72h at 37 °C. Tests were done in triplicate. MTT stock of 5 mg/mL was prepared in cell medium, the resulting solution was filtered and sterilized. Medium was removed from cells followed by a wash with 200 µl/well phosphate buffered saline (PBS). MTT solution was then added at 20 µl/well and incubated in the dark at 37 °C for 4 h. MTT solution was removed and cells were again washed with 200 µl of PBS. MTT dye was solubilized with 200 µl/well of DMSO with agitation. Absorbance was read at 540 using an EnVision multi-label plate reader (PerkinElmer,Beaconsfield,Buckinghamshire, UK). The above experiment was conducted at University of South Australia, Australia.

The cytotoxicity assay that was used as a guide for the isolation of an active compound was performed at the Department of Pharmaceutical Biosciences, Uppsala University, Uppsala Sweden. Regrouped fractions' (F1-F6) of the EtOAc fraction, where evaluated for cytotoxicity activity using the fluorometric microculture cytotoxicity assay (FMCA) (Lindhagen et al., 2008), which is based on monitoring of fluorescence arising from fluorescein that is produced as a result of fluorescein diacetate (FDA) hydrolysis by cells with intact cell membranes. The histiocytic human lymphoma cell line U-937 GTB suspended in growth medium was dispensed into the test sample containing microtiter plates.

Test sample was added at of concentration of 100 µg/ml to each well that was seeded with 200 µl of cell suspension, containing approximately 20,000 cells, to give a total volume of 200 µl per well. The plates were then incubated for 72 h at 37 °C in 5% CO2 atmosphere. The plates were then centrifuged at 1000 rpm for 5 min at 37 °C, the medium was removed by aspiration, and the cells were washed with phosphate buffered saline (PBS) 80.0 mg of NaCl, 2.0 mg of KCl, 14.4 mg of Na2HPO4 and 2.4 mg of KH2PO4 in 10.0 mL of distilled water. FDA (10 mg FDA

dissolved in 1 mL 100% DMSO, w/v) was added to preheated (37 °C) Q2-buffer (40 mL of 125mM NaCl, 10 mL of 25mM Hepes added up to 400 mL with MQ-H2O, pH 7.4). A portion of this solution (100 µl) was then added to each well and incubated for 40 min at 37°C. Fluorescence at 538 nm in each well was measured (Thermoscientific Varioskan Flash) with excitation at 485 nm.

The fluorescence in each well is proportional to the number of living cells and the cytotoxic activity is thus inversely proportional to fluorescence intensity and expressed in terms of Survival index (SI). Survival index is calculated as fluorescence in the experimental wells, articulated as a percentage of the control wells after the fluorescence of the blank wells is subtracted. The experiment was done in duplicates and reported as mean ±SD of the two independent experiments.

Spectroscopic analysis

Compound 1: colorless compound, HRESI-MS *[M+Na]* + *m/z* 658.2861 ^{1}H NMR (CD_3OD) 2.21; 2.33(H-1, m), 2.37(H-2, m), 5.14 (H-3,br d), 5.36(H-5, s), 5.44br (H-7,s), 5.06br(H-8 d), 1.48(H-9, dd), 1.82(H-11, dd), 5.36(H-12, d), 5.44(H-14, s), 0.93(H-16, d), 3.37(H-17 OH, s) 4.29 (H-17a, d), 4.40 (H-17b, br d), 1.21(H-18,s), 1.08 (H-19,s), 4.64(20a, d), 4.88(H-20 b, d), 2.53(H-2' sextet), 1.56 (H-3'a m), 1.75(H-3'b m), 0.94 (H-4',t), 1.23(H-5' d), acetyl protons 2.02,2.08,2.13,2.15 s. ^{13}C-NMR (CD_3OD) (C-1)38.1, (C-2) 29.1, (C-3)76.3, (C-4) 78.0, (C-5)120.9, (C-6)137.9, (C-7)71.8, (C-8)72.8, (C-9)30.9, (C-10) 22.9, (C-11) 26.4, (C-12)131.3, (C-13) 124.5, (C-14)71.7, (C-15)78.1, (C-16)16.2, (C-17) 59.2, (C-18)27.5, (C-19) 14.2, (C-20)

60.7, (C-2') 41.4, (C-3')26.4, (C-4')10.5, (C-5')15.7, (acetyplCH3)18.9,19.1,19.5,19.6, (acetyl carbonyl carbon)169.6,170.5,170.8, 175.15.

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