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Isolation and Characterization of a Pregnane Steroid from the Antiproliferative Active Extract of *Caralluma speciosa* (N.E.Br.) N.E.Br. Stem Latex

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This is to certify that the thesis prepared by Kalkidan Derese entitled: "**Isolation and Characterization of a Pregnane Steroid from the Antiproliferative Active Extract of *Caralluma speciosa* Stem Latex**" (N.E.Br.) N.E.Br. Partial Fulfillment of the Requirements for the Degree of Master of Science in Pharmacognosy complies with the regulation of the university and meets the accepted standards concerning originality and quality.

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List of Abbreviations

^{13}C-NMR	Carbon-13 nuclear magnetic resonance
^{1}H-NMR	Proton nuclear magnetic resonance
A427	Lung Cancer
DNA	Deoxyribonucleic acid
GI50	Growth inhibition of 50% of cells
HPLC	High-Performance Liquid Chromatography
MCF-7	Breast Adenocarcinoma
PPM	Parts per million
ROS	Reactive oxygen species
SiSoN	Cervical Cancer cell line (Normal)
SiSoR	Cervical Cancer cell line (Resistant to Cisplatin)
TLC	Thin layer chromatography
TOF-MS	Time of flight-mass spectrophotometer
TCIMs	Traditional, complementary, and integrative medicines
UV	Ultraviolet
WHO	World Health Organization

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Abstract

Isolation and Characterization of a Pregnane Steroid from the Antiproliferative Active Extract of *Caralluma speciosa* Stem Latex (N.E.Br.) N.E.Br.

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Addis Ababa University, 2024

Cancer is a broad term for a large diverse group of diseases that can affect any body part, standing as one of the leading global causes of death. In response to the challenges posed by cancers, researchers are actively investigating natural alternatives, specifically plant-derived compounds, seeking safer and more effective chemotherapeutic options to address the limitations of current treatments. In the traditional medical practices of Ethiopia, the stem latex of *Caralluma speciosa* (N.E.Br.) N.E.Br. stands for its traditional use in treating cancer. Thus, this study aimed at evaluating the antiproliferative activity of the latex and isolating its constituents. *In vitro* crystal violet cell proliferation assay was used to determine the antiproliferative/cytotoxic activity of the 80% methanolic extract of *C. speciosa* against four cancer cell lines. The latex extract exhibited cytotoxic effects against breast cancer cell line (MCF-7) and normal cervical cancer cell line (SiSoN) with Treated versus Control corrected percentage [$(T/C)_{corr.}$ (%)] values of -1.07 and -0.95, respectively. Additionally, the latex demonstrated antiproliferative effects on lung cancer cell line **(**A427**)** and cervical cancer cells resistant to cisplatin **(**SiSoR**)** with $(T/C)_{corr.}$ (%) values of 0.43 and 1.07, respectively. Subsequently, a pregnane glycoside was isolated from the 80% methanol extract of the latex using chromatographic techniques including HPLC. The structure was tentatively identified as 20-*O*-benzoyl-12-*O*-acetyl-14-hydroxy-20-pregn-5-ene-3-*O*-*β*-D-cymaropyranosyl-(1→4)-*β*-D-canaropyranosyl-(1→4)-*β*-D-glucopyranoside through 1D and 2D NMR spectral data. Although the antiproliferative activity of the isolated compound was not determined, considering the reported anticancer activity of pregnane glycosides, it was evident that the isolated compound could have contributed to the antiproliferative/cytotoxic activity of latex of *C. speciosa*. The present findings also support the traditional use of the plant for the treatment of cancer.

Keywords: *Caralluma speciosa*, stem latex, cancer cell lines, antiproliferative, pregnane glycoside

1. Introduction

1.1. Cancer

Cancer is an illness that occurs when abnormal cells grow uncontrollably, go beyond their usual boundaries, and invade other body areas (Brown *et al*., 2023). Damage to the DNA, which is the genetic code of each cell, causes cancer cells to develop (Leong *et al*., 2022). Normally, the body can repair damaged DNA, but in cancer cells, this does not happen. About 10% of all cancer cases can be caused by inherited DNA (You and Jones, 2012). In most cases, an individual's DNA can be damaged due to exposure to mutagen in their surroundings or random cellular events (Amente *et al.*, 2021). Cancer cells can spread to nearby organs and tissues and, in rare cases, even travel through the blood or lymphatic systems to other parts of the body (Leong *et al*., 2022).

There are numerous distinct cancers, and each has a unique set of signs and prognoses as well as available treatments (Jitwasinkul and Charoensuksai, 2018). The development of cancer can start anywhere in the body. Most of the time, it forms a solid tumor. However, some types of cancer can be referred to as liquid tumors (Mathai *et al*., 2019). There are four main types of cancer formed by the different kinds of cells in the body (Mattiuzzi and Lippi, 2019). For instance, carcinoma is the most common kind and the second leading cause of cancer-related death, which arises from the cells found on the internal and external surfaces of the body (Llovet *et al*., 2022). Sarcomas are the rarer kind, which arises from the tissues found in the supporting structures, such as bones, cartilage, fat, and muscle (Atarbashi-Moghadam *et al*., 2019). The Lymphomas are cancer cells that start in the lymph nodes and the tissues of the immune system (Najibi and Mooney, 2020) and leukemias are cancers of the blood cells that develop in the bone marrow and accumulate in the bloodstream (Saini *et al*., 2020).

1.1.1 Epidemiology

Worldwide, the incidence and mortality of cancer are rising at an accelerated pace, positioning cancer as the leading cause of death (Cai and Liu, 2021).The number of new cancer cases is predicted to increase globally by about 50% over the following two decades (Mao *et al*., 2022). The most commonly diagnosed cancers among black men are prostate (37%), lung and bronchus (12%), and colon and rectum (9%) (Giaquinto *et al.*, 2022). Moreover half (54%) of all cancer diagnoses among black people are related to the four most frequent cancers: lung, colon, prostate, and breast. Black men and women have a lifetime probability of 37% and 34%, respectively, of receiving a cancer diagnosis, while white men and women have a lifetime probability of 40% and 39%, respectively (DeSantis *et al.*, 2019).

After being surpassed by lung cancer since the mid-1990s due to the cigarette epidemic, breast cancer once again took the top spot in terms of cancer mortality among black women in 2019 (Giaquinto *et al*., 2022). Successful global cancer care faces many challenges, especially in low- and middle-income countries (LMICs), especially for those under 65 years old (Beddoe *et al*., 2016). For these nations, premature mortality and lost years of productivity have a greater economic impact (Bray *et al*., 2021).

The availability of standard treatments such as surgery, chemotherapy, and radiation therapy as well as supportive care services, including cancer screening facilities, and skilled medical personnel, can be exceedingly restricted in these areas (Haileselassie *et al*., 2019). Along their cancer journeys, patients have used affordable, culturally relevant traditional medical methods like acupuncture, yoga, meditation, and herbal remedies. These practices are all categorized as being a part of complementary and alternative medicine (CAM) (Hill *et al.*, 2019). Incorporating traditional, complementary, and integrative medicines (TCIMs) into national health systems at all

levels, including primary care, specialized care, and hospital care, has been encouraged and strengthened by the World Health Organization's (WHO) traditional medicine strategy since 2002 (Savatagi *et al*., 2022).

1.2 Plant metabolites with anti-cancer activity

Plants are known to produce a diverse range of secondary metabolites that possess valuable pharmacological properties (Kabera *et al*., 2014). Plants have been utilized as medicines for at least 60,000 years (Buyel, 2018). Since it led to the discovery of the anticancer vinca alkaloids vinblastine and vincristine as well as the isolation of podophyllotoxins, the natural precursor to etoposide, the 1950s-era hunt for anticancer drugs from plants has been successful (Cragg and Pezzuto, 2016). Irinotecan from *Camptotheca acuminata* and taxol from *Taxus brevifolia* are two further naturally produced anticancer medications (Shirolkar *et al.*, 2022).

In the last several decades, there has been an increase in the hunt for new plant-derived medications (Dehelean *et al*., 2021). It is believed that up to 60% of currently used anticancer drugs are naturally derived compounds from plants, oceans, and microbes (Abdel-Razek *et al*., 2020). About 114,000 extracts from an estimated 35,000 plant samples have been tested by the National Cancer Institute (NCI) of the United States of America against various tumor systems (Sithranga Boopathy and Kathiresan, 2010). Some examples include vinblastine and vincristine (*Catharanthus roseus*) (Kumar *et al*., 2013), epipodophyllotoxin, an isomer of podophyllotoxin (*Podophyllum peltatum* roots) (Shah *et al.*, 2021), paclitaxel (*Taxus baccata, T. brevifolia, T. canadensis*) (Yang *et al*., 2020), camptothecin (*Camptotheca acuminata*) (Fan *et al.*, 2022), flavopiridol (*Dysoxylum binectariferum*), and ipomeanol (*Ipomoea batatas*) (Shah *et al.*, 2013).

According to estimates by Fridlender *et al*. (2015), the two natural compounds paclitaxel and camptothecin represent roughly one-third and one-fourth of the global anticancer market, respectively. The most promising secondary metabolites obtained from plants for the treatment of cancer are phenolic chemicals, such as flavonoids (Tungmunnithum *et al*., 2018).

Pharmacological therapy is still one of the principal treatments for many forms of cancer. Despite the wide range of anticancer drugs now on the market, new anticancer medicines are still required. First, the use of some anticancer medications is constrained since they are frequently linked to substantial adverse effects. Second, cancer cells frequently develop a tolerance to the anticancer drugs that are prescribed (Cao *et al.*, 2020), which necessitates switching to a different pharmacological regimen. The diversity of anticancer drugs will grow, offering more options for treating the disease and ultimately raising the standard of living for cancer patients (Anand *et al*., 2023). Plants are a valuable natural resource for the screening of novel compounds with desired biological activities because they are sources of biologically active chemicals with a variety of molecular configurations (Lautie *et al.*, 2020). Many plant-derived substances have entered clinical studies or received FDA approval for the treatment of various diseases over the years (Thomas *et al.*, 2021).

1.3 The genus *Caralluma* R.Br.

The genus *Caralluma* belongs to the subtribe Stapeliinae (tribe Ceropegiae, subfamily Asclepiadoideae and family Apocynaceae) (Singh *et al.*, 2016). It is a xerophytic plant which includes about 120 taxa, widely distributed in Africa, Asia (Afghanistan, India, Pakistan, Sri Lanka and Iran), Arabian Peninsula, Southeast Europe and Canary Islands (Malladi *et al.*, 2018).

The word "*Caralluma*" originated from the Arabian word "qarh al-luhum" which means wound in the flesh or abscess (Adnan *et al.*, 2014). *Caralluma* plants are morphologically erect, creeping as

well scrambling succulent herbs with tetragonal branches, which are 20-30 cm tall, 4-angled, fleshy, green, and tapering to a point. The leaves are minute, present only on young branches, soon falling off, and leaving a tooth-like projection on the angles. The flowers are borne at the end of branches, singly or 2–3 together on short stalks. Flowers are like wheels, 2 cm across (Ramanjaneyulu *et al*., 2016). The petals are narrow, purple with yellow markings, and margins frilly with hairs. The fruits are 10–12 cm long and cylindric with one of the pairs often suppressed (Asmi *et al*., 2017). *Caralluma* plants have the foulest odor of any succulent plants (Adnan *et al.*, 2014).

The Crassulacean acid metabolism (CAM) is a survival mechanism used by *Caralluma* species in their native habitat (Masrahi *et al.*, 2012). The research in this area showed that the stomatal density, size, and area of the pore on the stem of *Caralluma* species are all low. According to Masrahi *et al*. (2011) stomatal characteristics have long been known to identify plants with the Crassulacean acid metabolism pathway.

1.3.1 Ethnobotanical uses

Arabic and Indian traditional medicine has employed members of the genus *Caralluma* to cure a wide range of illnesses, including diabetes, cancer, TB, snake and scorpion stings, skin rashes, scabies, fever, and inflammation (Pawade and Shinde, 2018). The boiled stem of *Caralluma edulis* has long been used in Iran and Pakistan to cure a variety of illnesses, including leprosy, rheumatism, gastrointestinal issues, hypertension, and Alzheimer's disease (Ansari *et al*., 2022). In India the water extract of the tuber of *C. adscendens* is used to lower blood sugar levels, whilst the sliced stems mixed with salt are consumed orally to induce diuresis (Kumar *et al.*, 2014). According to Malladi *et al.* (2018), *C. adscendens* has been used traditionally for its abilities to treat wounds, prevent obesity, and treat dermatitis. Two stem pieces of *C. laciantha* are applied

topically to the problem area for four days to treat skin infections; the stem pieces are crushed with some lime and cherry before application (Adnan *et al.*, 2014). The root of *C. umbellata* is burned in fire and consumed for 5 days daily on an empty stomach to cure ulcers and abdominal pain (Adnan *et al*., 2014).

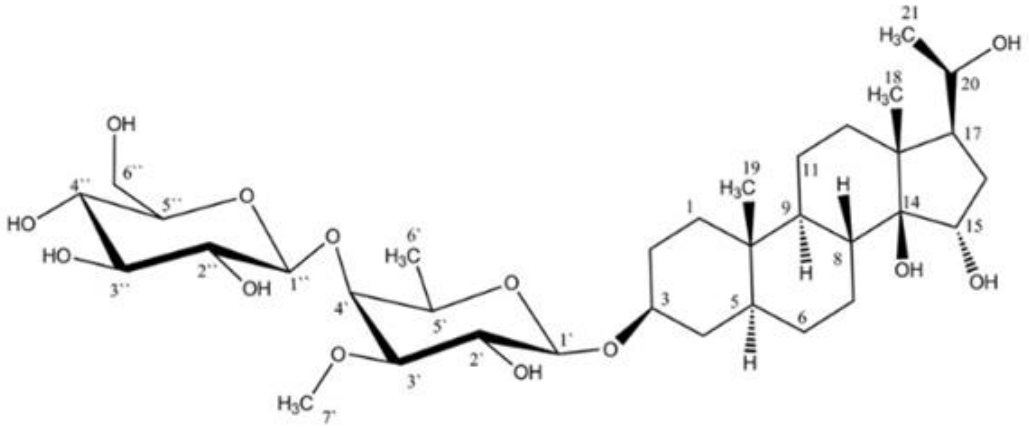
The juicy stem of *C. hiberculata* is consumed as a vegetable and used as a stomachic, carminative, and rheumatism remedy (Prabakaran and Kalimuthu, 2013). Stomach disorders have long been treated with *C. umbellata* (Jayaprakash *et al*., 2023). *Caralluma aucheriana* stems are used for liver disorders and as a cooling agent for sunburns and itchy skin in Saudi Arabia, India, Nigeria, and East Africa (Farouk *et al*., 2016). *Caralluma fimbriata*, traditionally referred to as "famine food" by tribal Indians, is consumed as a pickle or vegetable (Jayawardena *et al*., 2021). Its use to stifle hunger and quench thirst during prolonged hunting sessions has been documented *(Rao et al.*, 2021). *Caralluma tuberculata* has been eaten in rural India for centuries, raw, as a vegetable with spices, or preserved in chutneys and pickles, and is often found as a roadside shrub or boundary marker (Padwal *et al*., 2016). It is also used for its purported ability to suppress hunger and appetite and enhance stamina (Astell, 2016). Iran, Pakistan, Saudi Arabia, and South Africa have all historically employed *C. fimbriata* to filter blood (Anwar *et al*., 2022). The most prevalent *Caralluma* species in Yemen, *C. penicillata*, has been used in Yemeni traditional medicine to cure gastric ulcers and reduce inflammation (Albaser *et al*., 2014).

According to the herbalist's instructions, a decoction or maceration of the dried aerial portions of *C. dalzielii* is prepared and consumed in folklore medicine to cure infertility (Ugwah-Oguejiofor *et al.*, 2020). *Caralluma tuberculata* has been used in traditional medicine for a variety of conditions, such as dysentery, jaundice, constipation, stomach pain, freckles and pimples, hepatitis B and C, diabetes, blood purification, liver ailments, rheumatism, febrifuge, gastric problems,

paralysis, inflammation, and cancer (Baig *et al*., 2021a). It is also chewed to lower high blood pressure.

1.3.2 Phytochemical constituents

The genus *Caralluma* is characterized by the presence of pregnane glycosides, megastigmane glycosides, and various esters. Of these, pregnane glycosides are the main phytochemical components of the genus (Vanitha *et al.*, 2019). A typical example of these secondary metabolites is retrospinoside (**1**) 3-*O*-[β-D-glucopyranosyl-(1 → 4)-β-D-(3-*O*-methyl-6-desoxygalacto-pyranosy)]-14,15,20-trihydroxy-4β-pregnane is a polyoxy pregnane glycoside isolated from the aerial parts of *C. retrospiciens* (Ehrenb.) N. E. Br (Elsebai and Mohamed, 2015).



(**1**)

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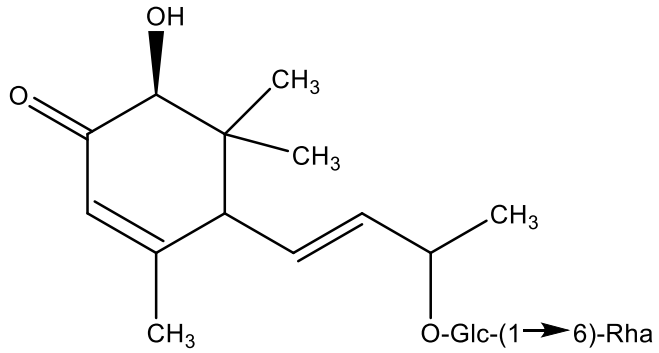
Figure 1. The structure of retrospinoside, a pregnane glycoside isolated from *Caralluma retrospiciens*

Megastigmane glycosides, one of the breakdown products of carotenoids, do also occur in various *Caralluma* species. (9R)-2β,9-Dihydroxymegastigma-4,7-dien-3-one-9-*O*-α-l-rhamnopyranosyl-

(1 → 6)- β-D-glucopyranoside (**2**) is the first megastigmane glycoside isolated from the methanol extract of the whole plant of *C.a negevensis* Zohary (Bader *et al*., 2003).



(2)

Figure 2. The structure of a megastigmane glycoside isolated from the *Caralluma negevensis*

1.3.3 Pharmacological activity

Members of the genus *Caralluma* have been reported to have anti-inflammatory, antitumor (Zakaria *et al*., 2001; Ugwah-Oguejiofor *et al*., 2013), anticancer, cytoprotective, antiulcer (Al-Faifi *et al*., 2016; Albaser et al., 2014b), antinociceptive (Firdoos *et al*., 2017), antioxidant, hypolipidemic (Khasawneh *et al*., 2014a; Maheshu *et al*., 2014; Tatiya *et al*., 2010), antihyperglycemic (Abdel-Sattar *et al*., 2017), antidiabetic (Ait Dra *et al*., 2019), treating paralysis and joint pains, antipyretic (Khan and Khatoon, 2008), antidepressant (Magaji *et al*., 2017), and antibacterial (Amrati *et al*., 2021) properties.

1.4 *Caralluma speciosa* (N.E.Br.) N.E.Br.

1.4.1 Description and distribution

Caralluma speciosa (N.E.Br.) N.E.Br., an uncommon succulent, grows quickly and readily on its own roots and can reach a height of one meter. It has stems that are 25–35 mm wide, upright, or ascending, adventitiously rooted, olive- to greyish green, with edges that quickly turn into light brown calluses. The pedicels grow up to 20 mm long with multi-flowered terminal umbel-like inflorescences. It has a deeply campanulate smooth corolla, approximately 30 mm long and 32 mm wide; 25–27 mm long and yellow on the inside. The lobes are triangular, purplish black, ascending or spreading, with numerous flexible, club-shaped hairs along borders. The outer lobes of the yellow, glabrous corona each have two erect, slender horns (Figure 1). *Caralluma speciosa* is widespread in Djibouti, Ethiopia, Sudan, Tanzania, Kenya, and Uganda (Kiros *et al*., 2023).

Figure 3. Picture of *Caralluma speciosa* (N.E.Br.) N.E.Br. in its natural habitat (Photographed by Kalkidan Derese around Gachena, Afar region, northeastern Ethiopia in March 2019)

Ethnobotanical use

In the prehistoric Harla and Dengego valleys of eastern Ethiopia, the stem of *C. speciosa* crushed with *Gloriosa superba* is used to treat skin cysts and tumors, and the latex as anti-poison and for the treatment of wound and itching skin (Belayneh and Bussa, 2014). In the Afar region,

northeastern Ethiopia, traditional healers use *C. speciosa* for cancer treatment (Haji Umed Buruhan, personal communication, March 2019), although they claim that it can cause a spontaneous abortion, which may lead to death if taken by a pregnant woman.

1.5 Statement of the problem

The current standard cancer treatments, such as surgery, chemotherapy, and radiotherapy are commonly employed, but they come with drawbacks in terms of effectiveness and toxicity to healthy tissues. Drug resistance, where cancer cells become resistant to common medications like chemotherapy, poses a significant challenge in cancer treatment, contributing to a considerable number of cancer-related deaths.

The stem latex of *C. speciosa* has been used to treat cancer for centuries in Ethiopia, yet this has not been experimentally validated. The overall purpose of this study is therefore to test if the plant possesses antiproliferative/cytotoxic activity, which is a particularly important screening test to select plant extracts with antitumoral potential for future studies.

2. Objectives

2.1. General objective

- To evaluate the antiproliferative/cytotoxic activities of the stem latex extract *Caralluma speciosa* and isolate the compound (s) from the active extract

2.2. Specific objectives

- To determine the antiproliferative/cytotoxic activities of the stem latex extract of *C. speciosa*;
- To isolate compounds from the stem latex extract of *C. speciosa*; and
- To elucidate the structure of the isolated compound (s).

3. Materials and Methods

3.1 Materials

3.1.1 Plant material.

The stem latex of *C. speciosa* was collected in March 2019 from Gachena, Afar region, eastern Ethiopia. The identity of the plant was confirmed by Mr. Melaku Wondafrash at The National Herbarium, Department of Plant Biology and Biodiversity Management, College of Natural Sciences, Addis Ababa University (AAU), where a botanical specimen was deposited (collection number: KD001/2019) for future reference.

3.1.2 Chemicals and instruments

The chemicals used in the study include methanol, chloroform, *n*-butanol, and acetic acid all from Sigma-Aldrich Co., MO, USA. Analytical TLC was performed using pre-coated silica gel 60 F254 plates (aluminium-backed, 200 µm, Merck KGaA, Darmstadt, Germany). Instruments used were Rotary evaporator (BUCHI Rotavapor™ R-300, Switzerland), UV cabinet (CAMAG, Switzerland), Evolution 60S UV-visible spectrophotometer (Thermo Fisher Scientific, Madison, USA), Shimadzu LC-2010A HT Liquid Chromatograph (Shimadzu, Japan), and Bruker Avance III 400 MHz Nuclear Magnetic Resonance (NMR) spectrometer (Bruker, Germany).

3.2 Methods

3.2.1 Collection and extraction of latex

The latex was collected in a screw-cap glass reagent bottle from the stem of a living *C. speciosa* plant by cutting with a blade and scraping the latex from the stem with a clean spatula. Following the collection, it was transported to Pharmacognosy laboratory of the School of Pharmacy, College of health Sciences, AAU and stored in a refrigerator. The latex (5g) was macerated in 80%

methanol (50 ml) at room temperature for 72 h, with occasional shaking and stirring. The extract was first filtered with gauze, followed by Whatman No. 1 filter paper. This was repeated twice, and the combined filtrates were concentrated using a rotary evaporator (Heidolph Instruments GmbH and Co., Germany) to remove the organic solvent. The remaining aqueous solution was dried in an oven at a temperature not exceeding 40 °C.

3.2.2 Isolation of compounds

Silica gel column chromatography was employed for fractionation and isolation of compounds, whilst analytical TLC was performed to monitor progress of the separation by column chromatography. HPLC was used to purify the compounds obtained from column chromatography.

3.2.2.1 Thin layer chromatography

Fractions obtained from column chromatography were subjected to analytical thin-layer chromatography (TLC) employing chloroform: methanol (4:1) and ethyl acetate: methanol: water (40:8:3) as mobile phases. The TLC chromatograms were visualized under ultraviolet light of wavelengths 254 nm and 366 nm. Subsequently, spots were assigned codes based on their descending order of Rf values.

3.2.2.2 Column chromatography

The column was packed by wet packing. Silica gel slurry was prepared by adding 100 g of the adsorbent (silica gel 60, 0.0623-0.200 mm) to 400 ml of chloroform, a little at a time, while swirling. The slurry was then carefully poured into the column so that the stationary bed is even and there are no bubbles, and pre-eluted with chloroform for 1 h.

A total of 4 g of dried hydroalcoholic extract was loaded onto the column. Elution was carried out by using $CHCl_3$–MeOH gradients as eluent i.e., increasing the amount of MeOH in $CHCl_3$ to afford 140 fractions. Ten ml fractions were collected as follows: [(1–35), $CHCl_3$ (100%)], [(36–54), $CHCl_3$:MeOH (95:5)], [(55–60), $CHCl_3$:MeOH (90:10)], [(61–69), $CHCl_3$:MeOH (85:15)], [(70–80), $CHCl_3$:MeOH (80:20)], [(81–90), $CHCl_3$:MeOH (75:25)], [(91–100), $CHCl_3$:MeOH (70:30], [(101–110), $CHCl_3$:MeOH (65:35)], [(111–120), $CHCl_3$:MeOH (60:40)], [(121–130), $CHCl_3$: MeOH [(55:45), [131–140)], $CHCl_3$:MeOH (50:50)]. These fractions were pooled according to their TLC profiles into three subfractions labelled as CS–1 (43–54), CS–2 (82–87), and CS–3 (94–98). The three subfractions were dried and weighed separately.

3.2.2.3 High performance liquid chromatography (HPLC)

CS-3 (10 mg) was initially dissolved in 1 mL of methanol. Subsequently, 10 μL of the solution was subjected to reversed-phase preparative HPLC for separation using Agilent C_{18} (size: 250 × 10 mm) analytical column. The chromatographic run utilized a mobile phase with solvents containing 0.01% acetic acid. The flow rate was set at 1 mL/min, with the temperature maintained at 40°C, and the detection was performed using a photodiode array detector. CS-3 was collected under the following conditions: Mobile phase gradient of 88-92% MeOH/H_2O with a 15-min run time.

3.2.3 Structural elucidation

The isolated compound was characterized on the basis of ^{1}H and ^{13}C NMR spectral assignments. NMR spectra were recorded on an FT-NMR spectrometer operating at 400 MHz for ^{1}H and 101 MHz for ^{13}C at room temperature using DMSO as a solvent. A region from 0 to 15 ppm for ^{1}H and 0 to 205 ppm for ^{13}C was employed for scanning. Signals were referred to internal standard tetramethylsilane (TMS). Chemical shifts were reported in δ unit (ppm) and coupling constants (*J*) are expressed in Hz. Multiplicities of ^{1}H NMR signals are indicated as *s* (singlet), *d* (doublet), *t* (triplet), *q* (quartet), *dd* (doublet of doublets), *dt* (doublet of triplets) *m* (multiplet).

3.2.4 Antiproliferative activity test

3.2.4.1 Cell culture

MCF-7, A427, SiSoN, and SiSoR are the cancer cell lines used to test the antiproliferative activity of the test substances. These cell lines were consistently kept in culture flasks of 75 cm^2 (Sarstedt, Nümbrecht, Germany), at 37 °C in a humid environment with 5% CO_2. The culture medium used for the cells was 90% RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma-Aldrich, Munich, Germany), 40 mg/L streptomycin, and 30 mg/L penicillin. The cells were passaged after being incubated at 37 °C in a 5% CO_2 humidified incubator (Heracell, Thermo Fisher Scientific, Waltham, MA, USA).

Table 1. Cancer cell lines used for activity testing

Adherent cell lines	Corresponding organ/tissue
MCF-7	Breast adenocarcinoma
A427	Lung cancer
SiSoN	Cervical cancer cell line (Normal)
SiSoR	Cervical cancer cell line (Resistant to cisplatin)

3.2.4.2 Crystal violet cell proliferation assay

The crystal violet test was utilized for screening of the test substances. The method by Feoktistova et al. (2016) was employed to determine the antiproliferative activity of the extract on adherent cell lines. In summary, every cancer cell line was deposited at a density of 1000 cells/well into 96-well microtiter plates and allowed to adhere to the plate surface for a full day. The following day, a series of five dilutions were obtained by serially diluting the extract stock solution (20 µg/mL in dimethylsulfoxide) twice to reach the required concentration range. The dilutions and stock solutions were added directly to the medium after being diluted five hundred times. Each well received 100 µL aliquots from the working dilutions. As a solvent, 0.1% (v/v) DMSO was employed. An extra 72 h were spent incubating the plates at 37 °C.

The culture medium was disposed of after 96 h and replaced with 1% glutaraldehyde buffer saline for 20 min. Dulbecco's buffer solution (pH 7.4) was then used to store the mixture at 4 °C. The day of staining involved removing the buffer solution and staining the cells for 30 min with 0.02% crystal violet in 100 µL of deionized water per well. The extra color was disposed of by giving the plates a 15-min soak in new water. The cell-bound dye was redissolved in 70% (v/v) ethanol/water, and a Spectramax 384 Plus plate reader (Molecular Devices, Sunnyvale, CA, USA) or a Sunrise plate reader (Tecan; Männedorf, Switzerland) was used to determine the optical density at $\lambda = 570$ nm. The corrected percent growth values, e.g., Treated versus Control corrected percentage [$T/C_{corr.}$ (%)] was calculated with the following equation:

$$(T/C)_{corr.}\ (\%) = \frac{OD_T - OD_C,0}{OD_C - OD_C,0} \times 100$$

where OD_T is the mean optical density (OD) of the treated cells, OD_C is the mean OD of the controls, and $OD_C,0$ is the mean OD of the seeded cells at the time the drug was introduced

4. Results and Discussion

4.1 Extraction yield

Extraction of *C. speciosa* latex by maceration in 80% methanol afforded a white amorphous solid with a percentage yield of 5% (w/w).

4.2 Effect of extract on cancer cell lines proliferation

Antiproliferative screening tests are important to select plant extracts with antitumoral potential for future studies. In eastern Ethiopia, the stem of *C. speciosa* is used in folk medicine for tumor treatment (Belayneh and Bussa, 2014). Furthermore, in the Afar region, northeastern Ethiopia, traditional healers claim that the stem of the plant is a herbal remedy for cancer (Haji Umed Buruhan, personal communication, March 2019). However, to date, there appear to have been no scientific studies on its potential anticancer activity against cancerous cell lines. Thus, in the present study the i*n vitro* antiproliferative activity of the 80% methanolic extract of *C. speciosa* was evaluated against A427, MCF-7, SiSoN, and SiSoR human cancer cell lines by using the crystal violet cell proliferation assay. As shown in Table 2, at a concentration of 20 μg/ml the extract demonstrated negative $T/C_{corr.}$ values against MCF-7 and SiSoN indicating a relevant cytotoxic activity. However, the extract showed antiproliferative activity when tested against A427 and SiSoR cancer cell lines. According to the US National Cancer Institute, a plant extract with an IC_{50} value of less than 20 mg/mL on cancer cell lines is considered a promising anticancer agent (Pandey *et al*., 2017). This implies that the hydroalcoholic latex extract of *C. speciosa* is a promising chemotherapeutic candidate. However, since anticancer agents have to be selective to cancer cells, their effects on normal cells have to be studied.

Table 2. Antiproliferative/cytotoxic activity of the 80% methanol stem latex extract of *Caralluma speciosa* (20 µg/mL) after 96 h on MCF-7, A427, SiSoN, and SiSoR cell lines in primary screening. Testing was conducted with the crystal violet cell proliferation assay.

Test substance	$T/C_{corr.}$ (%), SEM			
	MCF-7	A427	SiSoN	SiSoR
80% methanol extract	-1.07, 0.22	0.43, 0.83	-0.95, 0.51	1.07, 0.824

MCF7: Breast adenocarcinoma, A427: Lung carcinoma, SiSoN: Cervical cancer cell line (Normal), SiSoR: Cervical cancer cell line (Resistant to cisplatin); T/C_{corr} (%): Treated versus Control corrected percentage; SEM: Standard error of the mean.

Previous studies have demonstrated that various plants belonging to the genus *Caralluma* possess genuine antiproliferative and cytotoxic activities. For example, the chloroform fraction of *C. quadrangular* was shown to have cytotoxic activity against MCF-7 breast cancer cells (Abdallah *et al*., 2013). Similarly, the ethanol extract of the whole fresh plant of *C. tuberculata* showed concentration-dependent growth inhibition of the MCF-7, MDA-MB-468 (human breast estrogen-independent adenocarcinoma), and Caco-2 (colorectal adenocarcinoma) cancer cell lines after 24 h (Waheed *et al*., 2011).

Owing to the potent *in vitro* cytotoxic and antiproliferative activities of *C. speciosa* latex extract against different cancer cell lines, it was deemed prudent to carry out a phytochemical investigation of the extract to isolate compound(s) with cytotoxic or antiproliferative activity.

4.3 Isolation of a compound

The 80% methanolic stem latex extract of *C. speciosa* was first inspected by TLC (Figure 4). It was then subjected to column chromatography on silica gel using $CHCl_3$:MeOH (increasing polarity) as eluents. Fractions (94–98) that were obtained by eluting with $CHCl_3$:MeOH (70:30)

showed one spot on silica gel TLC. These fractions were combined and evaporated to dryness to afford 420 mg of a crystalline substance labeled as CS-3.

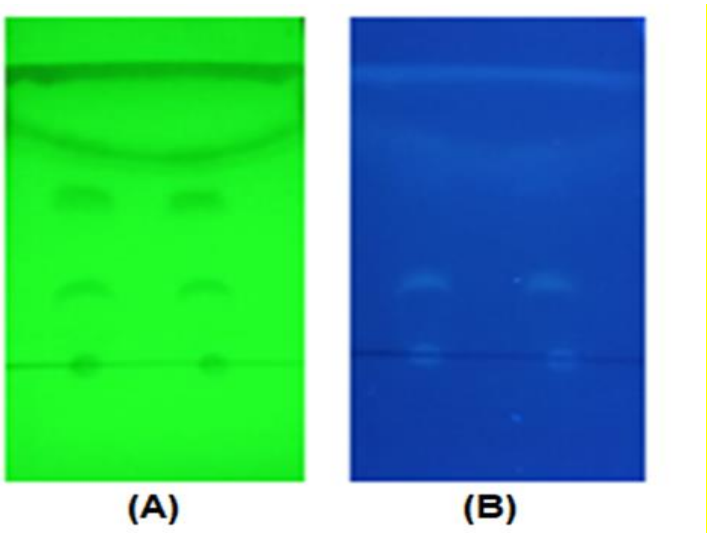


Figure 4. TLC chromatogram of the 80% methanolic extract of stem latex of *Caralluma* speciosa in chloroform: methanol (4:1) solvent system. (A) Viewed under UV light of 254 nm and (B) viewed under UV light of 366 nm.

CS-3 was purified by a reversed phase HPLC using Agilent C_{18} columns employing a MeOH-H_2O gradient elution. A repetitive analytical-HPLC runs afforded 7 mg of a white crystalline compound. HPLC chromatograms of CS-3 in the hydoalcoholic extract of *C. speciosa* and after purification are depicted in Figures 5 and 6, respectively.

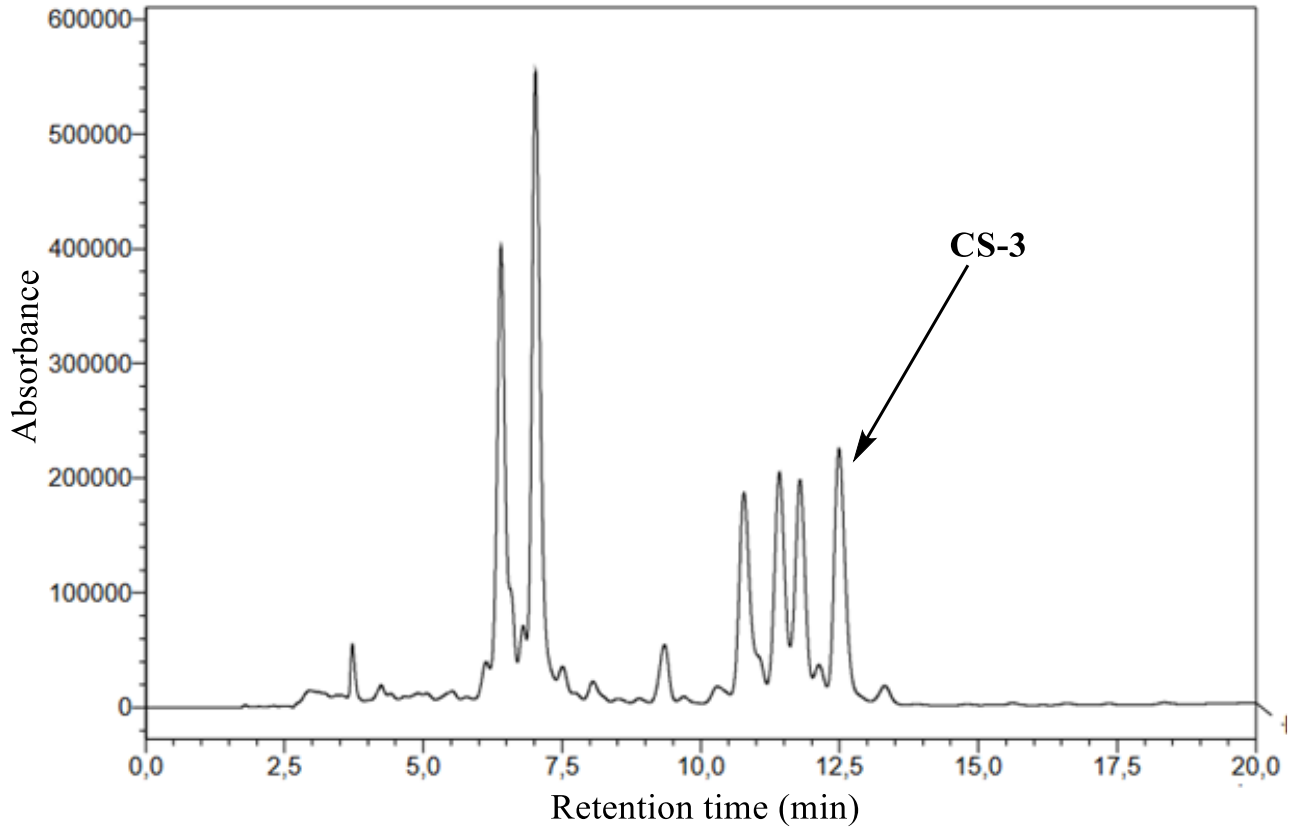


Figure 5. Reversed phase HPLC chromatogram showing CS-3 in the hydroalcoholic extract of *Caralluma speciosa* stem latex (Mobile phase gradient 88-92% MeOH/H_2O containing 0.01% acetic acid; Agilent C18 analytical column; Flow rate: 1 mL/min.

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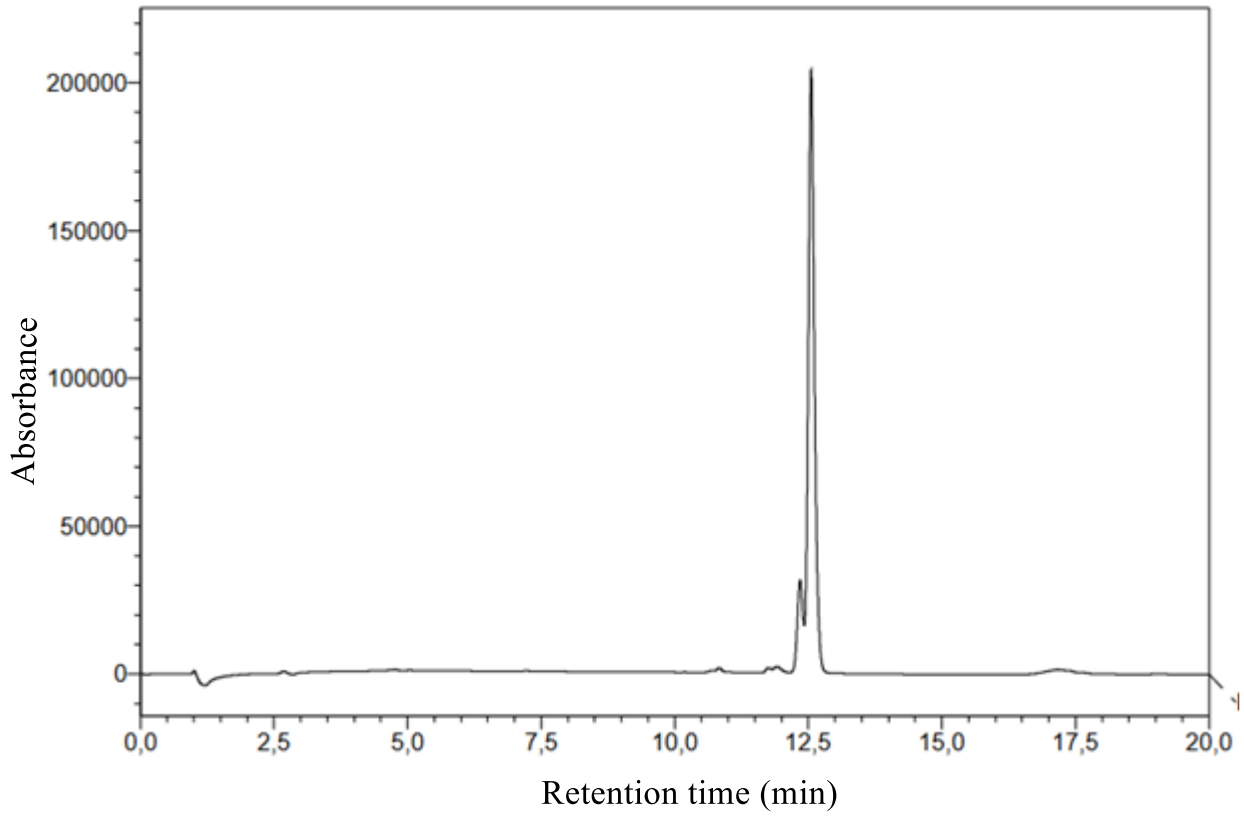

Figure 6. Reversed-phase HPLC chromatogram of CS-3 after purification (Mobile phase: Gradient of 88-92% methanol/water containing 0.01% acetic acid; Agilent C18 analytical column; Flow rate: 1 mL/min.

4.4 Characterization of CS-3

Structural elucidation of the isolated compound was accomplished through the analysis of its 1D (^{1}H, ^{13}C) and 2D-NMR spectral data and by comparing its spectral data with those reported in the literature for similar compounds.

The ^{13}C-NMR spectrum of CS-3 (Appendix I) revealed that the compound has 49 carbon atoms, corresponding to six aromatic carbons (δ 130.8, δ 129.8 × 2, δ129.75 × 2, δ 132.8), two olefinic (δ 140.67, δ 123.15), seven quaternary (δ 140.67,38.47, δ 51.39,84.22, 168.07,130.82,174.43), two carbonyl (δ 174.43, δ 168.07), and one methoxy (δ 58.6), along with two oxymethine (δ 75.01, δ

70.25) and six methyl groups (δ 10.44, δ 19.57, δ 19.98, δ 22.8, δ 18.43, δ 18.31). Close examination of both ^{1}H and ^{13}C-NMR spectra suggested that CS-3 is a pregnane steroid. The ^{13}C-NMR analysis revealed carbon signals at δ 168.07 (C-1'), δ 138.02 (C-2'), δ 129.98 (C-3' and C-7'), δ 129.75 (C-4' and C-6'), and δ 132.8 (C-5'), indicating the presence of a benzoyl group in CS-3. Additionally, signals at δ 174.43 (C-1'') and δ 22.85 (C-2'') were observed unmistakably suggesting an acetyl group as part of the structure in compound CS-3. Furthermore, a sugar chain comprising three sugar units connected to C-3 (δ 79.7) of the aglycone was evident by the presence of three anomeric carbon signals resonating at δ 97.43 (C-1'''), δ 101.29 (C-1'''') and δ 106.27 (C-1''''). The existence of a double bond in CS-3 was identified through olefinic signals observed at δ 123.15 (C-6) and δ 140.67 (C-5) in the ^{13}C-NMR spectrum of the same compound. Tables 3 and 4 provide a comprehensive summary of carbon chemical shift assignments.

The ^{1}H-NMR spectrum of CS-3 (Appendix II) further verified the presence of a benzoyl group, as indicated by three sets of aromatic protons resonating at H-3'7' (δ 8.08, H, *dd*), H-4'6' (δ 7.51, H, *m*), and H-5' (δ 7.64, H, *m*). Additionally, the existence of an acetyl group was confirmed by the ^{1}H-NMR spectrum of CS-3, which displayed a singlet signal integrating for three protons and resonating at δ 2.01.

The ^{1}H-NMR spectrum revealed the presence of an olefinic methine proton (=CH-) signal resonating at δ 5.48 (2H, t). Additionally, the proton spectrum exhibited three anomeric protons of a sugar chain at δ 4.79 (H-1, *dd*), δ 4.58 (H-1, *dd*), and δ 4.32 (1H, *d*). The complete ^{1}H-NMR spectral data are summarized in Table 3.

A benzoyl group at C-20 and an acetyl group at C-12 were detected in the NMR data, along with a straight sugar chain made up of three sugar units attached to the aglycone's C-3 position. The downfield shift of the corresponding protons at H-3 (δ 3.51), H-12 (δ 4.41), and H-20 (δ 4.79) and

carbons at C-3 (δ 79.87), C-12 (δ 70.25), and C-20 (δ 75.01), confirmed that those were the sites of glycosylation and acylation, furthermore, the distinct long-range coupling between the signal of carbonyl carbons of benzoyl and acyl groups and H-20 and H-12, respectively, of the aglycone moiety, confirmed acylation at C-12 and C-20 (Abdallah et al., 2013). The NMR spectra of CS-3 showed close similarity with the spectra of the compound, which was previously isolated from *C. quadrangula* (Abdallah *et al*., 2013).The only difference is the presence of benzoyl group instead the acetyl group at C-12 in *C. quadrangula*, which showed an upfield shift for C-12 (δ 70.25) relative to the same carbon in *C. quadrangula* (δ 77.5). This was further corroborated by comparing the data with those of similar pregnane glycosides (Al-Massarani *et al*., 2012). Assignment of the proton signals is depicted in Tables 3 and 4.

Table 3. 1H-NMR and 13C-NMR chemical shifts of the aglycone part of CS-3

Carbon numbering	CS-3	(Abdallah et al., 2013)	CS-3	(Abdallah et al., 2013)
1	1.06, 1.15 *m*	1.13, 1.73 *m*	38.15	37.1
2	1.65, 1.49 *m*	1.56, 1.91 *m*	28.32	29.4
3	3.51 *m*	3.57 m	79.87	77.7
4	2.15, 2.25 *m*	2.23, 2.40 *m*	39.91	38.7
5			140.67	139.6
6	5.48 *m*	5.43 *d*	123.15	121.4
7	2.18,2.10 *m*	1.91, 2.28 *m*	27.48	26.9
8	1.28 *m*	1.78 *m*	36.59	37.3
9		1.33 *m*	44.8	43.2
10			38.47	37.3
11	1.57, 1.69 *m*	1.58, 1.80 *m*	27.18	26.2
12	4.41 *t*	4.84 *dd*	70.25	77.5
13			51.39	51.9
14			84.22	86.6
15	1.73, 1.65 *m*	1.73, 1.90 *m*	33.04	32.1
16	2.1, 1.78 *m*	1.65, 2.01 *m*	26.84	24.7
17	1.96 m	2.31 *m*	49.79	49.5
18	0.89 *s*	1.17 *s*	10.44	9.4
19	1.20 *s*	0.97 *s*	19.57	19.3
20	5.40 *m*	5.14-5.31 *m*	75.01	74.3
21	1.30 *d*	1.23 *d*	19.98	19.5
		C- 20 benzoyl group		
1′			168.07	165.9
2′			130.82	130.8
3′, 7′	8.08 *dd*	8.12 *dd*	129.98	129.8
4′, 6′	7.51 *m*	7.49 *m*	129.75	128.5
5	7.64 *m*	7.65 *m*	132.8	133
		C -12 acetyl group		
1″			174.43	
2″	2.01 *s*		22.85	

Table 4. ^{1}H and ^{13}C NMR of sugar moieties of CS-3

	Literature		**CS-3**	
Cymarose				
position	**δH**	**δC**	**δH**	**δC**
1‴	4.72 – 4.90 *dd*	95.0	4.79 dd	97.43
2a‴	1.57 *m*	35.9	1.78 *dd*	36.89
2b‴	2.07 *m*	-	1.74 *dd*	-
3‴	3.68 *q*	77.2	3.54 *dt*	79.2
4‴	3.22 *dd*	82.7	3.23 *d*	84
5‴	3.75 *m*	77.2	3.9 *m*	78.84
6‴	1.21 *d*	18.1	1.09 *d*	18.43
OCH_3	3.34 *s*	57.3	3.41 *s*	58.6
Canarose				
1⁗	4.49- 4.66 *dd* (*J*=9.6,1.8)	101.0	4.58 dd (J = 9.7,2.0 Hz)	101.29
2a⁗	1.62 *m*	38.4	1.75 *dd*	38.62
2b⁗	2.26 *m*	-	2.0 *dd*	-
3⁗	3.44 *m*	69.5	3.9 *dt*	68.3
4⁗	3.83 *dd*	88.2	3.3 *dd*	88.51
5⁗	3.18 *m*	70.4	3.9 *m*	70.13
6⁗	1.26 *d*	17.8	1.01 *d*	18.31
Glucose				
1⁗′	4.23-4.40 *d* (*J* = 7.8 Hz)	104.3	4.32 *d* (*J* = 8.0 Hz)	106.27
2⁗′	2.98 *m*	73.3	2.99 *dd*	73.2
3⁗′	3.08 *m*	76.6	3.23 *dd*	76.7
4⁗′	3.02 *m*	69.9	3.03 *dd*	71.5
5⁗′	3.11 *m*	76.5	3.6 *m*	75.5
6a⁗′	3.41 *m*	61.0	3.57 *d*	61.21
6b⁗′	1.62 *m*	-	3.52 *d*	-

Based on the spectroscopic data provided above and a comparison with literature data, the structure of CS-3 was tentatively identified 20-*O*-benzoyl-12-*O*-acetyl-14-hydroxy-20-pregn-5-ene-3-*O*-*β*-D-cymaropyranosyl-(1→4)-*β*-D-canaropyranosyl-(1→4)-*β*-D-glucopyranoside (Figure 4) (Al-Massarani et al., 2012; Abdallah et al., 2013). A perusal of the literature unveils that the compound has not been isolated before.

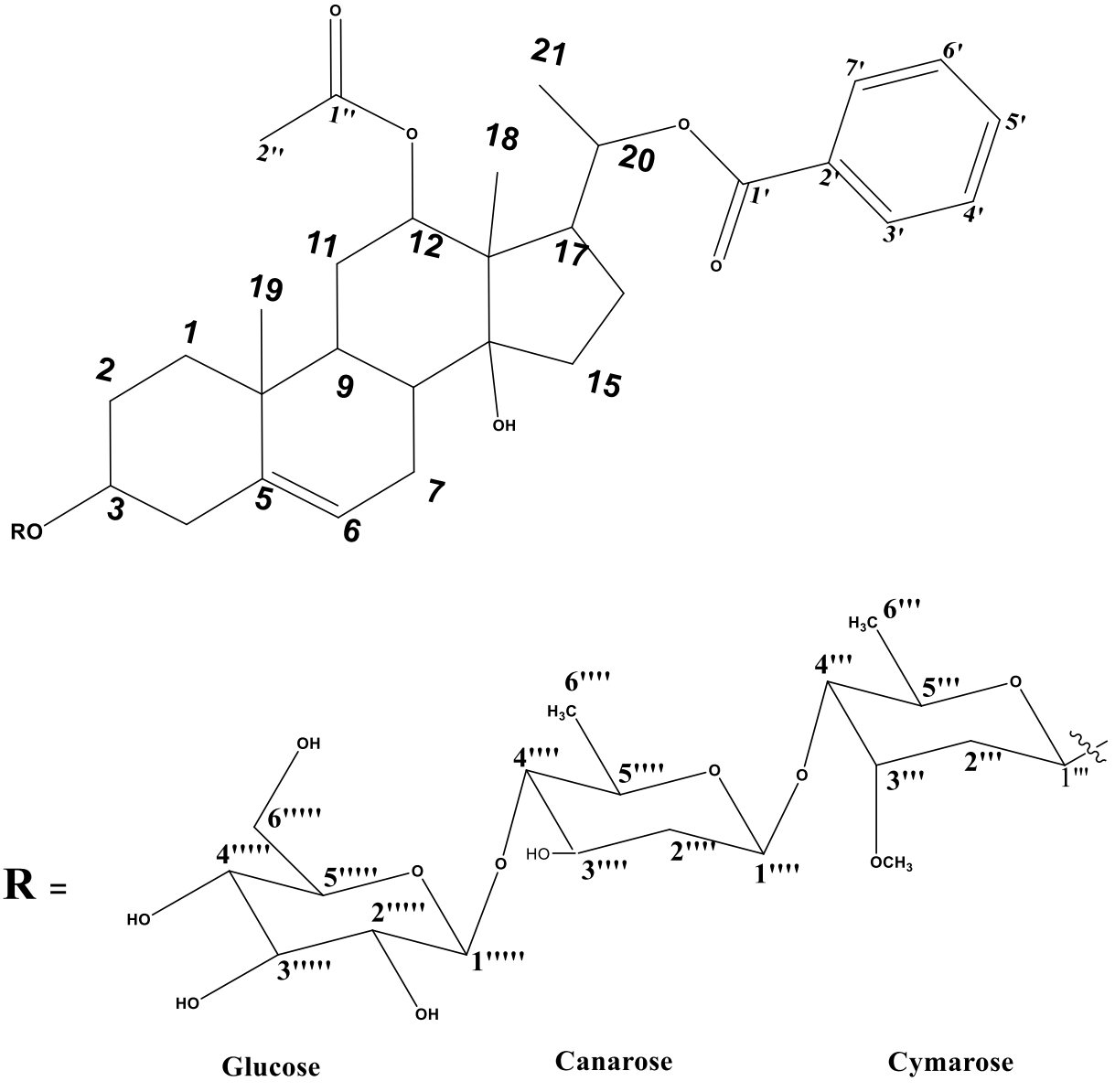

Figure 7. Structural formula of CS-3

5. Conclusion

The findings of the present work revealed that at a concentration of 20 μg/mL, the 80% methanol stem latex extract of *C. speciosa* possesses genuine *in vitro* antiproliferative activity against lung carcinoma (A427) and cervical cancer resistant to cisplatin (SiSoR), whilst at the same concentration of the extract was toxic to breast (MCF-7) and normal cervical (SiSoN) cancer cell lines. These findings uphold the traditional use of the plant for the treatment of cancer. The present study also resulted in the isolation of a compound tentatively identified as a novel pregnane glycoside 20-*O*-benzoyl-12-*O*-acetyl-14-hydroxy-20-pregn-5-ene-3-*O*-*β*-D-cymaropyranosyl-(1→4)-*β*-D-canaropyranosyl-(1→4)-*β*-D-glucopyranoside. Taking into consideration literature reports on genuine anticancer activities of pregnane glycosides, it can be surmised that the cell growth inhibition and toxicity caused by the stem latex of *C. speciosa* are in part or in full associated with the presence of acylated pregnane glycosides in the plant.

6. Recommendations

The following recommendations are forwarded

- ✓ Acute and chronic toxicity of the latex extract must be studied;
- ✓ Antiproliferative/cytotoxic activity of the isolated compound must be determined;
- ✓ If the isolated compound is found to be active, the mechanism of its antiproliferative activity on molecular targets must be ascertained to precisely pinpoint the mode of action of isolated compounds; and
- ✓ Antiproliferative/cytotoxic properties of the other pregnane glycosides present in the latex must be assessed.

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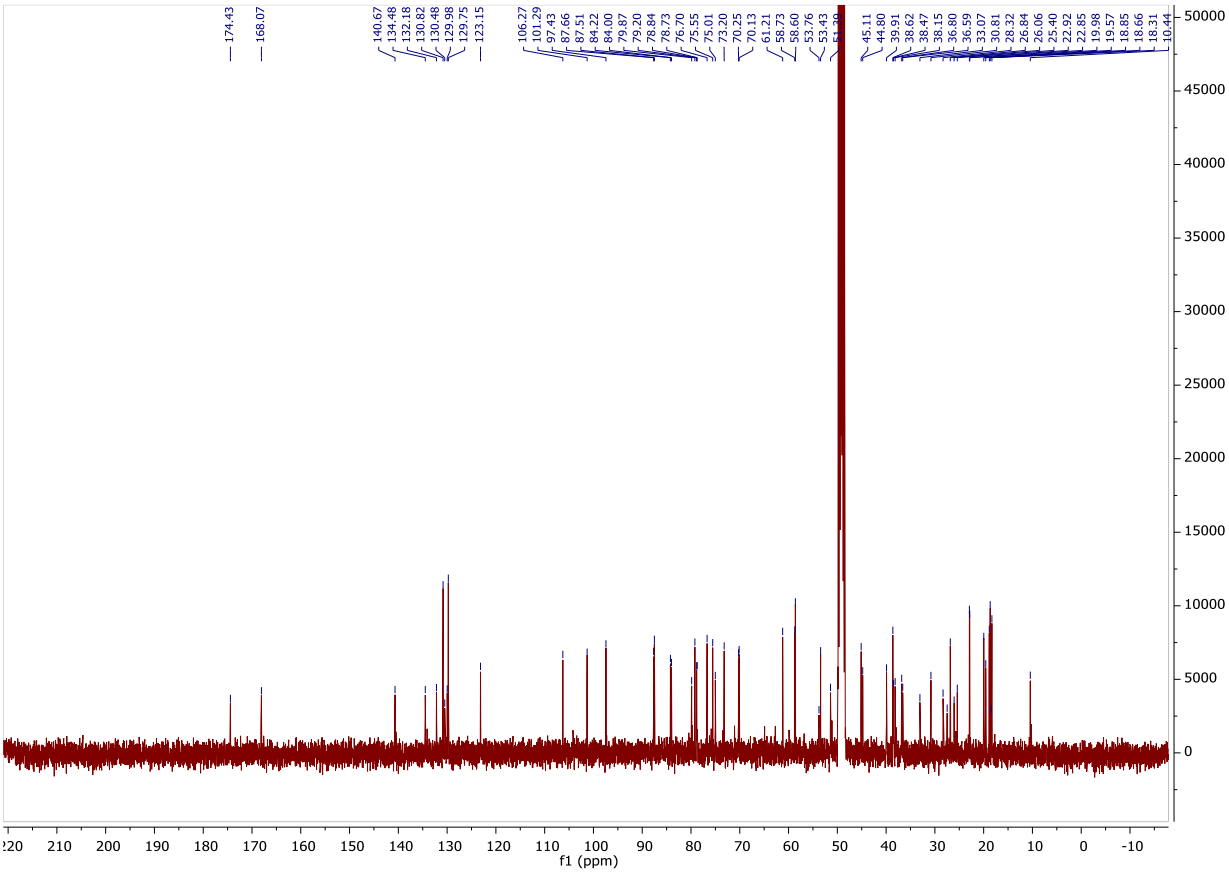
Appendices

Appendix 1. ^{13}C-NMR of CS-3



Appendix II. ^{1}H-NMR spectrum of CS-3