Saudi Journal of Biological Sciences 25 (2018) 1826–1833

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

The binding proximity of methyl β-lilacinobioside isolated from *Caralluma retrospiciens* with topoisomerase II attributes apoptosis in breast cancer cell line

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ARTICLE INFO

Article history:
Received 21 January 2018
Revised 22 February 2018
Accepted 25 February 2018
Available online 27 February 2018

Keywords:
Caralluma retrospiciens
Methyl β-lilacinobioside
Anticancer
MCF-7
Molecular docking

ABSTRACT

The alterations in somatic genomes that controls the mechanism of cell division as a main cause of cancer, and then the drug that specifically toxic to the cancer cells further complicates the process of the development of the widely effective potential anticancer drug. The side effects of the drug as well as the radiotherapy used for the treatment of cancer is severe; therefore, the search of the natural products from the sources of wild plants having anticancer potential is become immense importance today. The ethno-medicinal survey undertaken in Al-Fayfa and Wadi-E-Damad region of southern Saudi Arabia revealed that the *Caralluma retrospiciens* (Ehrenb.) N.E.Br. (family Apocynaceae) is being used for the treatment of cancer by the native inhabitants. The biological evaluation of anticancer potential of bioassay-guided fractionations of methanolic extract of whole plant of *C. retrospiciens* against human breast adenocarcinoma cell line (MCF-7) followed by characterization using spectroscopic methods confirmed the presence of methyl β-lilacinobioside, a novel active constituent reported for the first time from *C. retrospiciens*, is capable of inhibition of cell proliferation and induction of apoptosis in MCF-7 cells by regulating ROS mediated autophagy, and thus validated the folkloric claim. Based on a small-scale computational target screening, Topoisomerase II was identified as the potential binding target of methyl β-lilacinobioside.
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1. Introduction

A normal cell become cancerous when the abnormal gene expression occurs due to the cellular DNA that control the mechanism of cell division corrupted (Bertram, 2000; Harrington, 2016). The burden of cancer at the global level is high, which accounts for about one in every seven death worldwide (ACS, 2017). The multidisciplinary scientific investigations are making continuous efforts to scrap cancer; however, the perfect cure is yet to come into medicine world (Hait, 2010; Sanghani et al., 2012). The natural products have historically and continuously being explored for promising new leads in pharmaceutical development (Mann, 2002; Newman and Cragg, 2012). The arid plant biodiversity of Saudi Arabia had been used in folk medicine since long time (Rahman et al., 2004) might be due to abundant production and accumulation of the medicinally important secondary metabolites in the wild plants that grows in a harsh stress (Harlev et al., 2012).

The genus *Caralluma* R. Br. (family Apocynaceae) comprises approximately 58 species, belongs to a group of stem-succulents commonly known as stapeliads, mainly distributed in arid to semi-arid areas of Arabia, Africa and Peninsular India. *Caralluma*

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2018.02.017
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retrospiciens (Ehrenb.) N.E.Br. [-synonym *Caralluma russelliana* (Courbai ex Brongn.) Cufod.] is a wild stapeliads, distributed in Eritrea, Ethiopia, Kenya, Saudi Arabia, Uganda and Yemen (Bruyns et al., 2010). *C. retrospiciens* is commonly known as '*mali*' in Arabic (Fig. 1), occurs in dry places of Abha and Jizan region of southern Saudi Arabia (Collenette, 1999). During the survey for the documentation of ethno-medicinal plants of anticancer potential, the first author came across the use of *C. retrospiciens* for the treatment of cancer by the native inhabitants of Al-Fayfa and Wadi-E-Damad region of southern Saudi Arabia. The pregnanes and pregnane glycosides rich members of the family Apocynaceae (Abdel-Sattar et al., 2007) including *Caralluma* (Abdel-Mogib and Raghib, 2013; Elsebai and Ietidal, 2015) recently caught wide attention because of its anticancer potential (Rajendran and Rajendran, 2004; Wen et al., 2016). Hence, the present study aims to unravel the anticancer active constituent of *C. retrospiciens*.

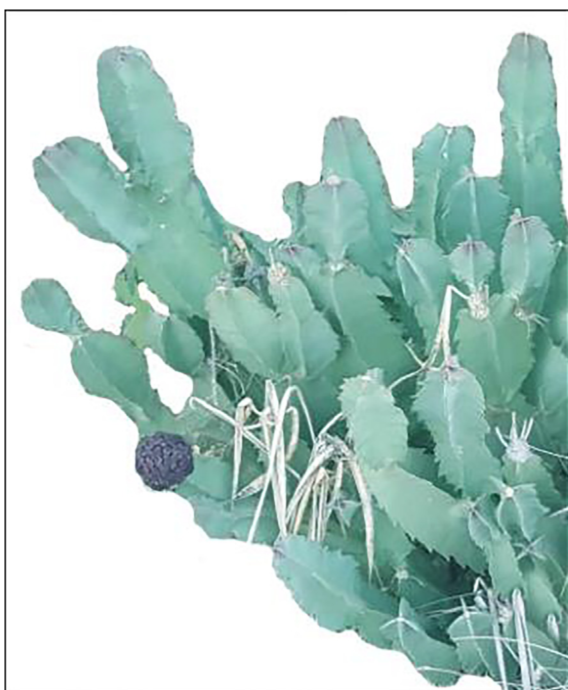


Fig. 1. Habit of *Caralluma retrospiciens* (Ehrenb.) N.E.Br. [-synonym *Caralluma russelliana* (Courbai ex Brongn.) Cufod.] (family Apocynaceae).

Table 1
^{1}H NMR and ^{13}C NMR spectral data of the isolated compound (500 MHz, Methanol-d_4).

Position	δ_H	δ_C
1	4.69 (*m*)	99.3
2	2.16 (*m*), 1.52 (*m*)	35.6
3	3.56 (*m*)	78.3
4	3.61 (*dd*, *J* = 3.0, 9.5 Hz)	83.9
5	3.67 (*d*, *J* = 1.5 Hz)	68.1
6	1.32 (*d*, *J* = 6 Hz)	18.7
OMe	3.39 (*s*)	57.5
OMe	3.30 (*s*)	55.0
1	4.71 (d, *J* = 8 Hz)	102.3
2	3.32 (*m*)	73.5
3	3.24 (*t*)	84.3
4	3.16 (*dd*, *J* = 3.0, 9.5 Hz)	75.1
5	3.65 (*m*)	71.2
6	1.23 (*d*, *J* = 6 Hz)	18.2
OMe	3.60 (*s*)	62.5

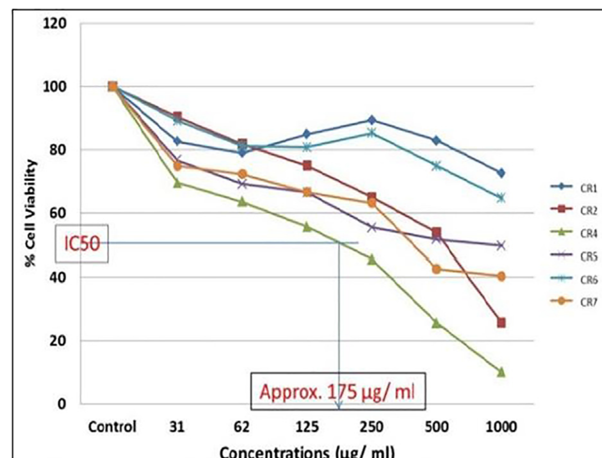
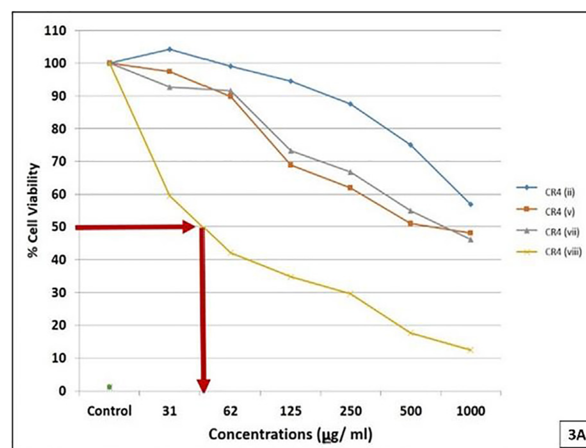


Fig. 2. The inhibition of MCF-7 cell proliferation by the treatment of six different concentration i.e. the serial dilution of 1 mg/ml crude methanolic extract (CR1), precipitate (CR2), hexance extract (CR3), chloroform extract (CR4), Ethyl acetate extract (CR5), butanol extract (CR6), and an aqueous extract (CR7) of *C. retrospiciens* for 24 h. (The surface of culture plate wells of CR3 treatment were covered with dense granulated particles might be the reason for complete cell growth inhibition in its all six concentration; therefore, CR3 was excluded from further toxicity assessment, and the data has not been incorporated in the graph.). A dose-dependent decrease in the percentage of cell viability was observed in the range of 10–90%, the compound CR4 shows highest toxicity (IC_{50} approximately 175 µg/ml) compared to the rest tested compounds.



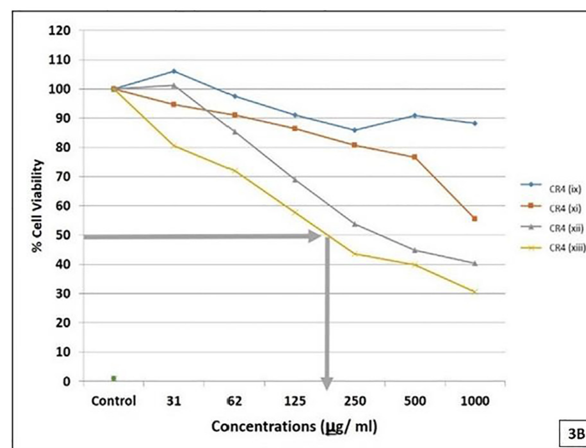


Fig. 3. (A and B) The inhibition of MCF-7 cell proliferation by the treatment of six different concentration of serial dilution of 1 mg/ml of the treatment of 10 different fractionates of CR4 i.e. CR4(i), CR4(ii), CR4(v), CR4(vii), CR4(viii), CR4(ix), CR4(x), CR4 (xi), CR4(xii) and CR4(xiii). The cell growth inhibition (IC_{50} approximately – 30 µg/ml) was occurred in the case of the cells treated with the fractionated compound CR4(viii).

2. Materials and methods

2.1. Plant material

The whole aerial part of *C. retrospiciens* was collected in January 2015 from the Al-Fayfa region of southern Saudi Arabia. The collected plant was taxonomically identified following the flora of Saudi Arabia (Collenette, 1999; Chaudhary, 2001). A voucher specimen (FMA-127) was prepared, and deposited at the Herbarium of the Department of Botany and Microbiology, King Saud University, Riyadh, Saudi Arabia (KSUH) for the record and reference. The correct botanical nomenclature was confirmed from the Tropicos® (http://www.tropicos.org/) and The Plant List (http://www.theplantlist.org/) database.

2.2. Extraction, fractionation, purification and characterization of anticancer active constituent

The air-dried and powdered aerial part of *C. retrospiciens* (1 KG) was extracted with methanol (95% methanol) by maceration for two weeks at 37 °C. The obtained methanolic extract (CR1) was filtered and concentrated under reduced pressure at 40 °C using a rotary evaporator. The dried methanolic extract (70 g) was subsequently re-dissolved in 40% methanol to obtain a precipitate (CR2), and partitioned successively for several times with *n*-hexane (3 × 200 ml), chloroform (3 × 200 ml) and *n*-butanol (3 × 200 ml) to provide the corresponding extracts. Each extract namely, methanolic extract (CR1), precipitate (CR2), hexance extract (CR3), chloroform extract (CR4), Ethyl acetate extract (CR5), butanol extract (CR6) and aqueous extract (CR7) was tested for its cytotoxic activity.

The fractionation of chloroform extracts (CR4) gave 10 fractions [CR4(i)-CR4(xiii)], were tested for their cytotoxic activity. 200 mg CR4(viii) was subjected for further purification on Sephadex LH20 column with methanol: water (9:1) as a solvent which yielded into two main sub fractions [Fraction CR4(viii)a and CR4 (viii)b]. Fraction CR4(viii)a yielded a major compound when subjected to further purification on the chromatotron (centrifugal TLC) (silica gel 60, 0.04–0.06 mm, 1 mm and acetonitril:chloroform, 2:8) finally recovered 20 mg of a viscous compound. Different spectroscopic methods such as Ultraviolet Spectroscopy (UV), Infrared Spectroscopy (IR), Mass Spectroscopy (MS) and Nuclear Magnetic Resonance (1D NMR, 2D NMR) were used for the structural identification and elucidation of CR4(viii).

2.3. Determination of the inhibition of cell proliferation and induction of apoptosis

The cytotoxicity of test compounds were evaluated according to the previously described methods (Ali et al., 2014; Abul Farah et al., 2016). In brief, the MCF-7 cells were cultured as adherent layers in T-25, T-75 culture flasks, 96-well or 6 well flat bottom culture plate depending upon the requirement of the designed experiment. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Mossman, 1983) were performed to analyze the cytotoxic activity of the test compounds at the concentration of six serial dilution of 1 mg/ml to find the IC_{50} value. The morphological changes in the MCF-7 cells were observed using inverted microscope to determine the alterations induced in the cells treated with test compounds for 24 h. Further, the cells were treated below the IC_{50} concertation, and annexin-V FITC apoptosis induction assay (Evens et al., 2004) using BD FACSCalibur flow cytometer, DNA ladder assay Singh et al. (1988), reactive oxygen species (ROS) (Wang and Joseph, 1999) and autophagy (Paglin et al., 2001) detection were performed following previously described method (Abul Farah et al., 2016). 50 μM concentration of Doxorubicin was dissolved in complete cell culture medium, and used as a positive control in all the experiments.

2.4. Molecular docking simulation for the determination of the potential drug target of anticancer active constituent

To determine the potential drug target of anticancer active constituent, a small group of candidate drug targets (Table 1) were

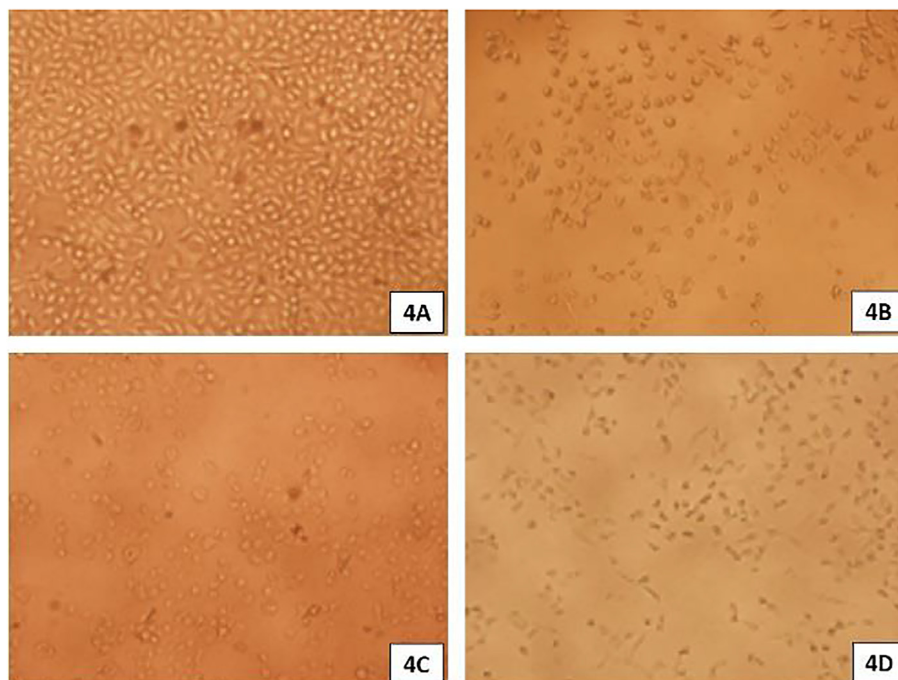


Fig. 4. (A–D) The morphological changes observed in MCF-7 cells. Control (A), 50 mM Doxorubicin used as positive control (B), 1 mg/ml CR4 (C), and 30 μg/ml CR4(viii) (D) for 24 h.

carefully selected, and were evaluated by molecular docking (Bai et al., 2013) based on their binding affinities with the molecular docking: compound. The coordinates anticancer active constituent was initially built and optimized using Chem3D (Mills, N.) with MM2 force field. Its atomic partial charge was assigned by means of the Gasteiger–Marsili method. The structures of candidate targets were parameterized according to the Amber ff99 force field. Then the compound were docked to each of the targets and the most energetically favorable binding pose was kept.

3. Results and discussion

3.1. MTT assay for the recognition of anticancer active constituent

The cytotoxicity of compounds isolated from *C. retrospiciens* were determined by the MTT assay in order to acquire information on cell death, survival and metabolic activities in MCF-7 cells. The MCF-7 cells were treated with different concentrations of CR1-CR7 in ranging 1.0, 0.50, 0.25, 0.12, 0.06 and 0.03 mg/ml for 24 h (Fig. 2). A dose-dependent decrease in the cell viability percentage was observed; consequently, it was shown that anticancer activity resided predominantly in the chloroform extracts (CR4) ($IC_{50} \sim 1$ 75 μg/ml) compared to the rest of the tested compounds (Fig. 2). Hence, the chloroform extract (CR4) was chosen for further purification. Further, the MCF-7 cells were treated with 10 different sub-fractions i.e. CR4(i)-CR4(xiii). The complete cell growth inhibition ($IC_{50} \sim 30$ μg/ml) was noted in the case of the cells treated with the fraction CR4(viii) (Fig. 3A and B). There was no any significant changes were noted in the morphology in control cells; however, the cell shrinkage, loss of cell adhesion, decreased cell density which are the characteristics of apoptotic cell death were evident in MCF-7 cells treated with CR4(viii) (Fig. 4A–D).

The MCF-7 cell line is commonly used in plant bioprospecting studies. The *in vitro* MTT assay provides the basic information (cell death, survival and metabolic activities) of cytotoxicity of any test compound. Moreover, the extract of several species of the genus *Caralluma* have also previously been reported to be cytotoxic in *in vitro* studies (Deepak et al., 1997; Ramesh et al., 1999; Al-Harbi et al., 1994; Al-Faifi et al., 2016; Wen et al., 2016; Ashwini

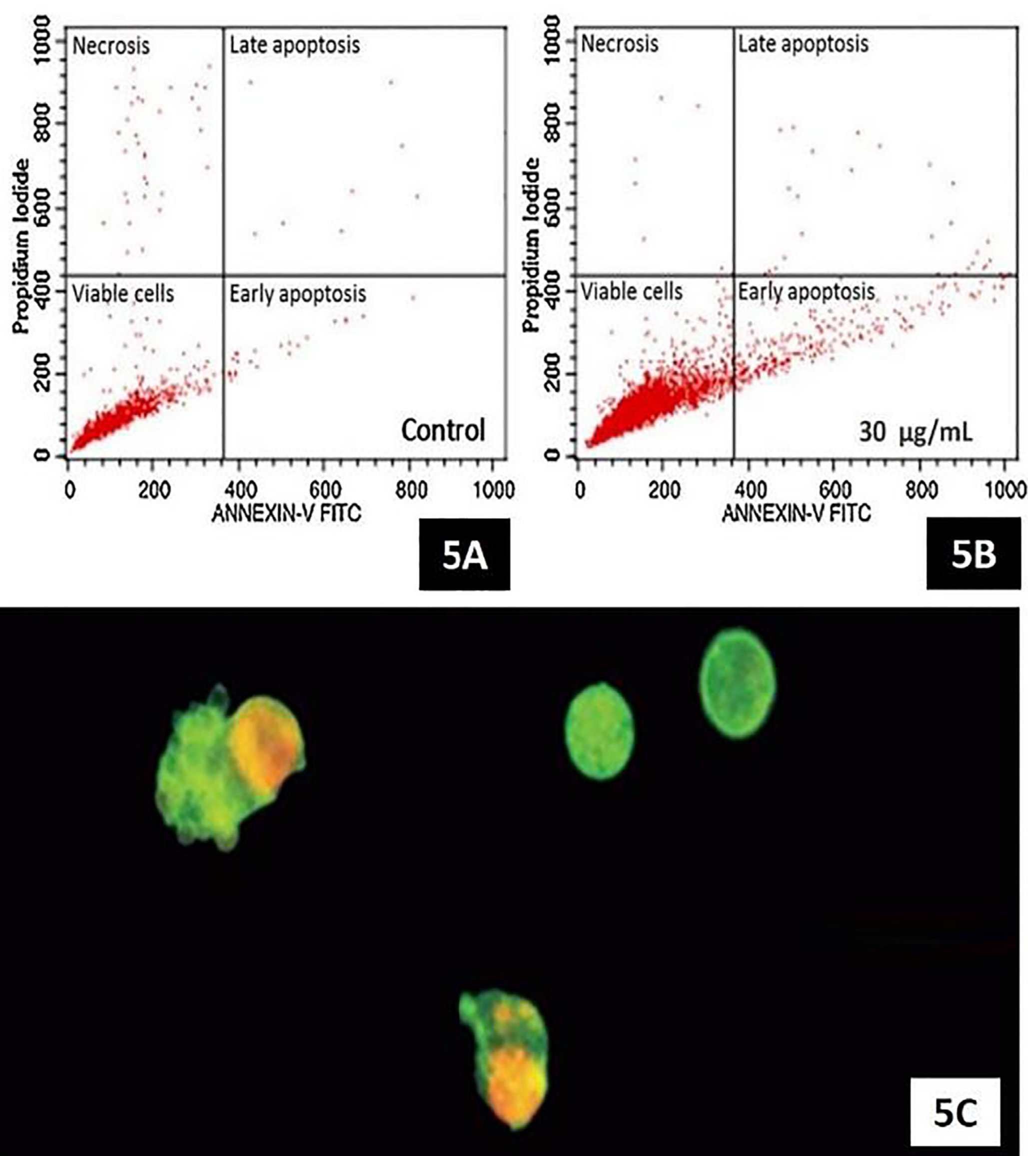


Fig. 5. (A–C) Apoptosis analysis of MCF-7 cells treated with 30 μg/ml of CR4(viii) (B) for 24 h using annexin-V FITC and propidium iodide staining under flow cytometer, Control (A). Apoptotic cells observed by annexin-V FITC and propidium iodide staining under fluorescence microscopy (C).

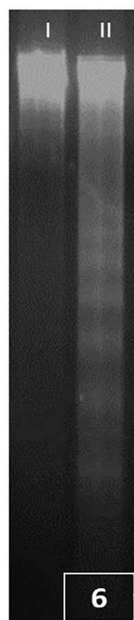


Fig. 6. Analysis of genomic DNA fragmentation in MCF-7 cells after the treatment of 30 μg/ml of CR4(viii) for 24 h (lane II), control (lane I).

et al., 2017). The present results also provide conclusive evidence of the cytotoxic potential in the extract of *C. retrospiciens.*

3.2. The induction of apoptosis by regulating ROS mediated autophagy

The representative dot plots of the annexin V/PI stained MCF-7 cells treated with 30 μg/ml CR4(viii) for 24 h showed 2–3% dead cells or undergoing towards apoptosis, while about 15% cells were found to be in early and late apoptosis stages (Fig. 5A and B). As compared to control, the cells treated with 30 μg/ml CR4(viii) for 24 h showed a ladder pattern DNA band under agarose gel electrophoresis (Fig. 6), an increase in the ROS level as reflected by the enhanced intensity of the green fluorescence (Fig. 7A and B), and more acidic vesicular organelles were observed in the cytoplasmic region (Fig. 8A and B).

The induction of apoptosis in cancer cells by the natural product is one of the useful strategy in the cancer drug development (Hu and Kavanagh, 2003; Kwon et al., 2006; Gonçalves et al., 2010; Lee and Park, 2010). The potential ability of autophagy to modulate cell death makes the process of autophagy an important therapeutic target of cancer (Corcelle et al., 2009). The pure compound from plant extracts have been shown to anticancer activity either ROS independent or through high ROS content/ROS induction (Cohen et al., 2017), the ROS generation triggers the production of autophagy (Scherz-Shouval and Elazar, 2007; Ling et al., 2011).

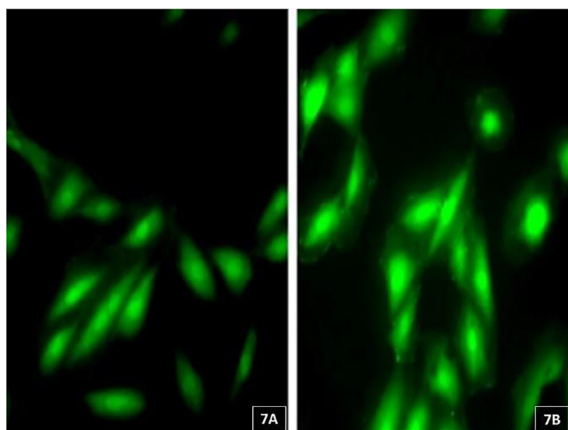


Fig. 7. (A and B) Detection of intracellular ROS by fluorescence microscopy. Control (A), cells after the treatment of 30 μg/ml of CR4(viii) for 24 h (B).

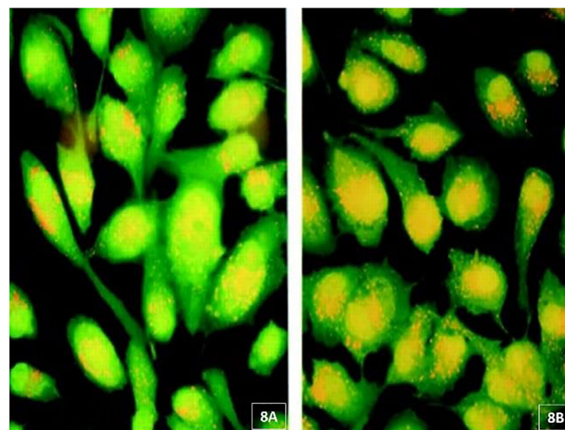


Fig. 8. (A and B) Detection of autophagy by fluorescence microscopy. Control (A), cells after the treatment of 30 μg/ml of CR4(viii) for 24 h (B).

3.3. Spectroscopic characterization

From the active fraction CR4(viii), an amorphous powder (20 mg) was isolated. It appeared as a gray spot after spraying with *p*-anisaldehyde–sulfuric acid reagent. 1D and 2D NMR experiments proved that the isolated compound represents a sugar moiety of a bioside (Table 1). Based on ^{1}H NMR spectrum (Table 1), which showed two signals for two anomeric protons at δ_H 4.69 and 4.71 (Table 1) connected to their corresponding anomeric carbons in the HSQC spectrum (Table 1) at δ_C 99.3 and 102.3 respectively, it was confirmed that the isolated compound is a sugar consisting of two molecules (Table 1). In addition, two methyl doublets

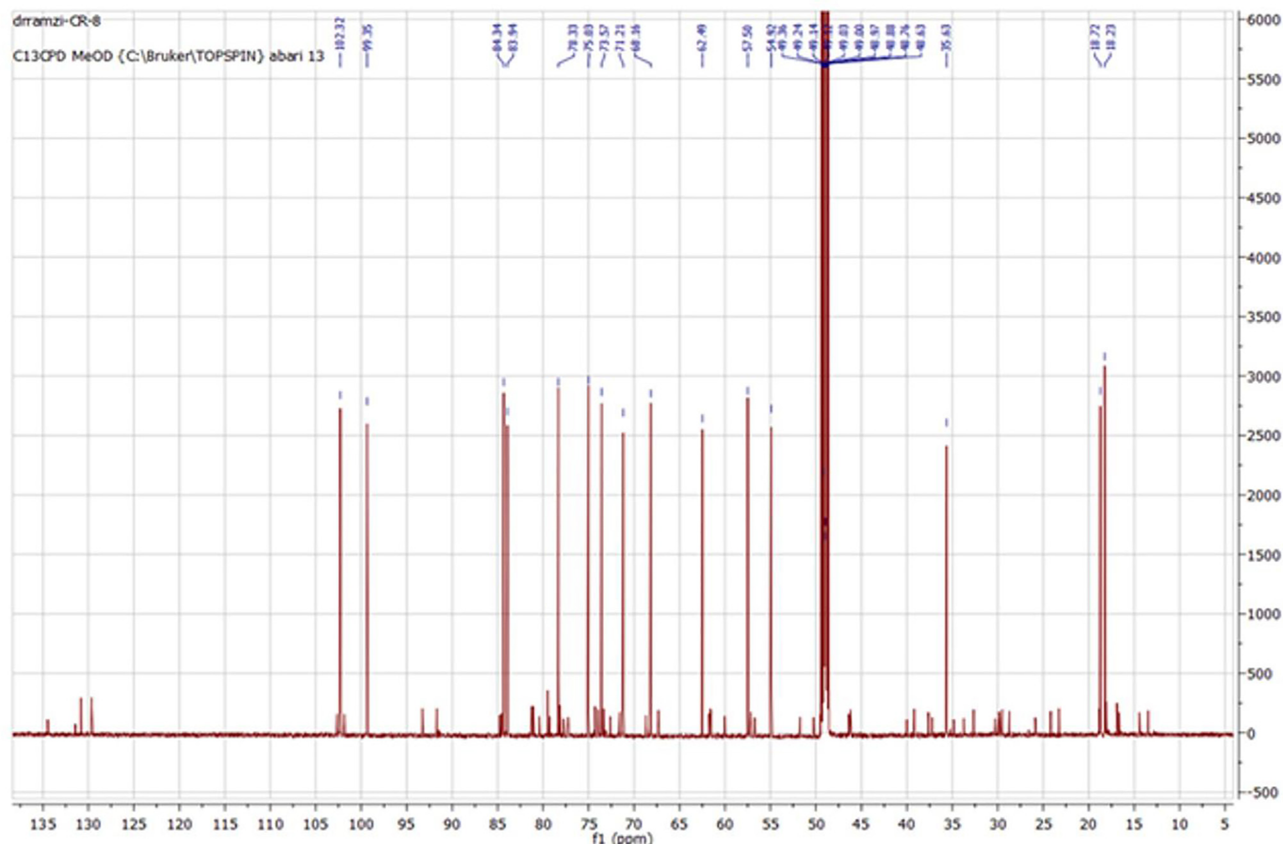


Fig. 9. ^{1}H NMR spectra of the isolated compound in Methanol-d_4.

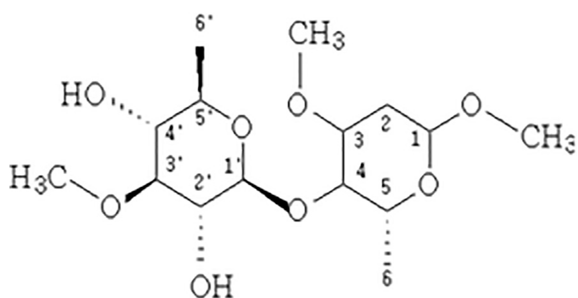


Fig. 10. Chemical structure of methyl β-lilacinobioside [CR4(viii)].

appeared at δ_H 1.32 and 123 directly attached to their corresponding carbons at δ_C 18.7 and 18.1 respectively (Table 1) indicating the presence of 2 sugars of 6-deoxy hexoses. The DEPT spectrum showed the presence of a C-atom of a methylene at δ_C 35.6 connecting to two H-atoms at δ_H 2.16 and 1.52 indicating that one of the sugars is dideoxy hexose. Furthermore, three methyl singlets (3H, δ_H 3.39, 3.30 and 3.60) connected to quaternary carbons at 57.5, 55.0 and 62.5 respectively, indicating the presence of three methoxy groups (OCH_3) (Table 1).

The comparison of the ^{1}H and ^{13}C NMR data of sugar regions of the isolated compound (Fig. 9) with data reported in literature (Braca et al., 2002; Abdel-Sattar et al., 2007, 2008) confirmed the presence of one 1-methyl-cymarose and one 6-deoxy-O-Me-allose (Allomethylose) without connecting to any aglycone. The literature survey indicated that this compound is methyl β-lilacinobioside (Fig. 10) and reported here for the first time from *C. retrospiciens*.

The noticeable anticancer activity in the beginning was predicted that it could be attributed to one of the pregnan glycosides which were also previously isolated from different plant species, including species of *Caralluma* (Adnan et al., 2014; Hussain et al., 2015; Ibrahim et al., 2015). Interestingly, our results showed that the most anticancer active compound was attributed to this isolated sugar-bioside (methyl β-lilacinobioside). Similar sugar molecules namely 2-deoxy-D-glucose showed anticancer activity against head and neck carcinoma cells (Vibhuti et al., 2013). Vibhuti et al. (2013) reported that 2-Deoxy-D-glucose could be considered as a potential therapeutic agent that induces cell death (which could be linked to induced oxidative stress) selectively in tumors with p53 mutations (particularly in the proline rich region). In addition, it is reported that 2-deoxy-D-Glucose causes cytotoxicity in cancer cells by disrupting thiol metabolism. It is an effective component in therapeutic strategies. It targets the metabolism of cancer cells with glycolysis inhibitory activity (Oladghaffari et al., 2015). Moreover, the genus *Caralluma* contains cardenolides, flavone glycosides and esterified olyhydroxypregnane glycosides. The cardenolides, flavone glycosides and esterified olyhydroxypregnane glycosides have also previously been reported to have antitumor activity (Deepak et al., 1997; Ramesh et al., 1999; Al-Harbi et al., 1994; Al-Faifi et al., 2016; Wen et al., 2016; Ashwini et al., 2017).

3.4. Molecular docking simulation of methyl β-lilacinobioside with key proteins involved in the cell cycle and DNA replication

The tumour-suppressor protein p53 (-the guardian of the genome) is one of the main targets for cancer therapy (Joerger et al., 2015; Parrales and Iwakuma, 2015; Semenova et al., 2015). Tyrosine phosphorylation is an important signaling mechanism, is controlled by protein-tyrosine phosphatases (PTPs). The normal expression of PTP regulates cell proliferation and tumor

Table 2
The predicted binding energy and Docking Score against each target protein.

PDB ID	Predicted binding energy (kJ/mol)	Docking score
1T8I	−39.21	−6.442
1ZXM	−36.975	−8.623
2O2F	−26.718	−4.57
4RH5	−23.68	−3.284
5AB9	−30.883	−4.669

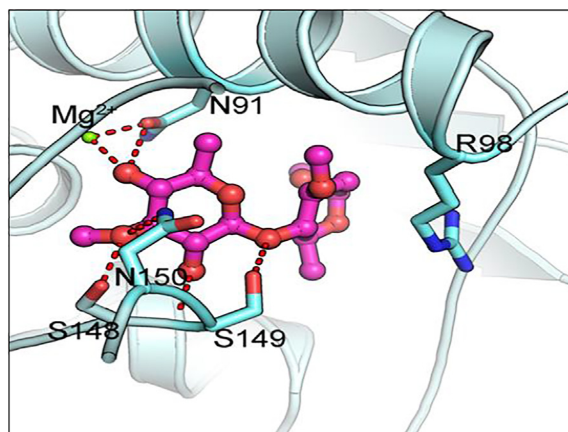


Fig. 11. The predicted binding pose of methyl β-lilacinobioside (magenta stick ball model) to topoisomerase II (cyan cartoon). Red dash line represent hydrogen bonds or ionic bond.

suppression, while the over expression of PTP leads to cancer (Ostman et al., 2006). Both the DNA topoisomerases I and II plays very important roles in structure and metabolism of DNA (Phosrithong and Ungwitayatorn, 2010). BCL-2 is an anti-apoptotic oncoprotein which affects neoplastic cell proliferation by preventing the cell death (Reed, 1994). The computational approaches which 'dock' small molecules with the structures of macromolecular targets protein provides information of drug receptor interaction, are being widely used in hit identification and lead optimization (Kitchen et al., 2004). Since the experimental results in the present study indicates that methyl β-lilacinobioside induces apoptosis in MCF-7 cells by regulating ROS mediated autophagy. Hence, we have selected target proteins Topoisomerase I (PDB ID: 1T8I), Topoisomerase II (PDB ID: 1ZXM), Bcl-2(PDB ID: 2O2F), protein tyrosine phosphatases and PTPs (PDBID: 4RH5) and p53 PDB ID: 5AB9) for molecular docking. The docking results (Table 2) suggests that topoisomerase II could be the potential binding target of methyl β-lilacinobioside with the binding affinity of −36.975 kJ/mol. As shown in Fig. 11, the hydrogen bond network and an ionic bond formed between the methyl β-lilacinobioside and residues of S148, N150, S149, N9 and magnesium ion could contribute the great potency of the compound. Moreover, the computational result indicates that the bioactive compound methyl β-lilacinobioside could bind to topoisomerase II with relatively high binding affinity, contributing to the major role of inhibiting the cancer cells.

Conflict of interest

The authors report no conflicts of interest in this work.

Acknowledgement

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the research group project No RGP-195.

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