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Cucurbitacins-type triterpene with potent activity on mouse embryonic fibroblast from *Cucumis prophetarum*, cucurbitaceae

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Abstract

Background: Higher plants are considered as a well-known source of the potent anticancer metabolites with diversity of chemical structures. For instance, taxol is an amazing diterpene alkaloid had been lunched since 1990. **Objective:** To isolate the major compounds from the fruit extract of *Cucumis prophetarum,* Cucurbitaceae, which are mainly responsible for the bioactivities as anticancer. **Materials and Methods:** Plant material was shady air dried, extracted with equal volume of chloroform/methanol, and fractionated with different adsorbents. The structures of obtained pure compounds were elucidated with different spectroscopic techniques employing 1D (^{1}H and ^{13}C) and 2D (COSY, HMQC and HMBC) NMR (Nuclear Magnetic Resonance Spectrometry) and ESI-MS (Eelectrospray Ionization Mass Spectrometry) spectroscopy. The pure isolates were tested towards human cancer cell lines, mouse embryonic fibroblast (NIH3T3) and virally transformed form (KA3IT). **Results:** Two cucurbitacins derivatives, dihydrocucurbitacin B (**1**) and cucurbitacin B (**2**), had been obtained. Compounds **1** and **2** showed potent inhibitory activities toward NIH3T3 and KA31T with IC $_{50}$ 0.2, 0.15, 2.5 and 2.0 µg/ml, respectively. **Conclusion:** The naturally cucurbitacin derivatives (dihydrocucurbitacin B and cucurbitacin B) showed potent activities towards NIH3T3 and KA31T, could be considered as a lead of discovering a new anticancer natural drug.

Keywords: *Cucumis prophetarum*, cucurbitacin B, dihydrocucurbitacin B, mouse embryonic fibroblast and virally transformed form

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Introduction

Generally, there are two different approaches used for discovering of antitumor compounds; bio-chemical approach and target-based approach. The first approach has gained a significant attention in the last decades. [1] This resulted in discovery of antitumor agents. For instance, Food and Drug Administration (FDA) approved imatinib mesilate as a first-line treatment for chronic myelogenous leukemia. [2] The use of high-throughput screening is aimed at discovery of anticancer agents employing mouse embryonic fibroblast (NIH3T3) and virally transformed form (KA3IT) cells. The selection of these cells based on that they are adherent, easily manipulated, and well characterized. [3],[4]

The Cucurbitaceae are mostly known prostrate or climbing herbaceous annuals plants comprising about 125 genera and 960 species, includes the melons and gourd crops like cucumbers. The family is predominantly distributed around the tropics, where edible fruits are grown. The diversity of the cucurbitacins' activities, especially cytotoxicity and antifeedants, is a good evidence for further investigations. [5],[6],[7] Recently, they were exploited for their antitumor properties, differential cytotoxicity toward renal, brain tumor, and melanoma cell lines, [8] inhibition of cell adhesion, [9] and finally, antifungal effects. [10] A computer survey includes science finder data base, indicated that a number of cucurbitacins were isolated from genus *Cucumis.* For example, cucurbitacins were isolated from *Cucumis prophetarum:* cucurbitacin (B, E, I, O, P, and Q1); dihydocucurbitacin (D and E), isocucurbitacin (B, D, and E) and dihydroisocucurbitacin (D and E). [11],[12],[13],[14],[15]

In continuation of our research program which interested in the isolation of the bioactive secondary metabolites from marine macro organisms or higher plants, collected from Saudi Arabia. [16],[17],[18] The fruits of *Cucumis prophetarum* L., belongs to family Cucurbitaceae, wild plant growing in the desert of Makah, 80 km from Jeddah Saudi Arabia. The total extract (Chloroform: Methanol [1: 1]) had been fractionated using different chromatographic techniques, led to purification of two cucurbitacins derivatives; dihydocucurbitacin B (**1**) and cucurbitacin B (**2**). The compounds 1 and 2 [Figure 1] showed potent inhibitory activities toward mouse embryonic fi broblast (NIH3T3) and virally transformed form (KA3IT) cells with IC_{50} 0.2, 0.15, 2.5, and 2.0 µg/ml, respectively.

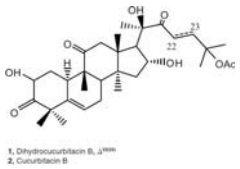

Figure 1: Structures of dihydocucurbitacin B (1) and cucurbitacin B (2)

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Materials and Methods

General procedure

Chromatographic material: silica gel type 60-120 mesh.was used form Column Chromatography (CC). Silica gel GF_{254} was used for Thin Layer Chromatography (TLC). Finally, silica gel 60 F_{254} was used for Preparative Thin Layer Chromatography (PTLC). Electron impact mass spectra were determined at 70 ev on a Kratos EIMS-25 instrument. All NMR spectra were recorded on (1D and 2D) NMR spectra were recorded on a Varian VI-500 MHz spectrometer. LC-CIMS was performed using an API 2000, LC MS/MS from Applied Biosystems/MDS Sciex. Bruker Avance 300 DPX and 500 DRX spectrometers in $CDCl_3$. Chemical shifts are given δ (ppm) relative to TMS as internal standard. The spray reagent used is: Anisaldehyde-sulphuric acid. A freshly prepared solution was made by adding concentrated sulphuric acid (1 ml) to a solution of anisaldehyde (0.5 ml) in acetic acid (50 ml).

Plant material

Cucumis prophetarum L. was collected from wild plants growing from the desert of Makah, Saudi Arabia. The fresh fruits were separated, air-dried, and powdered. A voucher sample was deposited at the Chemistry Department, Faculty of Science King Abdulaziz University, Jeddah, Saudi Arabia.

Extraction and purification

The fruits of *C. prophetarum* (500 gm) were extracted twice by chloroform: methanol (1:1) at room temperature. The extract was concentrated under reduced pressure and led to yellowish brown residue (25 gm). This material was chromatographed on a column of silica gel. The total extract was fractionated by NP silica (500 gm, 80 cm X 2.5 cm) employing pet. Ether/$CHCl_3$ /MeOH (50 ml each fraction). The fractionation was followed by TLC using anisaldehyde-sulphuric acid as spraying reagent. The fraction eluted by chloroform: methanol (9: 1) was collected and purified by Sephadex LH 20 using methanol: chloroform (3:1) followed by preparative TLC silica gel and chloroform: Methanol (9+1), led to **1** (200 mg) and **2** (100 mg)

Cytotoxicity bioassays

Cytotoxic assays [19],[20] were performed using two proliferating mouse cell lines, a normal fibroblast line NIH3T3 and a virally transformed form KA3IT. Samples of extract or pure compound (5 mg) were dissolved in 62.2 µl of Dimethyl sulfoxide (DMSO), and working solutions made by diluting 20 µl of the DMSO solution into 2 ml of sterile medium (Dulbecco's modified Eagle's medium, Sigma Chemical Co. St. Louis, MO, USA). Two-fold or 2.5-fold dilutions of the extracts of pure compounds from 200 µg/ml to 0.5 µg/ml were prepared in triplicate in the wells of 96-well culture trays (Falcon Micro Test III, # 3072, Becton Dickinson Labware, Lincoln Park, NJ, USA) in 200 µl of medium containing 5% (v/v) calf serum (Hyclone Laboratories, Logon, Utah, USA). Inoculums of 2 \ 103 cells were added to each well in a 100 µl aliquot of 10% calf serum in medium. The 96-well trays of cells were cultured under standard conditions until uninhibited cultures (control) became confluent. The contents of the wells were decanted, and each cell layer washed with a small amount of the medium. The wells were filled with formal saline (3.7% w/v formaldehyde in 0.15 ml NaCl), and allowed to stand at room temperature for at least 30 minutes. The trays was washed with tap water, and attached cells stained by adding two drops of 0.5% (w/v) crystal violet solution in 20% (v/v) aqueous methanol added to each well. The trays was washed with tap water, and the IC_{50} estimated visually as the approximate concentration that causes 50% reduction in the number of stained cells adhering to the bottom of the wells.

Results and Discussion

The chromatographic purification of the total extract obtained from fruits of *C. prophetarum* resulted in the isolation of two compounds belong to cucurbitacins-type triterpens; dihydocucurbitacin B (**1**) and cucurbitacin B (**2**). They identified by employing 1D (^{1}H and ^{13}C) and 2D (COSY, HMQC and HMBC) NMR, and ESI-MS spectroscopy. The pure isolates were tested towards human cancer cell lines, mouse embryonic fibroblast (NIH3T3) and virally transformed form (KA3IT). The compounds **1** and **2** showed cytotoxic activities toward NIH3T3 and KA31T with IC_{50} 0.2, 0.15, 2.5, and 2.0 µg/ml, respectively.

The structure elucidation of 1, commenced when the molecular formula of $C_{32}H_{48}O_{8}$ was established by LC-ESI-MS at 583 $[M^{+} + Na]$. This result was validated by HRESIMS, m/z 583.3245 $[M+ Na]^{+}$. The ^{13}C NMR spectra (^{1}H decoupled and DEPT) of 1 showed 32 resonances attributable to 9 X CH_3, 7 X CH_2, 6 X CH, and 10 X C [Table 1]. Three of the eight elements of unsaturation, as indicated by the molecular formula of 1, deduced to be a carbonyl group appeared at δ_c 213.9, 212.9, and 212.0, for C-3, C-11, and C-22, respectively, and an olefinic proton at δ_c 5.79 (J= 5.5) assigned for H-6 of the double bond at $\Delta^{(5)6}$. The molecule, thus, has four rings. As the ^{1}H and ^{13}C NMR data enabled all but three of the hydrogen atoms within 1 to be accounted for, it was evident that the remaining three protons were present as part of a hydroxyl functions. After association of all the protons with directly bonded carbons via 2D NMR (HMQC) spectral measurements, it was possible to deduce the structure of 1 by interpretation of the ^{1}H- ^{1}H COSY and ^{1}H - ^{13}C HMBC spectra. From the ^{1}H- ^{1}H COSY spectrum of 1, a ^{1}H- ^{1}H spin system between H-2 and H_2-1 and between H_2-1 and H-10 was observed. Long-range C-H correlations observed between the resonances of H-8 and those of, C-6, C-7, C-9, C-10 and C-19; between H_2-1 and C-2, C-3, C-5, C-8, C-9, and C-19; between H-2 and C-1, C-3, C-4, and C-10; between H-6 and C-4, C-5, C-7, C-8, and C-10; between H_3-29 and C-3, C-4, and C-5 and between H_3-30 and C-3, C-4, and C-5 established ring A and B, which are fused together. HMBC correlations, this time observed between H_3-28 and C-8, C-14, and C-15, as well as correlations between H-16 and C-13, C-14, C-15, and C-17, and also correlations between H_3-18 and C-12, C-13, C-14, C-17 were observed. Long-range C-H correlations observed between the resonances of H-12 and C-11, C-13, C-17, were observed. Ring C was established by deduction through the previous data. From the ^{1}H- ^{1}H COSY spectrum of 1, a ^{1}H- ^{1}H spin system between H_2-15 and H-14 and H_2-16 and between H_2-16 and H-17 were observed indicated the C-C bond between C-14 to C-15 and C-15 to C-16 and C-16 to C-17. Ring D was established based on ^{1}H- ^{1}H COSY and ^{1}H - ^{13}C HMBC spectra. The main skeleton of 1 was established as steroidal derivative. From ^{1}H- ^{1}H COSY, correlations were observed between H_2-23 and H_2-24. Extensive investigation of the HMBC correlation between H_3-21 and C-20 and C-22 (C=O) was observed. Long range correlations between C-22 and H_2-23 and H_2-24 led to establishing the side chain as iso-heptane derivative. This side chain is attached to the steroidal nucleus the connection between C-17 and C-20 based on HMBC correlations, between H_3-21 and C-17 and C-20. The positions of the three hydroxyl groups were assigned by examining the correlation obtained from the ^{1}H and ^{13}C NMR chemical shifts and supported by the ^{1}H- ^{1}H COSY, ^{1}H - ^{13}C HMBC spectral data. The remaining from the structure of 1, is the acetate moiety, which connected to C-25 based on the ^{13}C chemical shift. The spectral data of 1, is well fitted with published data with dihydocucurbitacin B, which was isolated from *Bryonia cretica*.[15] It is isolated from the first time from C. prophetarum of Saudi source.

Table 1: 1H ($CDCl_3$, 500 MHz) and $^{133,\ 125}$ MHz) spectral data of compound 1 and 2a

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The structure of **2** was constructed based on the molecular formula of $C_{32}H_{46}O_{8}Na$, which abstracted from the ESI-MS m/z 581 $[M^{+}+Na]$ and HRESI MS m/z 581.3114$[M^{+} + Na]$. After extensive studying of the ^{1}H and ^{13}C NMR spectral data indicated that the doublet at 4.42 (J =13.0, 6.0) and quartet at 4.35 (J = 8.0) were assigned for H-2 and H-16, respectively. A normal H_2-1 shift at 2.30 (1H, ddd, J = 13.0, 6.0, 3.0 Hz), 1.24 (1H, q, I = 13.0 Hz). An acetate signal was clear from ^{1}H and ^{13}C shifts at 2.08 and 170.2 ppm, respectively. The spectra also exhibited two double bond one

at 5.79 (d, J =5.5) and the other at 7.05(1 H, d, J = 16.0 Hz), 6.48 (1 H, d, J = 16.0 Hz), which were assigned for C-6 and C_{23} =C_{24} , respectively. The structures also have three carbonyl groups by ^{13} C NMR at 213.0, 212.2, and 202.4, for C-3, C-11 and C-22, respectively. The structure also has eight methyls at δ 1.57, 1.55, 1.44, 1.36, 1.34, 1.29, 1.08, and 0.98 ppm. It was clear from the spectral data of **2**, that it is cucurbitacin B, which was published before. [11],[12]

The potent activities of compounds **1** and **2** toward NIH3T3 and KA31 will open the gate for new era of discovering anticancer drug especially for the steroidal compounds, which lead to discovering of new anticancer natural drugs.

Conclusion

This manuscript investigates the fractionation of Cucumis prophetarum, Cucurbitaceae aiming at finding or discovering a bioactive metabolites. This study afforded dihydrocucurbitacin B (**1**) and cucurbitacin B (**2**) [Figure 1]. The structures of the two compounds were elucidated by spectroscopic analyses including: 1D (^{1} H and ^{13} C) and 2D (COSY, HMQC and HMBC) NMR, and ESI-MS spectroscopy. The cytotoxicity of **1** and **2** , towards human cancer cell lines, mouse embryonic fibroblast (NIH3T3) and virally transformed form (KA3IT) cells, has been estimated. The compound **1** and **2** had potent inhibitory activities toward NIH3T3 and KA31T with IC_{50} 0.2 and 0.15, 2.5, and 2.0 µg/ml, respectively.

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Figures

[Figure 1]

Tables

[Table 1]

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