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Original Research

Investigation of *in vitro* Wound Healing Activity of *Polygonatum orientale* Desf. Rhizome

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Abstract

In this study, we carried out a bioassay-guided fractionation of the most active extract *Polygonatum orientale* Desf. rhizome, in order to isolate and identify the fraction/s or compound/s responsible for wound healing activity. The wound healing process considered via scratch wound assay on NIH-3T3 fibroblasts. The results showed that the methanol extract and its fractions A5 and A6 showed excellent wound healing effect and were rich of bioactive glycoside compounds. Fraction A6 was selected for further fractionation and two sub-fractions B5 and B6 showed acceptable wound healing on fibroblasts. B5-P (sucrose) and B6-P were isolated as two active compounds from theses fractions that significantly reduced wound area, without any toxicity at very low concentrations (50-200 ng/mL). These results supported the traditional use of *P. orientale* rhizome for wounds treatment and showed that the accelerated wound healing activity of the rhizome is due to the presence of bioactive polar compounds.

Keywords: *Polygonatum orientale* rhizome; Fibroblasts; 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay; Scratch wound healing assay; Sucrose

Introduction

Iranian *Polygonatum* is a perennial herbaceous flowering plant with the scientific name of *Polygonatum orientale* Desf. (Asparagaceae). The common name of this plant is "Oriental Solomon's Seal", which is known as "Shaghaghol" in Iran. This genus usually grows in humid or semi-humid and shady forests of the northern region in Iran [1,2]. This plant has hard, bony, tubular, and yellowish cream color rhizomes with the thickness of a fingertip. The rhizomes have a slightly pungent taste and are used for making jam in Iran [3]. In Iranian traditional medicine, the rhizomes have been used for treatment of wounds, kidney stones, gout, rheumatoid arthritis, and diabetes [4,5]. Pharmacological studies have indicated that the anti-inflammatory, antioxidant, anti-aging, anti-diabetic, and anti-cancer effects of *Polygonatum* species are related to a variety of biologically active substanc-

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es such as flavonoids, homoisoflavanones, steroidal saponins, triterpenoid saponins, polysaccharides, and lectins [6,7]. Studies on *P. orientale* are very limited and just a few glycosylated saponin compounds have been isolated from the rhizome, including spirostanol glycoside, sceptrumgenin glycoside (polysceptroside), and akyrogenin glycoside (spiroakyroside) [8]. In a recent study, for the first time, the steroid compound 12α-hydroxystigmast-4-en-3-one and a fatty acid were isolated from *P. orientale* rhizome [5].

Wound healing is a dynamic process and a normal biological response to injury, which occurs through different overlapping phases, including hemostasis, inflammation, proliferation and maturation or remodeling [9]. To date, several medicinal herbs have been reported to show wound healing activity via several mechanisms, such as modulation in wound healing, fighting against infection, improving collagen deposition, and increasing fibroblasts and fibrocytes [10]. Migration and proliferation of fibroblasts during the proliferation phase is an important criterion for in vitro investigation of the wound healing mechanism [11]. Regarding the traditional uses of *P. orientale* rhizome, for the first time, we considered the wound healing activity of it, through bioassay guided fractionation procedures to define active fractions and compound/s.

Materials and Methods

General experimental procedures

NIH-3T3 mouse embryo fibroblast cell line was obtained from the National Cell Bank of Iran (NCBI), Pasteur Institute of Iran. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), Penicillin/streptomycin (Pen/Strep) and Trypsin were purchased from Gibco (Gaithersburg, USA). Phosphate Buffer Saline (PBS) was purchased from Invitrogen (Carlsbad, USA). 3-4, 5 dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT), trypan blue, formaldehyde, triton, allantoin, dimethyl sulfoxide (DMSO) and DAPI (4′,6- Diamidino-2-phenylindole dihydrochloride) were purchased from Sigma-Aldrich (Munich, Germany). All of the NMR spectra were obtained using a Varian-INOVA 500MHz spectrometer and are reported in ppm relative to tetramethylsilane (TMS) as internal standard. EI-MS spectrum was recorded on a quadrupole mass spectrometer (Agilent Technologies) with electron ionization at 70 eV. Melting point was determined on a Reichert-Jung hot-stage microscope. Silica gel (35-70 mesh) was used as the stationary phase in vacuum liquid chromatography (VLC) and 26 G (230-400 mesh) (Merck) was employed for loading the extract on VLC. Sephadex LH-20 (Fluka) was used for size exclusive chromatography (SEC) and thin layer chromatography (TLC) was performed on silica gel (Merck) pre-coated aluminum sheets.

Plant material and preparation of the extracts

Polygonatum orientale Desf. rhizomes were collected from Chalus toward Marzanabad, Mazandaran Province, Iran in Jun 2019. They were validated by prof. G.R. Amin and the voucher specimen PMP-1241 was deposited at the Herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. The rhizomes (Figure S1) were dried in shade, pulverized by an electric blender, and then passed through a 35-mesh sieve. From the powdered rhizomes (150 g), various extracts were prepared stepwise using increasingly polar solvents (chloroform, ethyl acetate, and methanol) by maceration for 48 h on a shaker. Each solvent was filtered and concentrated using a rotary evaporator at 40 °C to dryness and the dried extracts were stored at 4 °C for the next step. All extracts were further subjected to MTT assay and scratch wound assay according to the procedures described later.

Bioassay-guided fractionation and compound isolation

Based on the assay, the methanol extract of *P. orientale* rhizomes was subjected to bioassay-guided procedures. 20 g of the MeOH extract was fractionated using VLC packed with silica gel. The elution was performed with a stepwise gradient of $CHCl_3$–MeOH (10:0 to 1:9, v/v) that obtained 19 primary fractions. These fractions were monitored with TLC using $CHCl_3$–MeOH (2:1, v/v) as a mobile phase and the TLC zones were visualized after spraying with anisaldehyde-sulfuric acid (AS) reagent and heating at 100 °C for 5 min. Finally, these fractions were grouped into six fractions (A1–A6) according to the similarity of TLC profile. The cell viability and cell migration of six fractions were determined through MTT test and scratch wound assay, respectively. The most bioactive fraction A6 (1.5 g) was subjected to further purification by SEC using Sephadex LH-20 and methanol (100%) as the stationary and mobile phases which resulted in 18 primary sub-fractions. After monitoring fractions on TLC, using $CHCl_3$–MeOH–H_2O (15:10:4, v/v) as the mobile phase and spraying AS reagent, the fractions were pooled according to the similarity of the TLC profile to obtain six sub-fractions (B1–B6). Sub-fraction B5 and B6 showed acceptable wound healing on NIH-3T3 fibroblast cells and two bioactive B5-P (30 mg) and B6-P (4.5 mg) were purified by crystallization in MeOH from theses fractions, respectively. These compounds showed green/grayish color spot on TLC after spraying AS reagent. The fractionation procedure of *P. orientale* rhizome was summarized in the supplementary (Figure S2).

Cell culture and cell viability assay

The viability of NIH-3T3 fibroblasts was assessed by

MTT assay after being exposed to the samples [12]. The cells were cultured in complete culture medium DMEM with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin (pen/strep), and 100 IU/mL penicillin. The cell cultures were placed in an incubator at 37 °C under 5% CO2 and 97% humidity. The NIH-3T3 cells were seeded into 96-well plate at a density of 1×104 cells/well. They treated with varying concentrations of the extracts (5–200 ng/mL), fractions/sub-fractions (5–100 ng/mL) and compounds (5–200 ng/mL) that prepared in DMSO (less than 1% v/v in every well). After incubation for 48 hours, the medium was washed twice with phosphate buffer and then 50 µL of MTT (5 mg/mL) was added. The cells were incubated for another 3 hours at 37 °C. In the next step, purple crystals of formazan were dissolved in DMSO for 15 min and the absorption was read at 540 nm using microplate reader (Synergy, Bio Tek Instruments Inc. Germany). Then the background absorbance of the plates at 690 nm was subtracted from 540 nm. Data are represented as percent of control.

In vitro scratch-wound healing assay

The migration ability of NIH-3T3 fibroblasts into the wounded area was assessed using scratch assay method. The cells were seeded at 1×104 cells/well in 96-well plate along with DMEM, 2% FBS and 1% pen/strep. To prevent cells from going to proliferation and division states, a lower percentage of FBS was used in the culture medium to only examine the migration properties of fibroblasts. For formation a monolayer cell base with approximate 75% confluency, 24 hours incubation at 37 °C was done. Then, with a sterile 100 µL pipette tip, a linear scratch was created. After that, the cells were washed using PBS in order to remove residues and then exposed to safe dose of extracts/fractions/sub-fractions and different concentrations of active compound (5-200 ng/mL) for 48 hours [13]. Allantoin at 50 µg/mL concentration was used as a positive control [14]. In the following, the fixation of cells was performed with 3.7% formaldehyde for 10 min, and then the cells were permeated with 0.2% Triton for 5 minutes and staining by DAPI for 1 min. Finally, images were taken using Olympus BX51 fluorescence microscope (Japan) and the changes in the gap area were quantitatively measured using Image J software.

Statistical analyses

The statistical significance of the data was analyzed by one-way ANOVA, Tukey post hoc test with "Instat" software and the significance level was set at $P \leq 0.05$. The test results were expressed as mean ± SEM in Microsoft Office Excel 2010 software.

Results

Bioassay-guided fractionation and isolation sucrose

The nontoxic concentration values in MTT assay for MeOH (10 ng/mL), EtOAc (10 ng/mL), and CHCl3 (50 ng/mL) were chosen for in vitro scratch-wound healing assay on NIH/3T3 fibroblasts. Among the extracts treated cells, the MeOH extract showed the most cell migration rate (Figure 1). The MeOH extract decreased wound area significantly ($P < 0.01$) down to 80.35% of the control group. Due to the higher wound healing activity of MeOH extract, following investigation was carried out on it. The VLC of the MeOH extract yielded six fractions (A1–A6) and their effects on fibroblasts viability and migration were shown in figure 2. According to the results, A5 and A6 fractions at nontoxic concentration (5 ng/mL) were able to decrease significantly ($p < 0.01$) the wound area down to 81.55% and 81% of control after 48 hours, respectively. The TLC profiles of fractions A5 and A6 were visualized just after using reagent spraying AS. Based on the TLC profile, in the first step, A6 fraction was selected for further bioassays (data not shown). The SEC of A6 fraction yielded six sub-fractions (B1-B6). Figure 3 showed the effects of sub-fractions B1-B6 on fibroblasts viability and migration. As shown in figure 3b and c, the B5 and B6 have the best effects on the cells migration and wound area was reduced significantly ($p < 0.05$) down to 84.18% and 83.51%, respectively. Due to the TLC result, two compounds B5-P (30 mg) and B6-P (4.5 mg) were obtained through crystallization in the form of white crystals from sub-fractions B5 and B6, respectively. As B5-P and B6-P had no toxic effect on fibroblasts at concentrations 5–200 ng/mL, these concentrations were used for scratch wound assay. The results showed that B5-P significantly decreased the wound area down to 74.88% ($p < 0.001$), 79.12% ($p < 0.01$), and 83.17% ($p < 0.05$) within 48 hours at concentrations of 50, 100, and 200 ng/mL, respectively (Figure 4). In addition, B6-P was determined as a bioactive compound in the fibroblasts migration by decreasing wound area in all treatment groups, however, at 5 ng/mL the wound area was significantly decreased up to 79.32 % ($p < 0.01$) (Figure 6). Allantoin, as a positive control, reduced the wound area up to 63.33% at a concentration of 50 µg/mL ($p < 0.001$).

Structural elucidation of the active compound

The compound B5-P was obtained as white odorless crystals, mp 191.3–193.3 °C: Its molecular formula $C_{12}H_{22}O_{12}$ was identified by EI-MS, ^{13}C NMR and ^{1}H NMR. The EI-MS analysis revealed a small peak at m/z 341 (M-1) corresponding to its ion molecular

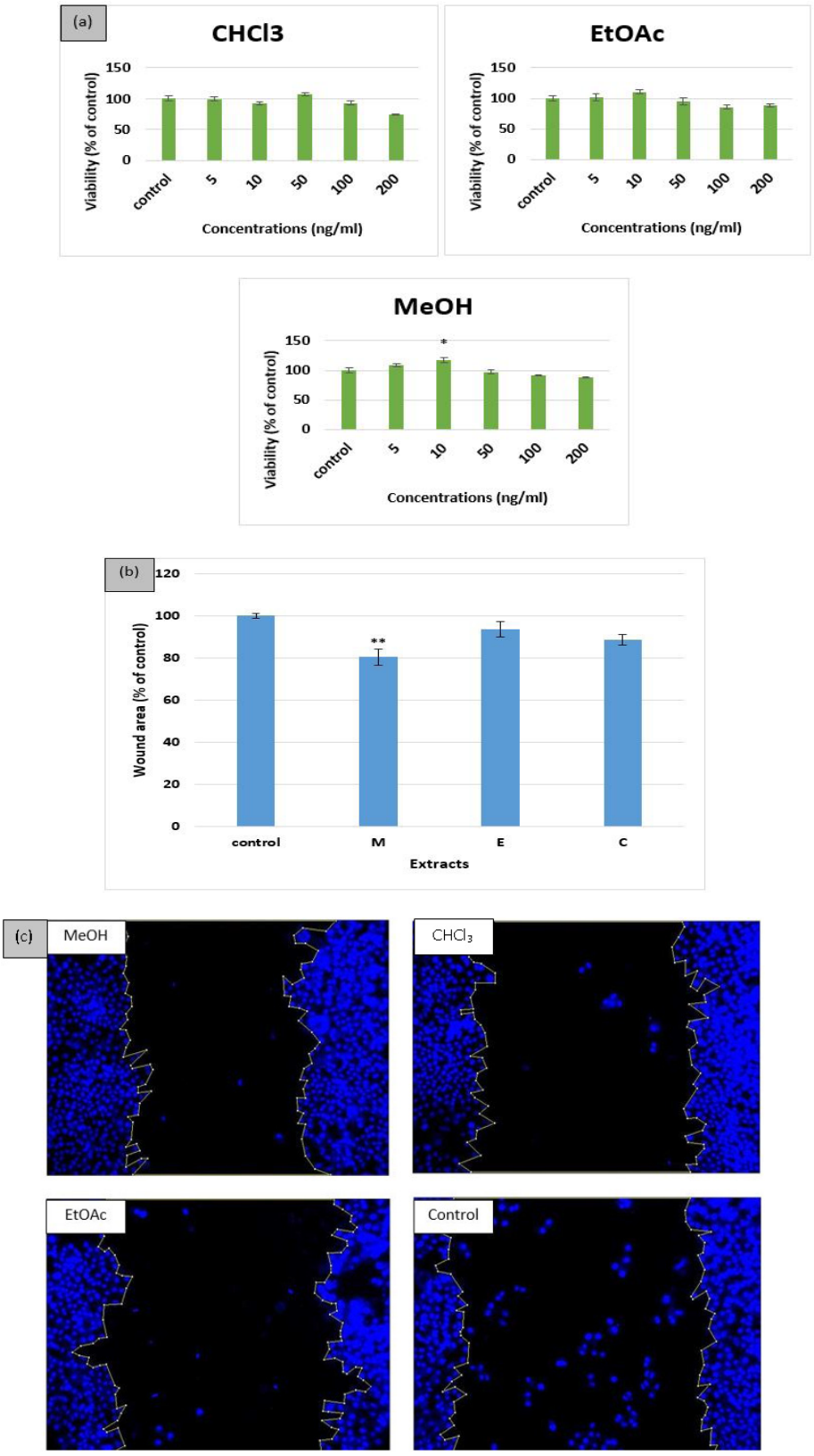


Figure 1. Effect of the $CHCl_3$, EtOAc and MeOH extracts from *P. orientale* on viability NIH-3T3 fibroblast cells using MTT assay (a). Quantitative (b) and qualitative (c) scratch-wound healing results of the fractions $CHCl_3$, EtOAc and MeOH after 48 h on NIH fibroblast cells exposing to nontoxic concentration values. Images of the fields were collected by fluorescence microscopy (magnification × 20) and analyzed with ImageJ software. * $p < 0.05$ and ** $p < 0.01$ show significant differences compared to control. Bars represent the mean ± S.E.M. of four experiment

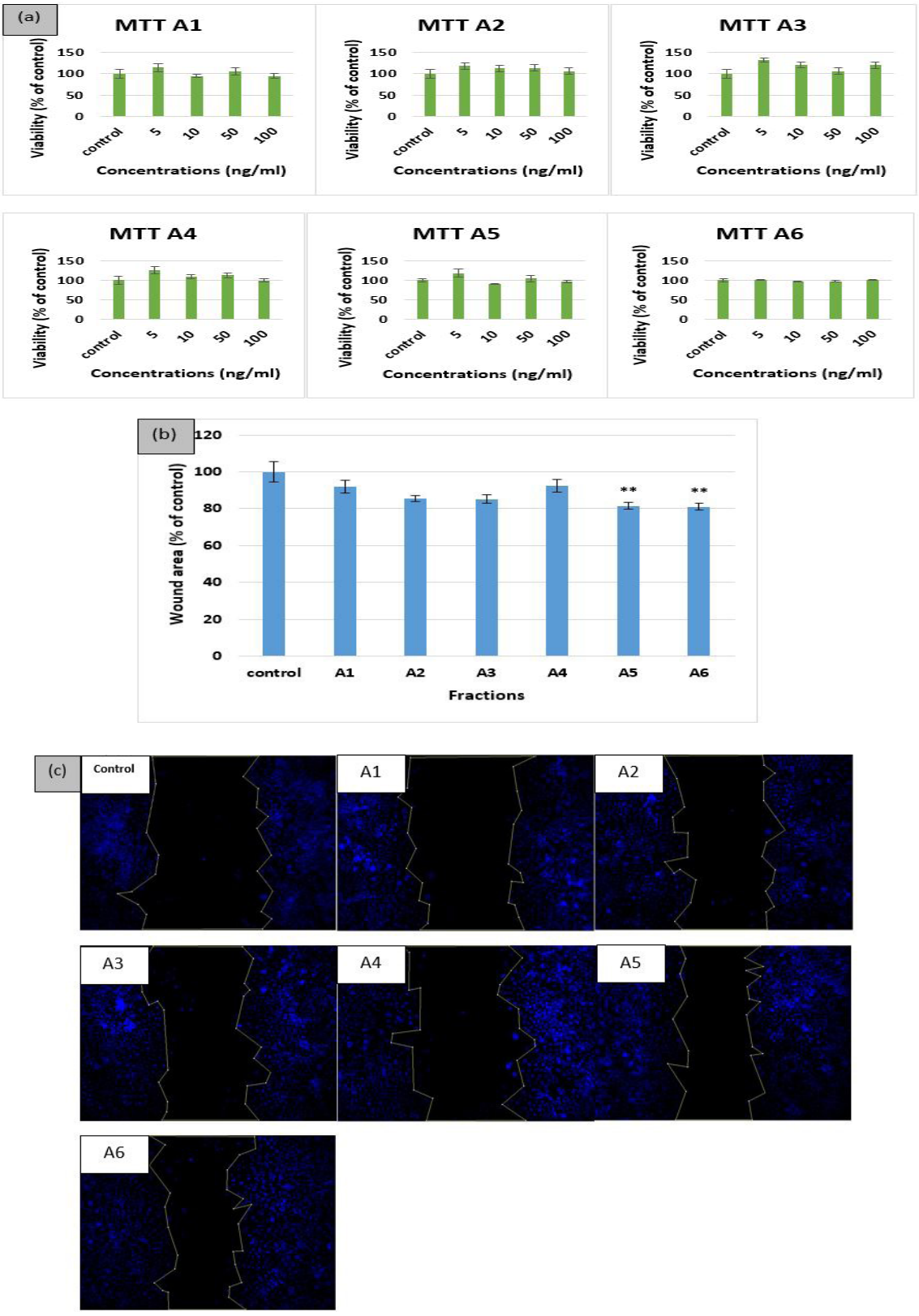


Figure 2. Effect of the fractions (A1-A6) on viability NIH-3T3 fibroblast cells using MTT assay (a). Quantitative (b) and qulitative (c) scratch-wound healing results of the fractions (A1-A6) after 48 h on NIH fibroblast cells exposing to nontoxic concentration values (b). Images of the fields were collected by fluorescence microscopy (magnification ×20) and analyzed with ImageJ software. ** $p < 0.01$ show significant difference compared to control. Bars represent the mean ± S.E.M. of four experiments.

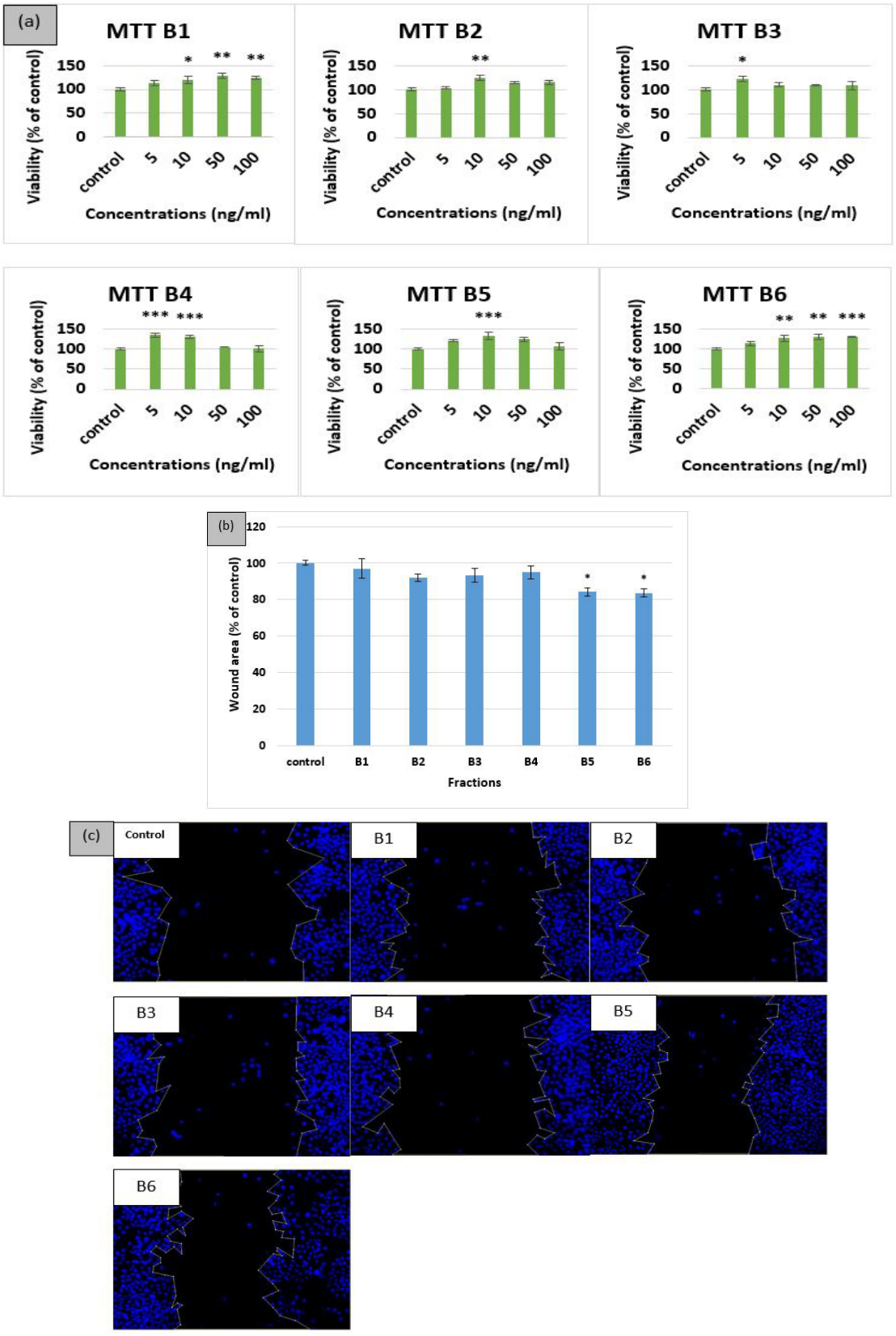


Figure 3. Effect of the sub- fractions (B1-B6) on viability NIH-3T3 fibroblast cells using MTT assay (a). Quantitative (b) and qulitative (c) scratch-wound healing results of the sub-fractions (B1-B6) after 48 h on NIH fibroblast cells exposing to nontoxic concentration values (b). Images of the fields were collected by fluorescence microscopy (magnification × 20) and analyzed with ImageJ software. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ show significant differences compared to control. Bars represent the mean ± S.E.M. of four experiments.

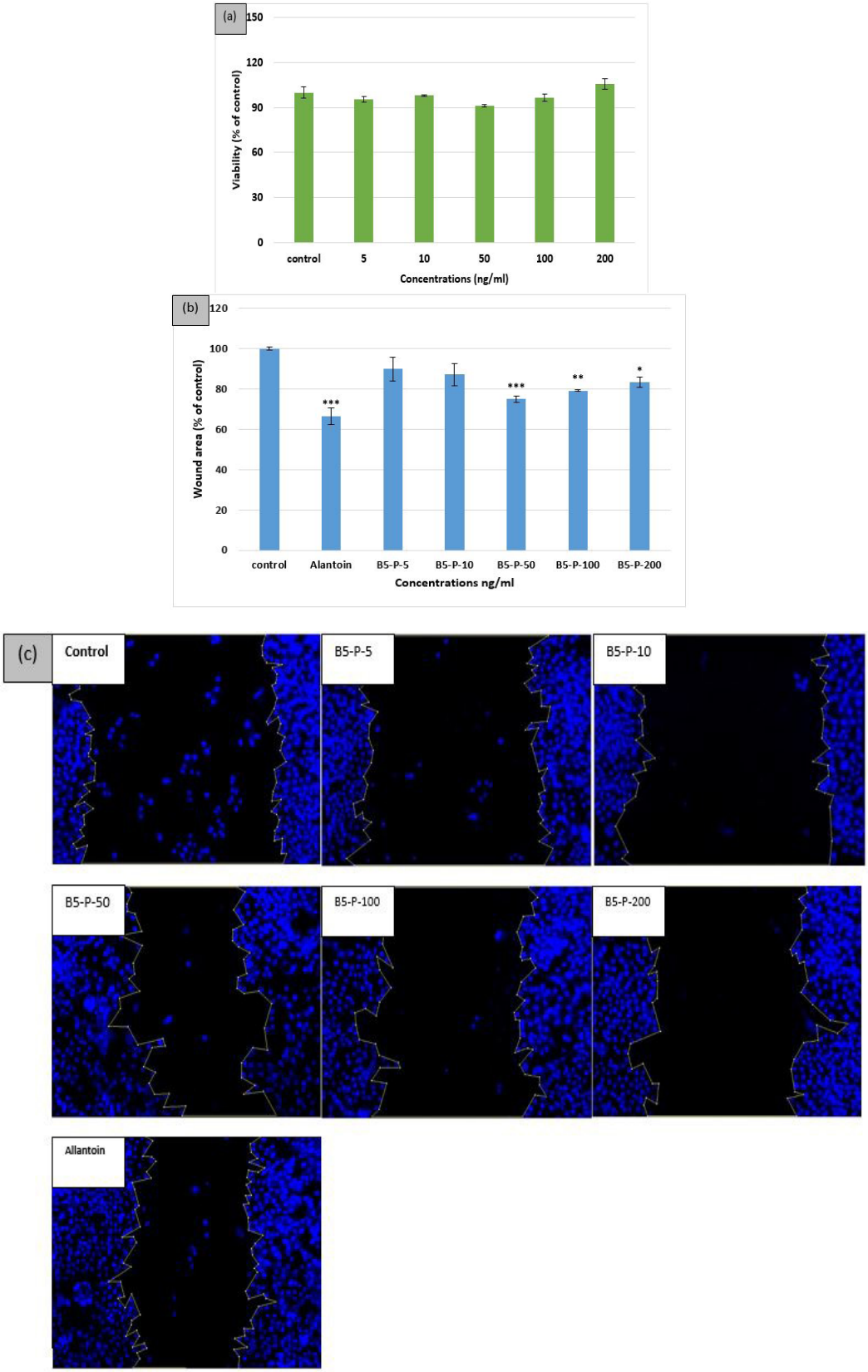
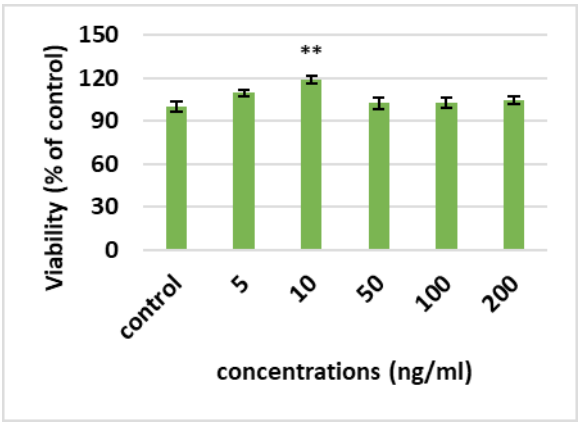
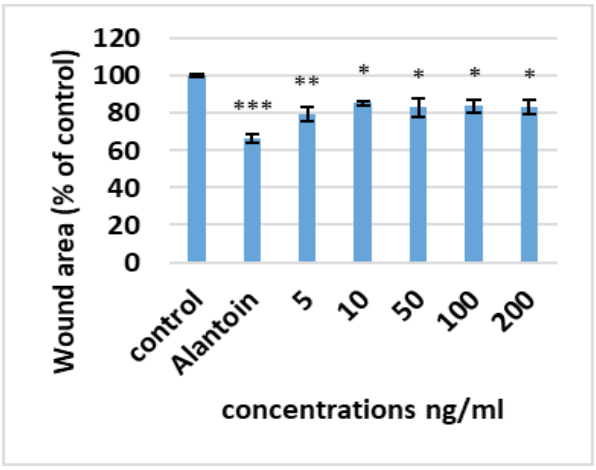


Figure 4. Effect of the B5-P on viability NIH fibroblast cells using MTT assay (a). Quantitative (b) and qualitative (c) scratch-wound healing results of the B5-P after 48 h on NIH fibroblast cells exposing to a concentration range of 5–200 ng/mL (b). Images of the fields were collected by fluorescence microscopy (magnification × 20) and analyzed with ImageJ software. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ show significant differences compared to control. Bars represent the mean ± S.E.M. of four experiments.





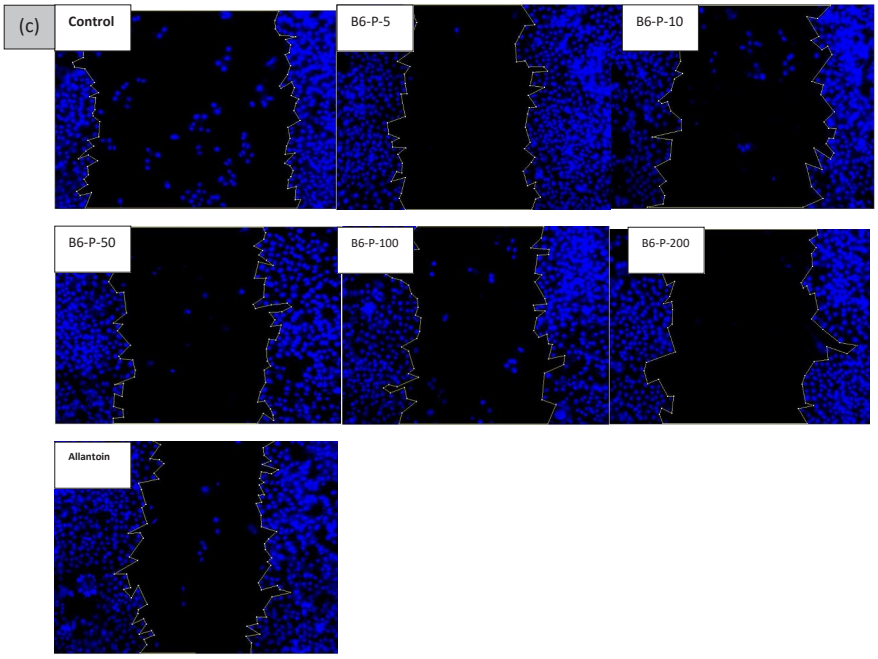


Figure 5. Effect of the B6-P on viability NIH fibroblast cells using MTT assay (a). Quantitative (b) and qualitative (c) scratch-wound healing results of the B6-P after 48 h on NIH fibroblast cells exposing to a concentration range of 5–200 ng/mL (b). Images of the fields were collected by fluorescence microscopy (magnification × 20) and analyzed with ImageJ software. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ show significant differences compared to control. Bars represent the mean ± S.E.M. of four experiments.

mass and the main fragment ions at m/z 321, 293, 221, 145, 97, 87, 73, 59 (Figure S3). The ^{1}H NMR spectrum (500MHz, CD_3OD+D_2O) of this molecule showed (Figure S4) the pattern of absorption bands of a disaccharide molecule including, the eight oxymethine and three oxymethylen signals between 3.4 and 5.4 ppm. The presence an anomeric proton at δH 5.4 ($d_{,J}$=5 Hz) suggested the existence of alpha-glycosidic linkage as well as signal at δC 92.16. The ^{13}C NMR spectrum revealed the presence of 12 carbons with a disaccharide pattern. ^{13}C NMR (125MHz, CD_3OD+D_2O): 103.88 (C-2'), 92.16 (C-1), 82.34 (C-5'), 77.88 (C-3'), 74.36 (C-4'), 73.2 (C-4), 72.98 (C-3), 71.86 (C-2), 69.89 (C-5), 62.60 (C-6'), 61.96 (C-1'), 60.76 (C-6) (Figure S5). The NMR data of B5-P were characteristic of a disaccharide structure (Figure S6) that was in accordance with the published data for sucrose [15-18]. The amount of B6-P was not sufficient for the characterization.

Discussion

In this study, the wound healing property of *P. orientale* rhizome was evaluated based on ITM and the latest pharmacological reports on the *Polygonatum* species. The bioassay-guided fractionation of *P. orientale* rhizome was performed using the scratch-wound healing assay on NIH-3T3 fibroblasts. Although the scratch-wound healing assay cannot be substituted for in vivo studies, it is a reliable, convenient and inexpensive method for analysis of fibroblasts or keratinocytes migration in vitro [19,20]. The effect of extracts/fractions/sub-fractions and isolated compounds from *P. orientale* rhizome on viability of NIH-3T3 fibroblasts was evaluated by MTT assay to select the nontoxic concentrations. The higher wound healing activity of the MeOH extract in comparison with other extracts suggested that the presence of polar phytochemicals significantly improved the healing potential. Thus, a bioassay-guided fractionation was performed on MeOH extract.

In the first stage of isolation, the six fractions (A1-A6) yielded by VLC were found to exert low or no cytotoxicity at the range of 5-100 ng/mL, and all the fractions were selected for wound healing activity. According to these results, an acceptable concentration (5 ng/mL) was determined to the scratch test from the pre-screening for cell viability. The fractions A5 and A6 revealed a significant wound contraction, however, in the first step; just A6 was selected for fractionation and separation of effective compound(s). The best concentration for each fraction was determined using the pre-screening viability test. Among the six sub-fractions, just B5 and B6 showed a similar significant percentage in wound contraction. Based on the TLC profile of sub-fractions B5 and B6, the observed wound healing effect can be attributed to their glycoside contents such as disaccharides, polysaccharides and other glycosides. Therefore, the wound healing effects of B5 and B6 could be synergistic. These bioactive sub-fractions were further purified by crystallization to yield the bioactive compounds B5-P (sucrose) and B6-P. Sucrose and B6-P were isolated as two bioactive compounds from MeOH extract. In addition, the efficacy of wound area closure revealed by sucrose and B6-P, in a limited concentration range, was comparable to that shown allantoin as a positive control.

Based on previous studies, mono and disaccharides such as glucose, galactose, xylose, fructose and sucrose have been found in *Polygonatum* species rhizome [7,21], but for the first time it was isolated from *P. orientale* rhizome. Sucrose as a disaccharide has shown to increase the migration of keratinocytes and fibroblasts in damaged tissue, and induce re-epithelialization, building new extracellular matrix and granulation tissue formation [22,23]. Previous studies confirmed that sucrose in its pure form, as a non-toxic compound, could help to treat a variety of wounds [24]. Sucrose by itself can be effective in the migration and growth of fibroblasts through increasing TGF-α (transforming growth factor alpha), which involved in fibroblasts proliferation. In addition, TGF-α stimulates collagen synthesis by activation epidermal growth factor. On the other hand, sugar content in honey stimulated TGF-β that involved in the reorganization of the extracellular matrix and collagen deposition [23]. As mentioned earlier, *Polygonatum* species are rich in saponins that can have many biological activities [6,25,26]. For example, the saponins from the root of Panax ginseng, are named ginsenosides, have shown wound healing effect through promotion matrix synthesis and re-epithelialization, and decreasing inflammatory reactions during the wound healing process [27]. According to the results, the presence of various bioactive glycosides in MeOH extract of *P. orientale* rhizome can have synergistic effect on wound healing through different mechanisms.

Conclusion

This is the first study to assess the effect of different extracts and fractions from *P. orientale* rhizome on viability and migration of NIH-3T3 fibroblasts in vitro. The results of this study supported the traditional uses of *P. orientale* rhizome for wounds treatment. Amongst all the extracts investigated in this study, the MeOH extract showed the highest wound healing activity. A bioassay-guided scratch wound assay of the MeOH extract confirmed that the fractions A5 and A6 are rich in bioactive glycosides and sucrose is one of the bioactive compounds that along with other active ingredients improve wound healing potential. Therefore, *P. orientale* rhizome could be considered as a new valuable source for obtaining natural wound healing agents and for further investigations, the structural elucidation and evaluation of wound healing activity of other isolated compounds from the effective fractions are recommended.

Conflict of Interests

The authors of this article announce that we have no conflict of interests.

Acknowledgements

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Supplementary

Figure S1. *Polygonatum orientale* Desf. Rhizomes

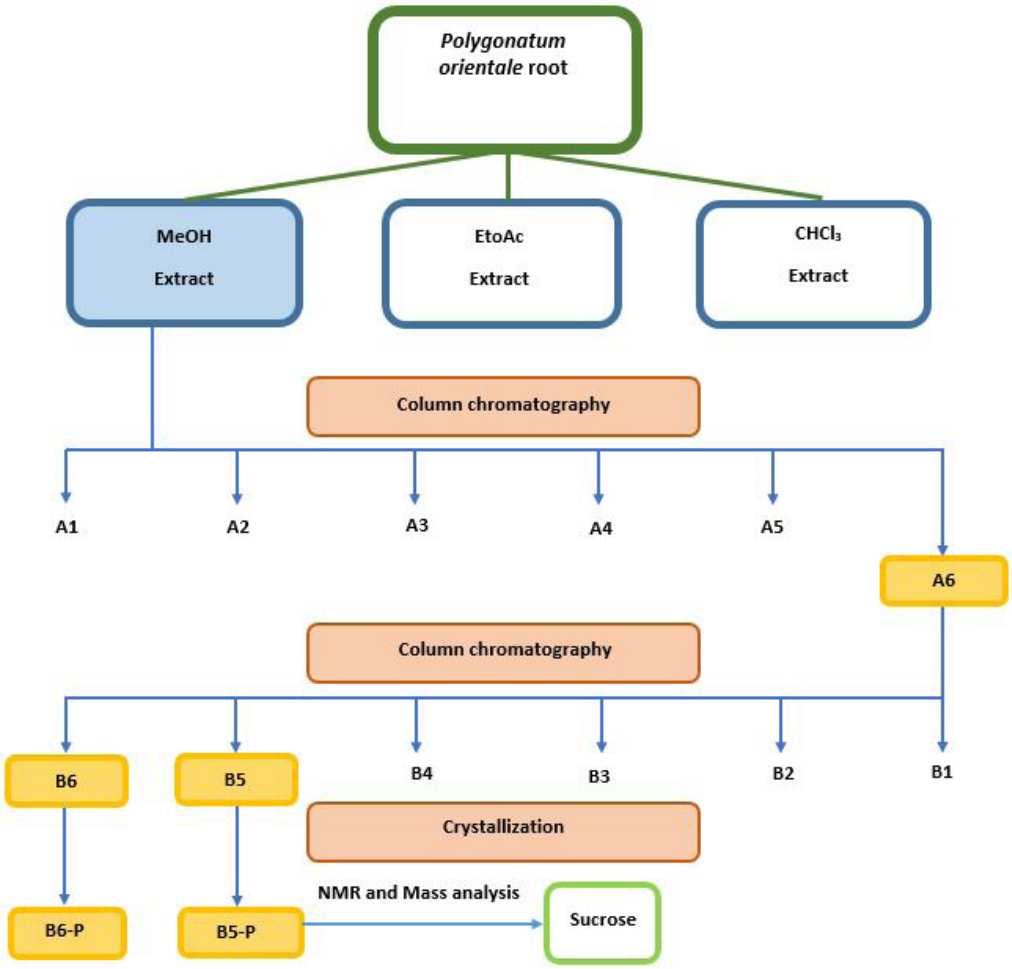

Figure S2. *S*cheme for the bioassay-guided fractionation of *Polygonatum orientale* rhizome extract

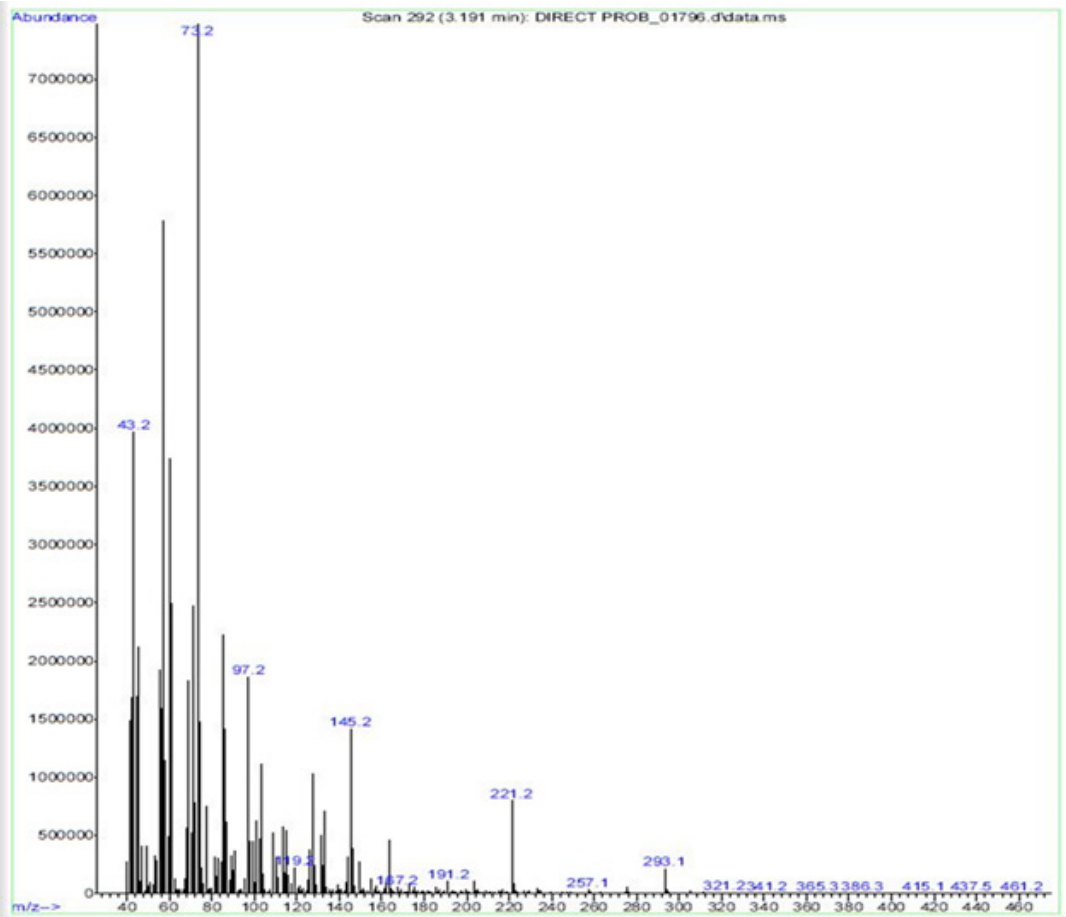

Figure S3. EI MS spectrum of Sucrose

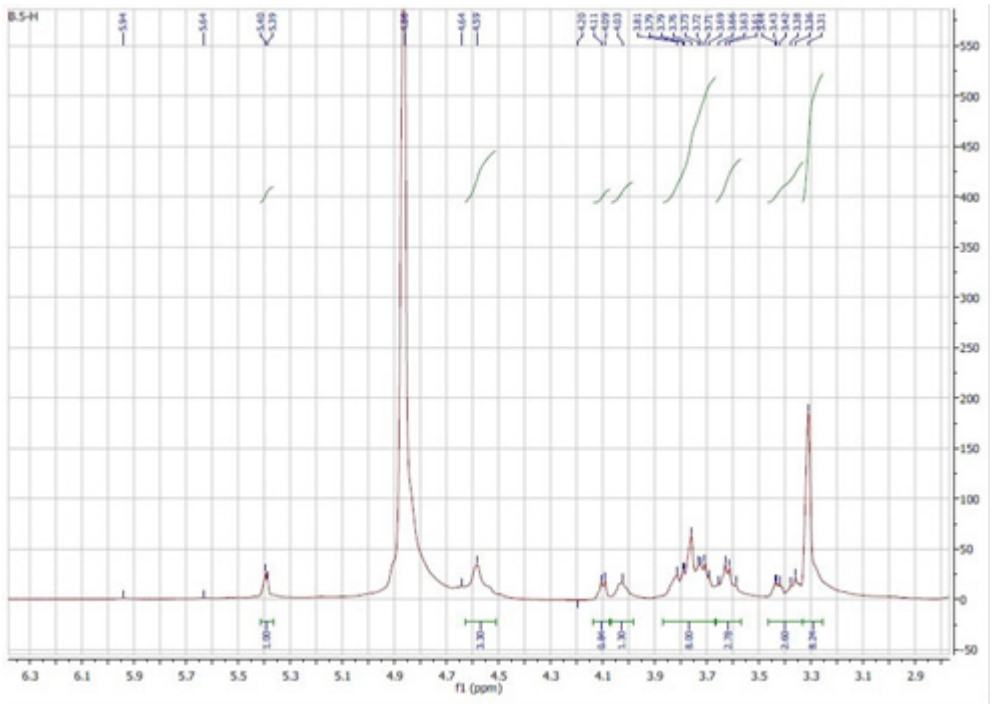

Figure S4. ^{1}H NMR (CD_3OD, 500 MHz) spectrum of Sucrose after adding one drop D_2O

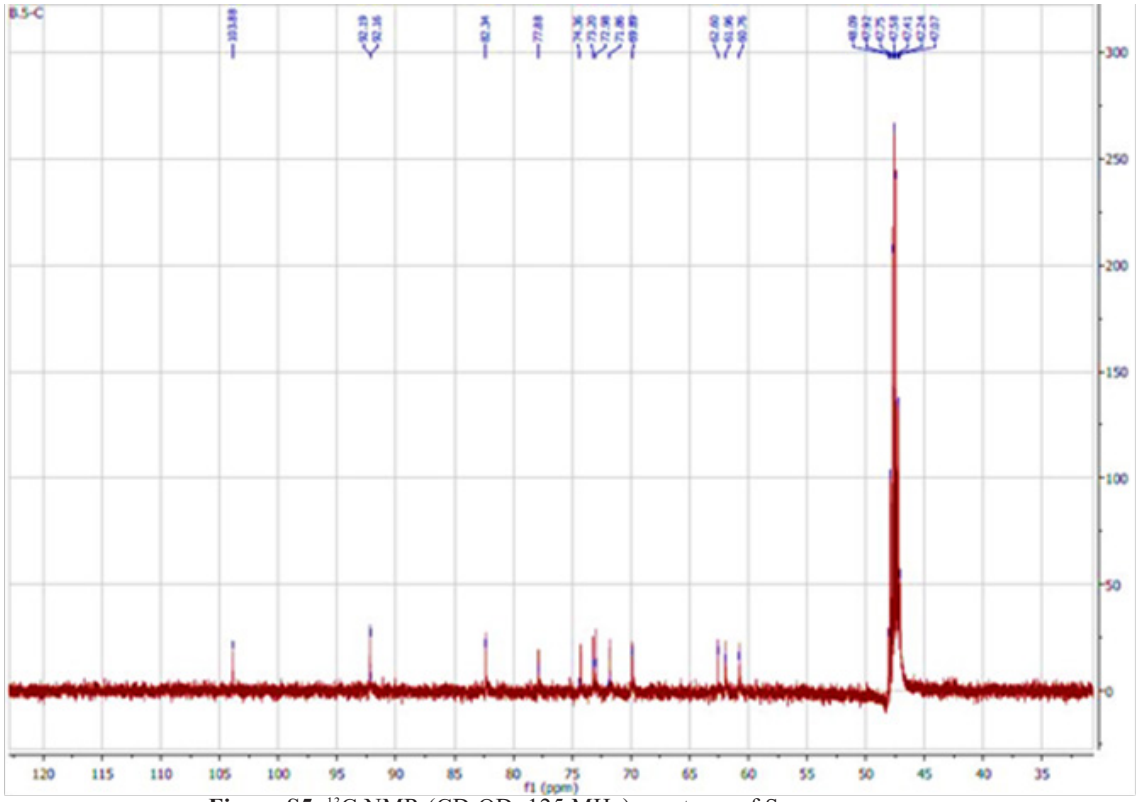

Figure S5. ^{13}C NMR (CD_3OD, 125 MHz) spectrum of Sucrose

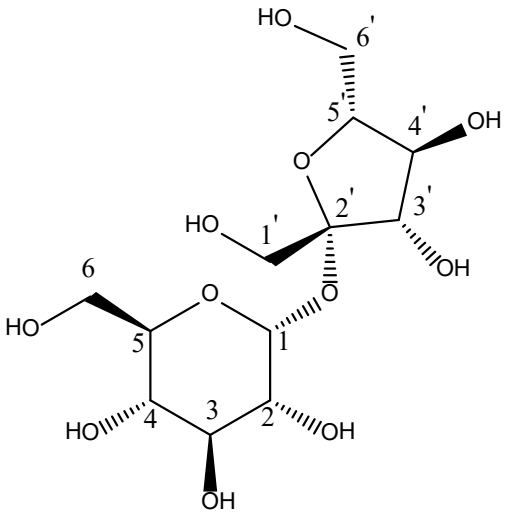

Figure S6. Sucrose molecular structure

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