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Absolute configuration and anti-cancer effect of prenylated flavonoids and flavonostilbenes from *Sophora pachycarpa*: Possible involvement of Wnt signaling pathway

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ABSTRACT

A new prenylated flavonostilbene, namely, alopecurone P together with three known compounds sophoraflavanone G, 2-(4-hydroxyphenyl)-2,3-dihydrobenzo[*b*]furan-3,4,6-triol and alopecurone J were characterized from the roots of *Sophora pachycarpa*. The absolute configuration of alopecurones J and P were characterized by comparison of experimental electronic circular dichroism (ECD) spectroscopy and simulated data using time-dependent density functional theory (TDDFT) for possible stereoisomers. The cytotoxic properties of isolated compounds have also been evaluated on two breast cancer cell lines (MCF-7 and MDA-MB-231) and normal cell line (NIH/3T3) using AlamarBlue®, flowcytometry and western blot assays. Alopecurone J and P showed cytotoxic effect on MCF-7 cell line through Wnt signaling pathway. It seems that the presence of lavandulyl substitution in C-8 position of flavanone structure increased the cytotoxic effect.

1. Introduction

The genus *Sophora* (Fabaceae) has 52 species in the world and is present in Iran as three species including *S. mollis* (Royle) Baker, *S. alopecuroides* L. and *S. pachycarpa* C. A. Mey. This genus is an abundant source of prenylated flavonoids [1–5] and alkaloids [6–8]. *Sophora* species have been used as a folk medicine in China, Japan, Thailand, Mongolia, etc. *S. flavescens* roots are most frequent in Chinese medicine. *S. tonkinensis* is also an important traditional Chinese herbal plant, named Shan-Dou-Gen in Chinese. The traditional uses of these herbs include the treatment of diarrhea, gastrointestinal hemorrhage, and eczema. Furthermore, their roots and rhizomes were used for the treatment of acute pharyngolaryngeal infections and sore throats [9]. Another important species of *Sophora* genus is *S. alopecuroides* that was used to treat fever, bacterial infection, heart disease, and rheumatism. The Xinjiang Chinese Herbal Medicine Handbook has reported that it exerts detoxification, pain relieving, and insecticidal effects. Local people use it to treat dysentery, stomachache, leucorrhea disorder, eczema, psoriasis, etc. [6,10–14]. Previous studies on the chemical constituents of *S. pachycarpa* revealed the presence of quinolizidine alkaloids [15] and prenylated flavonoids [16]. Cytotoxic and apoptogenic effects of methanol extract of the *S. pachycarpa* plant was demonstrated on HL-60 and MCF-7 cell line with IC_{50} value of 15.07 and 52.33 μg/ml [17].

In the current work, for the first time we reported the identification of two known flavonoids (sophoraflavanone G and 2-(4-hydroxyphenyl)-2,3-dihydrobenzo[*b*]furan-3,4,6-triol) and two flavonostilbenes alopecurone J and alopecurone P (a new alopecurone structure), isolated from the roots of *S. pachycarpa* (Fig. 1). In addition, we evaluated cytotoxic activity and possible mechanisms of action involved in their cytotoxicity. The absolute configurations of alopecurone J and P were also characterized by electronic circular dichroism (ECD) spectroscopy.

2. Results and discussion

2.1. Chemistry and absolute configuration determination

The methanol extract of *S. pachycarpa* roots was separated by silica

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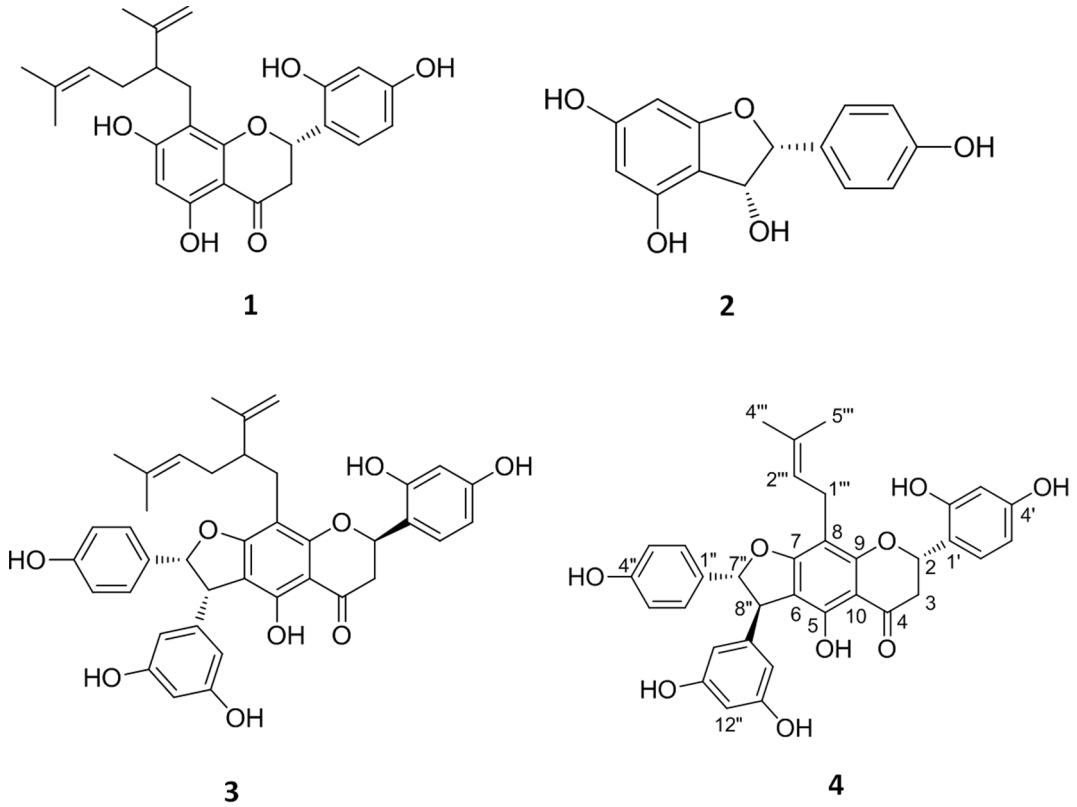

Fig. 1. Structures of sophoraflavanone G (**1**), and 2-(4-hydroxyphenyl)-2,3-dihydrobenzo[*b*]furan-3,4,6-triol (**2**), alopecurone J (**3**) and alopecurone P (**4**).

gel column chromatography, preparative thin layer chromatography, sephadex column and HPLC to give three known compounds and a new flavonostilbene. Sophoraflavanone G (**1**) [18] and 2-(4-hydroxyphenyl)-2,3-dihydrobenzo[*b*]furan-3,4,6-triol (**2**) [19] were reported in previous studies. The structure of these compounds were established by comparison their of the NMR spectral data with those of literature. The planar structure of two flavonostilbene compound **3** and **4** were established by analysis of NMR spectral data. Different types of alopecurones were reported previously [2,12,20]. According to the literatures, the orientation of resveratrol group at C-7″ and C-8″ can be as cis or trans [2]. Comparison of the ^{1}H NMR spectral data revealed a significant difference in the scalar coupling constant of H-7″ and H-8″. The coupling constant in alopecurone derivates with trans orientation is about 4.9–5.5 like alopecurone A, C, F, K and L while cis orientation the J-value of about 8.1–8.5 expected, such as alopecurone B and J. In compound **3**, the chemical shift and J-value of H-7″ [δ 5.90 (d, 8.1)] and H-8″ [δ 4.53 (d, 8.1)] indicated that the aryl group of the resveratrol residue was cis oriented, therefore the two configuration of 7″R, 8S″ or 7″S, 8R″ are predictable. The absolute stereochemistry at C-2 was different in alopecurone B and J but ^{1}H NMR spectral data do not show any difference. According to literatures, the $[\alpha]_D^{25}$ of alopecurone B is −1.5 but the $[\alpha]_D^{25}$ of alopecurone J is +20 [2,20]. The $[\alpha]_D^{25}$ of compound **3** was similar to alopecurone J. Consequently, these data suggested that compound **3** is alopecurone J. In the experimental ECD spectrum the compound **3** showed positive Cotton effects (CE) at 303, 220 nm and negative CEs around 245 and 210 nm, these effects are due to $\pi \rightarrow \pi^{*}$ transition of aromatic moieties. Comparison of experimental and calculated ECD data and those of published in literature confirmed that **3** is alopecurone J. Determination of chirality in C-2‴ in the lavandulyl are not possible by these data since this center is too remote from other chiral centers and chromophores and we couldn't detect any effect on ECD spectra and neither in NMR.

Compound **4**, obtained as a yellow powder, gave a $[M + Na]^{+}$ ion at *m/z* 605.1783 in the positive HRESIMS (calcd for $C_{34}H_{30}NaO_9$, 605.1788). ^{1}H NMR and ^{13}C NMR data of compound **4** were similar to alopecurone O [12], except a prenyl group that was located at C-8 in compound **4** instead of a 3-hydroxy-3-methylbutyl group in alopecurone O. The HMBC cross peaks of H-7″ (δ 5.52) to C-6 (δ 107.31) and H-8″ (δ 4.31) to C-7 (δ 166.59) indicated that the trihydroxystilbene moiety was fused at C-6 and C-7. Moreover, the HMBC cross peaks of H-1‴ (δ 3.22) to C-7 (δ 166.59) and C-8 (δ 103.02) suggested that the prenyl group was located at C-8. Furthermore, according to the NOESY cross peaks, H-8″ (δ 4.31) to H-2″ and H-6″ (δ 7.16), and H-7″ (δ 5.52) to H-10″ and H-14″ (δ 6.16). In addition, the similarity of ^{1}H chemical shifts and J-value of H-7″ [δ 5.52 (d, 5.0)] and H-8″ [δ 4.31 (d, 5.0)] confirmed the *trans* orientation position of aromatic rings of stilbene part (for more detail see Fig. 2). Comparison of calculated and experimental ECD spectral data suggested the configuration was 7″*R*, 8″*R*. The comparison of experimental and calculated ECD data for possible stereoisomers the 2*S*,7″*R*,8″*R* showed better match with three negative CEs around 310, 255 and 210 nm along with two positive CEs at 28and 230 nm and these value was in accordance with published data for similar class of compounds (Fig. 3) [21]. Consequently, these data suggested that compound **4** is a new alopecurone, namely, alopecurone P; $[\alpha]_D^{25}$ 300 (MeOH); UV (MeOH) λ_{Max} (log ε) 299 (3.212), 226 (3.56), 201 (3.96); CD (MeOH) $\Delta\varepsilon^{21}$ + 1 (340), −1.5 (307), −0.8 (290), −1.5 (250), +0.2 (240). Spectral data of compound **4** were reported in Table 1.

2.2. Cytotoxic activity

In the present work, *in vitro* cytotoxic properties of isolated compounds from *S. pachycarpa* and possible involved mechanisms of the cytotoxicity of these alopecurones were evaluated on two breast cancer cell lines (MCF-7 and MDA-MB-231) and a normal cell line (NIH/3T3) using AlamarBlue, flowcytometry and western blot assays.

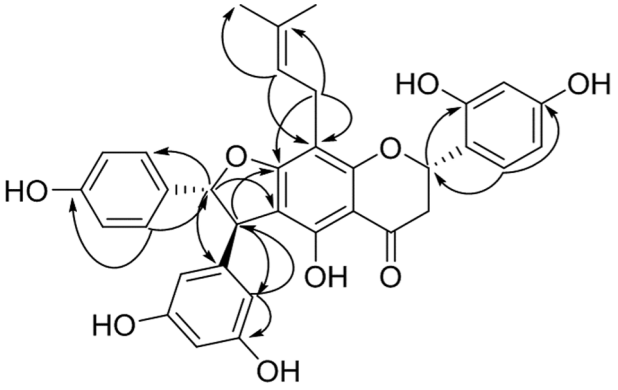

Fig. 2. HMBC key correlation of compound **4**.

2.2.1. Cell proliferation assay

Compounds **1–4** were tested for *in vitro* cytotoxic activity (MCF-7, MDA-MB-231 and NIH/3T3) (Table 2). Compounds **1** and **2** had weak significant cytotoxic effect in these cell lines. The IC_{50} values of compounds **3** in MCF-7, MDA-MB and NIH/3T3 cell lines was determined to be 8.25, 16.36 and 14.67 μg/ml, respectively. Compound **4** showed a moderate cytotoxicity. It seems that the presence of lavandulyl substitution in C-8 position of flavanone structure plays a key role for cytotoxic effect [22].

2.2.2. PI staining

The apoptotic rate and cell cycle analysis after treatment with compound **3** and **4** were determined by flow cytometry with propidium iodide (PI) to detect the so called sub-G1 peak. Small fragment of DNA can be eluted following incubation in a hypotonic phosphate–citrate buffer. PI is a quantitative DNA-binding color. When apoptosis occurred, cells missed DNA and took up less color and appeared to the left

Table 1
^{1}H and ^{13}C NMR spectroscopic data for compound 4.

Position	^{1}H NMR (300 MHz, acetone-d_6)	^{13}C NMR (75 MHz, acetone-d_6)
2	5.70 dd (13.2, 2.8)	74.77
3	eq 2.71 dd (17.1, 2.9) ax 3.13 dd (17.1, 13.3)	41.60
4	–	197.64
5	–	158.83
6	–	107.31
7	–	166.59
8	–	103.02
9	–	161.51
10	–	106.90
1′	–	116.61
2′	–	155.67
3′	6.47 d (2.3)	102.63
4′	–	158.78
5′	6.41 dd (8.4, 2.3)	106.90
6′	7.33 d (8.5)	127.87
1″	–	132.13
2″	7.16 d (8.5)	126.87
3″	6.82 d (8.6)	115.43
4″	–	157.70
5″	6.82 d (8.6)	115.43
6″	7.16 d (8.5)	126.87
7″	5.52 d (5.0)	94.32
8″	4.31 d (5.0)	54.15
9″	–	144.75
10″	6.16 d (2.1)	115.43
11″	–	158.83
12″	6.22 d (2.2)	101.28
13″	–	158.83
14″	6.16 d (2.1)	115.43
1‴	3.22 2H (m)	21.90
2‴	5.24 (*t*-like m)	122.22
3‴	–	131.09
4‴	1.60 3H (s)	25.06
5‴	1.56 3H (s)	17.03

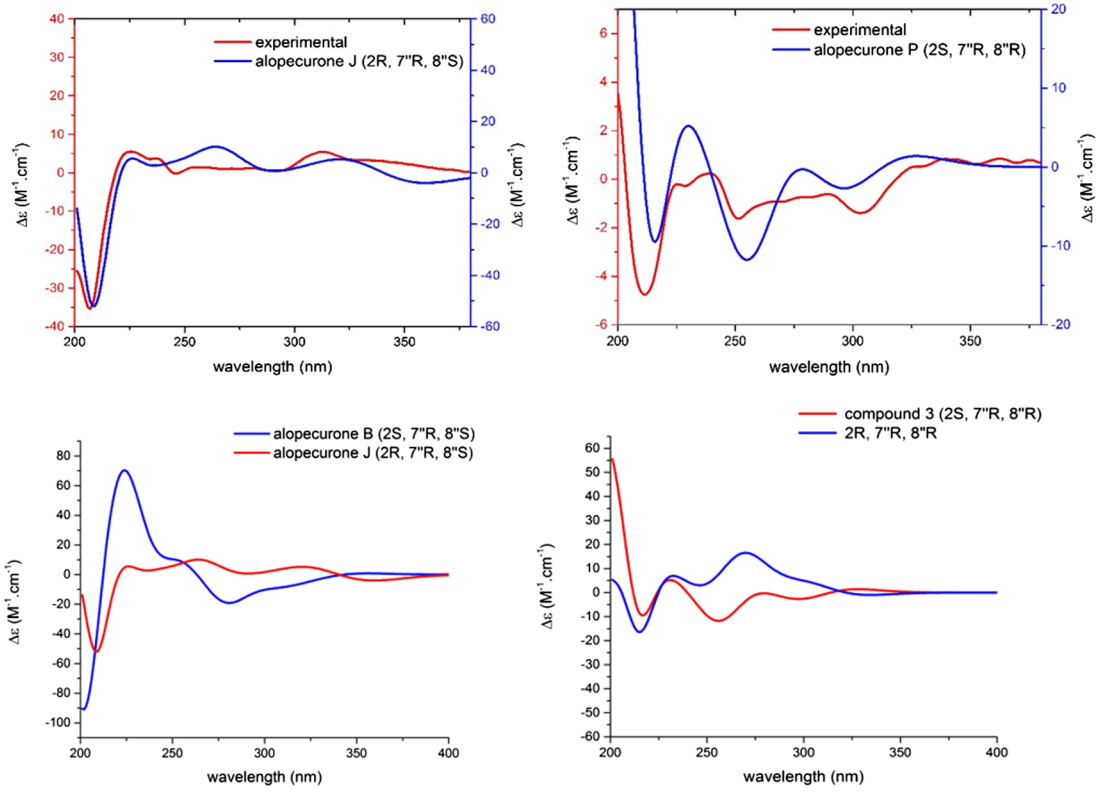

Fig. 3. Comparison of calculated ECD spectra by using TDDFT at B3LYP/6-31** level of theory in MeOH and experimental ECD spectra of compound **3** and **4**.

Table 2
IC_{50} values (μg/ml) for compounds **1–4** in MCF-7, MDA-MB-231 and NIH/3T3 cell lines.

Cell line	Compounds				
	1	**2**	**3**	**4**	Doxorubicin
MCF-7	40.86	92.51	8.25	23.51	1.03
MDA-MB-231	52.63	> 100	16.36	41.26	2.16
NIH/3T3	86.5	> 100	14.67	54.75	0.17

of the G1 peak. Comparison between sub-G1 peak of treated cells and untreated control cells in flow cytometry histogram demonstrated the induction of apoptosis in treated cells. PI stained low fluorescent fragmented DNA in apoptotic cells treated with different concentration of compounds **3** and **4** are a sign of apoptosis induction. Apoptotic cells lost most of their DNA content following incubation in the hypnotic buffer which contains triton X-100 appears as sub-G1 peak in the flow cytometry histogram (Fig. 4).

2.2.3. Western blot analysis

Western blot analysis of the cells treated with cytotoxic compounds was carried out to find out mechanism of action. The bax protein is a member of the bcl-2 family that promotes apoptosis. The ratio of bax to bcl-2 determines the susceptibility of a cell to apoptosis [23]. The caspases, especially caspase-3, are known to act downstream of bax/bcl-2 control and play a key role in the execution of apoptosis [24]. The bax expression in treatment with different concentration of compound **3** and **4** were higher than that of control. The bax/bcl-2 ratios were increased in the treatment group compared to control group. Pro caspase-3 expression was also decreased in treatment group compared to control. Regarding bax/bcl-2 ratio and caspase expression apoptosis were occurred in treatment with compound **3** and **4**. The Wnt signaling pathway, also called the APC/β-catenin/Tcf pathway, plays a fundamental role in human carcinogenesis [25]. It is well known that survivin are β-catenin/Tcf target genes [26]. Survivin plays a contributing role in regulating mitosis and cell division. Predictive and prognostic detection of survivin has been implemented in some types of cancer and strategies for targeting survivin as a new cancer treatment are underway [27]. Our findings revealed that compounds **3** and **4** decreased the expression of survivin and β-catenin and induced apoptosis in MCF-7 cells through Wnt signaling pathway (Fig. 5).

3. Conclusion

Two known prenylated flavonoids (sophoraflavanone G, 2-(4-hydroxyphenyl)-2,3-dihydrobenzo[*b*]furan-3,4,6- triol) and two flavonostilbenes were isolated from the roots of *S. pachycarpa*. Regarding to ^{1}H and ^{13}C NMR and ECD spectra the absolute configuration of alopecurone J and P were determined. Alopecurone J contains, a lavandulyl substitution instead of prenyl unit for alopecurone P. Different types of alopecurones including A, B, C, D, E, F, G [2], H, I, J, K, L [20], M, N and O [12] were produced by *S. alopecuroides*. Also alopecurones A and B were found in *S. pachycarpa* [16]. Alopecurone A that was isolated from *S. pachycarpa* with LC-MS technique exhibited remarkable cytotoxic effects with IC_{50} value of 26.43 on MCF-7 and 7.54 μg/mL on DU145 cell lines [28]. Furthermore, alopecurone A, B and D have potent inhibitory activity against multidrug resistance associated protein

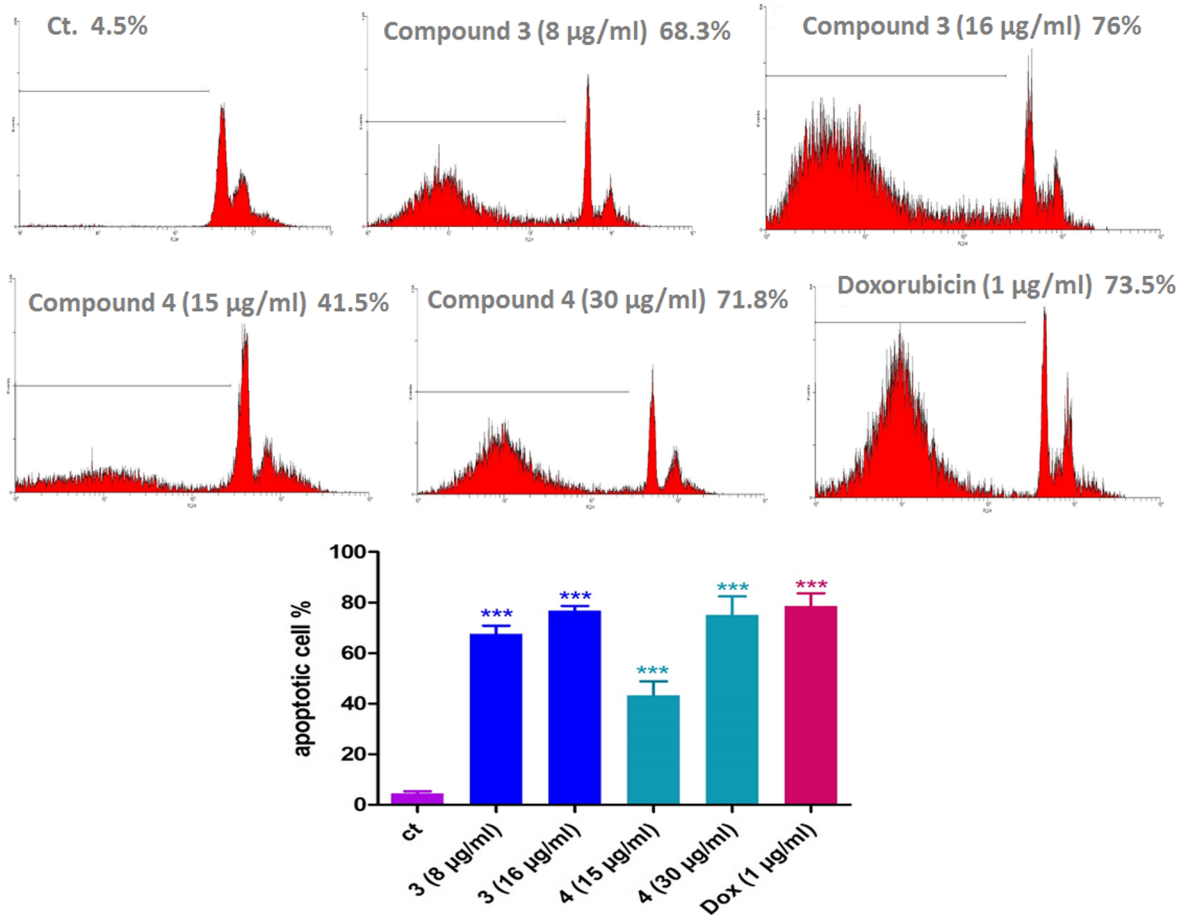

Fig. 4. PI staining and flow cytometry analysis of compound **3** (8 & 16 μg/ml), compound **4** (15, 30 μg/ml) and doxorubicin (1 μg/ml) induced apoptosis in MCF-7 cells.

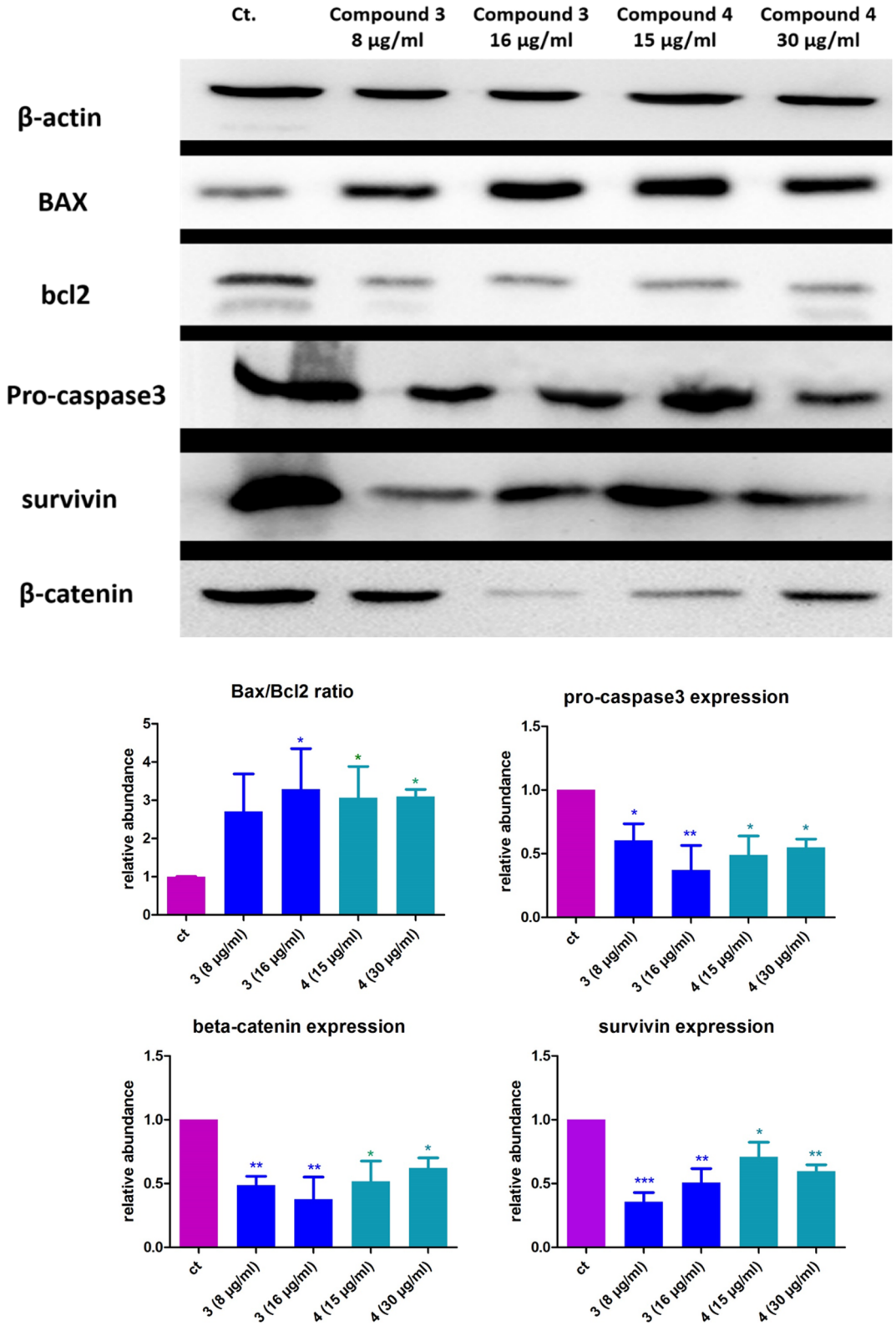

Fig. 5. Increase in level of Bax protein and decreased in bcl2 and pro-caspase 3 expressions in MCF-7 cell after 48 h exposure to different concentrations of compounds **3** and **4** demonstrate that apoptosis occurred. Decreased in the level of survivin and β-catenin showed possible mechanisms of actin. β-actin was used as a loading control.

1 (MRP1) and significantly decreased the IC_{50} value of doxorubicin on the MRP1-transfected U-2 OS cells (12-, 5- and 8-fold, respectively) at a non-toxic concentration [22]. In 2015 Xia et al. showed that alopecurone B at a non-toxic concentration enhances doxorubicin toxicity in MG-63 doxorubicin-resistance cells by inhibiting NF-kappaB signaling [29]. Alopecurones possess a broad range of biological activities. In this study, we determined the cytotoxic effect of alopecurone J and p and demonstrated that Wnt signaling, at least in part, might be the possible mechanism of cytotoxicity.

4. Materials and methods

4.1. General experimental procedures

Nuclear magnetic resonance (NMR) spectra were obtained using Bruker AVANCE III-300 spectrometer (Bruker, Germany). Chemical shifts are given as δ (ppm) using TMS as the internal standard. Column chromatography was conducted with Si gel 230–400 mesh (Merck, Germany). Analytical TLC was performed on Silica gel 60 F_{254} (Merck, Germany) and preparative TLC was performed on Silica gel 60 GF_{254} (Merck, Germany). Semipreparative HPLC was performed on a KNAUER liquid chromatography system with a quaternary pump (Smartline Pump 1000) and semi-prep C18 column (onyx monolithic; 100 × 10 mm). Diode array detector (Smartline DAD 2800) and EZ Chrom Elite software was used for detection and processing of data respectively, and Sephadex LH-20 (25–100 µm, Fluka). Ultraviolet (UV) spectra were recorded on a Shimadzu UV1650 spectrometer. Observation of plates was carried out under UV CAMAG spectrometer (CAMAG instruments, Berlin, Germany) at 254 nm. Optical rotations were recorded on a Polax-2L ATAGO, digital polarimeter at 25 °C (ATAGO Co. Ltd., Tokyo, Japan). High-resolution Electrospray ionization (HRESI) mass spectra data were acquired on an LTQ orbitrap XL (Thermo Fisher Scientific, America).

4.2. Plant material

The roots of *S. pachycarpa* were collected and identified in April 2015, from Khorasan-Razavi province, the road of Karde. A voucher specimen (No. 13247) was deposited in the herbarium of School of Pharmacy, Mashhad University of Medical Sciences. Mashhad, Iran.

4.3. Extraction and isolation

Total plant extract was obtained from the dried and milled roots of *S. pachycarpa* (500 g) with MeOH (for 3 days per extraction) using maceration method at ambient temperature. The combined extracts were concentrated to dryness under vacuum pressure to afford 50 g of a brown residue. Part of the resultant extract (35 g) was subjected to silica gel chromatography (230–400 mesh, 55 × 5 cm) using petroleum ether-ethyl acetate and ethyl acetate–methanol [(1:0 to 0:1 and 1:1, 0:1, v/v) × 2L] as a gradient solvent system to give ten fractions (Fr. 1–10). Fractions 4 and 6 were further purified using PTLC. Observation of plates at 254 nm to give **1** (50 mg, Rf 0.66). The solvent system was chloroform/methanol (3/0.5). Fraction 8 was further purified by Sephadex LH-20 CC (MeOH) and high-performance semi-preparative liquid chromatography with a gradient of MeOH–H2O (20:80, v/v) on an Onyx monolithic semi-prep C18 (100 × 10 mm) column to give **2** (12.0 mg) as a white powder, **3** (37.0 mg, Rt 6.8 min) and **4** (70 mg, Rt 7.15 min) as a yellow powders. The structures of the compounds were confirmed by comparison of ^{1}H- and ^{13}C- NMR spectra with those of previous reports [2,12,20].

4.4. ECD computational details

Conformational analysis for compounds, **3** and **4** was performed with MacroModel 9.1 software (Schrödinger LLC, New York) using the OPLS 2005 (Optimized Potential for Liquid Simulations) force field in H_2O. Conformers occurring within a 10 kcal/mol energy window from the global minimum were chosen for geometrical optimization and energy calculation using density function theory (DFT) with the B3LYP functional and the 6-31G(d,p) basis set in the MeOH using the SCRF-method with the Gaussian 09 program (Frisch et al., 2009) Vibrational analysis was made at the same level to confirm glabal minima. TD-DFT/B3LYP/6-31G (d,p) was conducted in methanol using the SCRF method, with the CPCM model. ECD curves calculated with a δ 0.25 ev using SpecDis v1.62 [30].

4.5. Cell cultures and treatment agent

The breast cancer cell lines MCF-7 and MDA-MB-231 and normal cell line NIH/3T3 were obtained from Pasteure institute (Tehran, Iran) and maintained in RPMI-1640 medium with 10% v/v fetal bovine serum and 100 u/ml penicillin and 100 mg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% of air. The stock solution of all extracts were prepared at 50 mg/ml in DMSO and kept at −20 °C.

4.6. Cell proliferation assay

The resazurin is the active ingredient of Alamar Blue®, which is a cell health indicator and uses the reducing power of live cells and is converted to resorufin. This reduction occurs in the live cytosol of the cells. Resazurin is a blue color and non-fluorescent compound without cytotoxic effect but resorufin is a red color and highly fluorescent compound. Viable cells convert resazurin to resorufin, this change increases the total fluorescence and color of the media surrounding cells [31]. About 10^4 MCF-7, MDA-MB and NIH cells were seeded in each well of a 96-microwell plate and treated with various concentrations of compounds **1–4**. After 44 h incubation, AlamarBlue® (17 µl) was added to each well according to the manufacturer's instructions. After further 4 h incubation, the cell viability was determined by measuring the absorbance at 600 nm in a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, USA). The cytotoxicity of compounds **1–4** were expressed as IC_{50}, which was calculated using Graph Pad Software (Graph Pad Prism version 5 software) and presented as mean ± SD of three independent experiments with three replicates for each compound.

4.7. PI staining

Apoptotic cells were determined using propidium iodide (PI) staining of treated cells followed by flow cytometry to detect the so-called sub-G1 peak [32]. Summarily, 4.5×10^4 MCF-7 cells were seeded in each well of a 24-well plate and treated with various concentrations of compounds 3 and 4 for 48 h. Floating and sticking cells were then collected and washed twice with PBS and incubated at 4 °C in the dark for 2 h with 400 µl of a hypotonic buffer (50 µg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100) before flow cytometric analysis using a FACScan flow cytometer (Partec GmbH, Münster, Germany) was conducted.

4.8. Western blotting analysis

About 10^6 MCF-7 cells were treated with 12.5, 25, 50 µM of alopecurone J and 12.5, 25, 75 of alopecurone P for 48 h. Cells were washed twice with cool PBS and resuspended in cool lysis buffer. Lysis buffer composition was 50 mM Tris-HCl (ph 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.2% SDS, 1% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride. This mixture was left on ice for 30 min. Then, the mixture was centrifuged in a microcentrifuge at 14,000 rpm for 20 min at 4 °C. The protein-containing supernatant was removed from a chilled test tube. The Bio-Rad Protein Assay kit was used to determine protein concentration. Equal amounts of proteins were loading in 12% SDS-page (W/V). After electrophoresis in stable voltage (120 V) for 1–2 h, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and probed with immunoblotting assay using different rabbit antibody as primary antibodies and anti-rabbit IgG as secondary antibody. Protein expression were detected by enhanced chemiluminescence (ECL) western blotting detection reagent. Images were quantified using Gel-pro Analyser V.6.0 Gel Analysis software (Media Cybernetics, InC, Bethesda, MD).

4.9. Statistical analysis

All results reported means ± SD from triplicate experiments performed in a parallel manner. Statistical differences between groups were analyzed by one-way ANOVA with subsequent Bonferroni post hoc tests. All comparisons were made relative to untreated controls and significance of difference is indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary material

^{1}H NMR, ^{13}C NMR, HMBC, HSQC and NOESY spectra of compound **4** and calculated ECD spectra of alopecurone A, B, J, K, F and L by using TDDFT at B3LYP/6-31** level of theory in MeOH can be found as Supporting Information. Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.01.051.

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