

Phytochemical analysis and antiproliferative activity of the aerial parts of *Scrophularia subaphylla*

Abbas Delazar^{1,2}, Solmaz Asnaashari³, Elhameh Nikkhah⁴, and Parina Asgharian^{1,2,5,*}

¹Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, I.R. Iran.

²Faculty of pharmacy, Tabriz University of Medical Sciences, Tabriz, I.R. Iran.

³Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, I.R. Iran.

⁴Research Center of Medicinal herbs, Maragheh University of Medical Sciences, Maragheh, I.R. Iran.

⁵Halal Research Center of IRI, FDA, Tehran, I.R. Iran.

Abstract

Scrophularia subaphylla (*S. subaphylla*) L., a medicinal plant from the Scrophulariaceae family, has been reported to possess potential profits in the treatment and prophylaxis of different diseases. Some phenolic compounds in this genus have been displayed decent effects on different types of cancer via multiple mechanisms. The current study aimed to bioassay guided isolation of cytotoxic constituents from the aerial parts of *S. subaphylla* against breast (MCF-7) and colon (HT-29) cancer cell lines as well as normal cells (L929). Different extracts of *S. subaphylla* were acquired by Soxhlet apparatus and then subjected to brine shrimp lethality test and MTT assay for assessing their cytotoxic characteristics. Cytotoxic extract subjected to further phytochemical fractionation using solid phase extraction, reversed-phase high pressure liquid chromatography (RP-HPLC), and one dimensional nuclear magnetic resonance (1D-NMR) spectroscopy. The biological activity of the isolated pure components, verbascoside and 3'-*O* rhamnosyl -4'-*O* para coumaryl 7- hydroxyl salidroside, was assessed using MTT assay against MCF-7 and HT-29 carcinoma cells. Two known phenylpropanoid compounds were isolated from this species. Their structures were elucidated by spectroscopic data (using ¹H-NMR and ¹³C-NMR) and compared with the previous literature. Both pure compounds in comparison with control group demonstrated significant antiproliferative activity against cancerous cells ($P < 0.001$). In our study, verbascoside and its derivative could inhibit proliferation of cancerous cells without any side effects on normal cells.

Keywords: : BSLT; MTT assay; Salidroside derivative; *Scrophularia subaphylla*; Verbascoside.

INTRODUCTION

According to a report of the world health organization (WHO), cancer is a common and crucial clinical problem nowadays. In the last decades, the incidence of cancer associated death has increased (1). Among all the types of cancers, breast and colon cancers are the most prominent, dangerous, and life threatening cancers due to their difficult detection and in some cases, ignorance of the early signs of these malignancies (2). While invasive and non-invasive medications are still applied for overcoming the genre of malignancies, some of the aforementioned strategies cause perniciousness and major undesired adverse effect on normal cells (3). On the other hand, because some cancerous cells exhibit resistance

to some of the anticancer agents and interventions through various mechanisms (4,5), multiple therapies and changing the treatment protocol for the patients have been suggested. Therefore, finding new anticancer compounds with minimum adverse effects and maximum efficacy against population of remaining cancerous cells seems imperative. Since the effectiveness of the natural products and secondary metabolites (6) in decreasing the occurrence of various types of cancers has been proven, there is a global search for novel natural components as anticancer agents.

Access this article online



Website: <http://rps.mui.ac.ir>

DOI: 10.4103/1735-5362.258495

*Corresponding author: P. Asgharian
Tel: +98-9141771694; Fax: +98-41333347581
Email: parina.asgharian@gmail.com

Among natural products, medicinal herbs have played a vital role both in prophylaxis and treatment of various forms of malignancies. *Scrophularia* genus (Scrophulariaceae) encompasses over 3000 species which are extensively dispensed from mid Asia including Iran to North America (7-10). *Scrophularia subaphylla* (*S. subaphylla*) is a well-known endemic medicinal species of Azerbaijan flora in Iran. Traditionally, some species of this genus have been ethno medically consumed as anti-inflammatory, anti-eczema, anti-erythema, removing mouth dryness, improving sore throat, and anticancer agents (8,9). Numerous investigators have isolated some chemical constituents like iridoid glycosides, phenyl propanoids, flavonoids, terpenoids, and saponins from *Scrophuraria umbrosa* (11,12). In addition, the mentioned secondary metabolites exhibited a number of biological activities such as antimicrobial, cardiovascular, protozoacidal, cytotoxic, antimalarial and wound healing effects (9-14). In this process, whereas the expression of caspase-3 mRNA increased, the expression of Bcl-2 decreased in cancer cell lines. (14-17). Despite the fact that, one publication has evaluated the volatile component of *S. subaphylla* (7), studies on antiproliferative activity as well as phytochemical constituents of this plant are not available. Previous studies have emphasized the significance of anticancer properties of *Scrophularia* species, which have been attributed to the presence of some main secondary metabolites. Herein, firstly we aimed to assess and compare the antiproliferative effect of various extracts of *S. subaphylla* on two human carcinoma cell lines of MCF-7 and HT29 and a normal cell line L929; and secondly the isolation and depiction of bioactive components of cytotoxic fraction of *S. subaphylla* was evaluated.

MATERIAL AND METHODS

Materials

Human breast carcinoma (MCF-7) and colon cancer (HT-29) cell lines were purchased from Pasteur Institute of Iran (Tehran, I.R. Iran). Roswell park memorial institute (RPMI) medium 1640, fetal bovine serum (FBS),

streptomycin and penicillin were provided from Gibco Invitrogen Corporation (UK). Pipettes, tissue culture flasks, 96-well plates, trypan-blue, and 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were from Sigma-Aldrich Co. (UK). Dimethyl sulfoxide (DMSO) was from Merck (Darmstadt, Germany). UV/visible spectrophotometer (Shimadzu, 2100; Japan).

Plant material

Aerial parts of *S. subaphylla* were collected from the mountains of Marand-Mishodagh, I.R. Iran in summer, 2013. The plant was identified by a botanist Dr Atefeh Ebrahimi and a voucher specimen under accession code (Tbz-fph 747) was stored in the herbarium of Tabriz University of Medical Sciences, Tabriz, I.R. Iran.

Preparation of extracts and Sep-Pak fractions

One hundred g of air-dried aerial parts of *S. subaphylla* were grounded and subjected to Soxhlet apparatus sequentially to make different extracts n-hexane, dichloromethane (DCM), and methanol (MeOH). All of the extracted solvents were evaporated by rotary evaporator at the 45 °C and under reduced pressure. Cytotoxic characteristic of the samples were assessed using brine shrimp lethality test (BSLT) and MTT assay. The MeOH and DCM extracts- showed the highest anti-proliferative activity against *Artemia salina* (*A. salina*) and cancerous cells. The MeOH extract was subjected to Sep-Pak to yield six different fractions. The solid phase extraction method was repeated for at least 4 times for obtaining sufficient amount of each fraction. Subsequently, after removing the solvents, the fractions were submitted to MTT test. Finally, 40% SPE fraction was subjected to reversed-phase high pressure liquid chromatography (RP-HPLC) for isolation of the chemical components.

Reversed phase preparative HPLC and determination of the chemical structures

The potent Sep-Pak fraction in MTT assay, were further analyzed using preparative HPLC eluting with a linear gradient of MeOH/water and a photo-diode array detector at a range of

190 to 400 nm. For isolation of pure compounds of 40:60 (MeOH:water) fraction, the superlative RP-HPLC solvent system was set as follows:

Mobile phase: 0-50 min, MeOH from 30 to 60% in H₂O; 50-62 min, 60% MeOH in H₂O; 62-67 min, MeOH from 60 to 30% in H₂O; 67-70 min MeOH 30% in H₂O. Subsequently, the solvents of 40% fraction were removed and the purity of collected compounds was controlled by analytical HPLC. Chemical structure of pure compounds were elucidated by one dimensional nuclear magnetic resonance (1D-NMR) (¹H-NMR and ¹³C-NMR spectroscopy techniques) in DMSO-d₆.

Cytotoxicity evaluation

The cytotoxicity of all extracts and fractions as well as two pure compounds was evaluated against MCF-7 and HT-29 as cancerous as well as L929 as normal cell lines. The cells were grown in flask by RPMI-1640 medium which supplemented with 10% heat inactivated FBS and incubated at condition of 37 °C temperature, 95% humidity, and 5% CO₂.

Evaluation of cytotoxic activity and cell viability were made by colorimetric MTT assay (18-19). For this purpose, cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) in logarithmic growth phase and cultured in 96-well plates (200 µL, 1.5 × 10⁴ cells/well). After 24 h, cells were treated with different concentrations of samples (1-1000 µg/mL), which had minimum amounts of DMSO (<1%). After 24 h, medium was removed from wells and 200 µL fresh medium (150 µL complete RPMI + 50 µL MTT solution 2 mg/mL in phosphate-buffered saline) was added to each well. The plate was shaken gradually and incubated for 4 h. Alive cell's mitochondria reduce yellow solution of MTT to blue formazan crystals. Finally, supernatant was emptied and the remained formazan crystals were dissolved in 150 µL DMSO. Cell viability was indicated by color intensity as well as comparing with the color of control. The color intensity was measured at 570 nm using a microplate reader (Bio Teck, Germany). DMSO 1% was considered as control group. The median

growth inhibitory concentration (IC₅₀) was calculated from a dose response curve plotted in the Graph Pad Prism software.

Antioxidant activity

The free radical scavenging ability of the samples was evaluated by the method based on the reduction of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) solutions in the presence of antioxidant. DPPH (8 mg) was dissolved in MeOH and chloroform (100 mL) to obtain concentration of 80 µg/mL. All the samples were dissolved in a suitable solvent to get a concentration of 1 mg/mL. Different concentrations (1.5 × 10⁻¹, 2.5 × 10⁻¹, 1.25 × 10⁻¹, 6.25 × 10⁻², 3.13 × 10⁻² and 1.56 × 10⁻² mg/mL) of all samples were made and then diluted with DPPH (5 mL). The samples were incubated for 30 min at 25 °C for any possible reaction to take place. Subsequently, the absorbance was read using spectrophotometer 160A (USA) at 517 nm against a blank (chloroform for nonpolar extracts and MeOH for polar fractions; furthermore, DPPH was added to both controls). RC₅₀ (50% scavenging activity of sample) values were calculated from the percentage reduction graph against samples concentration. The same procedure was repeated for positive control (quercetin). The percentage bleaching of DPPH by samples was calculated using the following equation:

$$I(\%) = 100 \times [(A_{\text{blank}} - A_s)/A_{\text{blank}}] \quad (1)$$

where, A_{blank} and A_s are the absorbances of the blank and the samples after 30 min, respectively. RC₅₀ was extrapolated from dose response curve (inhibition percentage against various concentrations). The tests were performed in triplicate (19,20).

Total phenolic contents

Phenolic contents of the *S. subaphylla* Sep-Pak fractions of MeOH extract were determined by Folin-Ciocalteu reagent, based on our previous report (21). Samples containing higher amounts of phenolic compounds were reduced with Folin-Ciocalteu reagent, which produced blue color. Briefly, 5 mL of Folin-Ciocalteu reagent (a 10% v/v in distilled water solution) and 4 mL of 1 M aqueous Na₂CO₃ were added to

0.5 mL solution of test samples. The mixtures were incubated for 15 min at room temperature with periodic shaking. The absorbance of the supernatants of solutions was evaluated at 765 nm against blank solution with a UV/visible spectrophotometer (Shimadzu, 2100; Japan). The standard curve was constructed with different concentrations of gallic acid solution as the standard. Finally, total phenolic values were expressed as milligrams of gallic acid equivalent per 100 g of powdered plant material. All tests were done in triplicate.

Total flavonoid contents

For determining the total flavonoid amount of Sep-Pak fractions of MeOH extract, concisely, 2 mL of samples were mixed with 400 μ L of water and 1 mL of reagent (mixture of 183 mg of $AlCl_3$ and 400 mg of sodium acetate), then were remained at 25 °C for 30 min for color formation. Consequently, the absorbance was measured at 415 nm using a UV/visible spectrophotometer. The standard curve was constructed using different solutions of rutin (1.25×10^{-1} , 6.25×10^{-2} , 3.13×10^{-2} , 1.56×10^{-2} , 7.81×10^{-3} , 3.9×10^{-3} mg/mL) as the positive standard (21).

General toxicity

BSLT, as a simple, low cost, high sensitive and convenient method, was applied for screening general toxicity based on the modified Meyer method and our previous publication. (14,22). Concisely, the hatching eggs *A. salina* which were obtained from Shilat Center, Tabriz, I.R. Iran, were prepared in 35% salt water and incubated under well aerated flask for 48 h. Subsequently, the different herbal extracts were dissolved in DMSO (not more than 0.05%) and normal saline to obtain various concentrations of samples. 1 mL of main prepared sample

solution along with 10 mL of seawater was added to each sterile vial. In addition, approximately, 10 nauplii as amateur shrimps were transferred in to the vials and incubated for 24 h. Finally, number of dead nauplii at each dosage was counted as percent of mortality of the extracts. The LC_{50} was estimated using linear regression analysis using excel software.

Statistical analysis

All statistical analyses were done using Graph Pad Prism 8.01 software, and significant differences between groups were analyzed using ANOVA. In this study, the experiments were performed in triplicates ($n = 3$) and all the data are presented as the mean \pm SD. P value < 0.05 was regarded as significant.

RESULTS

In the current research, BSLT, cytotoxic and free radical scavenging activities as well as TPC and TFC of samples were determined. Furthermore, chemical structures of potent anti-proliferative compounds obtained from the aerial parts of *S. subaphylla* were characterized and the results were illustrated in the Tables 1-4.

General toxicity

Preliminary BSLT bioassay was used for comparing the cytotoxic activities of the extracts with the positive control (Podophyllotoxin $LC_{50} = 2.69 \pm 0.006$ μ g/mL). Results (Table 1) demonstrated that, MeOH and DCM extracts were able to inhibit the growth of *A. salina* with LC_{50} of 68.20 ± 8.94 and 92.43 ± 4.27 μ g/mL, respectively. In the case of n-hexane extract no significant effect was observed.

Table 1. General toxicity of n-hexane, DCM and MeOH extracts of aerial parts of *Scrophularia subaphylla* against *Artemia salina*. Data are presented as mean \pm SD

Samples	LC_{50} (μ g/mL)
n-hexane	-
DCM	92.43 ± 4.27
MeOH	68.20 ± 8.94
Podophyllotoxin	2.69 ± 0.01

DCM, dichloromethane; MeOH, methanol.

Cytotoxic activity

Based on the BSLT findings, the DCM and MeOH extracts of *S. subaphylla* showed cytotoxic effects in comparison with n-hexane extract. Accordingly, DCM and MeOH extracts were selected for further anti-proliferative investigations against MCF-7, HT-29, and L929 as the cancerous and normal cell lines, respectively during 48 h period (Table 2). Hence, The MTT method was accomplished for assessing the cytotoxic activity. IC₅₀ values are shown in Table 2. According to the results Fig. 1, both MeOH and DCM extracts exhibited more cytotoxic effect on both HT-29 and MCF-7 cell

lines in comparison with control at 48 h incubation ($P < 0.001$). Moreover, the amount of IC₅₀ value of MeOH extract on MCF.7 cells ($241.90 \pm 30.10 \mu\text{g/mL}$) was lower than that of HT-29 cell line ($359.39 \pm 30.00 \mu\text{g/mL}$). Pure compounds, verbascoside and 3' *O* rhamnosyl -4' *O* para coumaryl 7- hydroxyl salidroside, in comparison with the control and other samples revealed considerable effects on cancerous cell lines ($P < 0.001$) (Fig. 1). Interestingly, L-929 as a normal cell line was not considerably affected by extracts and fractions as well as pure compounds.

Table 2. Antiproliferative activity of MeOH extracts, its Sep-Pak fractions, two pure isolated compounds, and DCM extract against MCF-7, HT-29, and L929 cell lines.

Samples	IC ₅₀ (μg/mL)		
	MCF-7	HT29	L929
DCM	300.8 ± 41.2	679.0 ± 53.2	> 600
MeOH	241.9 ± 30.1	359.3 ± 30.0	> 600
10% Sep-Pac	171.6 ± 45.3	401.2 ± 34.2	> 600
20% Sep-Pac	217.2 ± 50.2	494.9 ± 43.4	> 600
40% Sep-Pac	161.1 ± 23.2	220.3 ± 25.4	> 600
60% Sep-Pac	172.2 ± 26.3	251.8 ± 37.2	> 600
Verbasoside	0.39 ± 0.015	0.93 ± 0.06	> 600
3' <i>O</i> rhamnosyl -4' <i>O</i> para coumaryl 7- hydroxyl salidroside	0.53 ± 0.03	0.75 ± 0.04	> 600

DCM, dichloromethane; MeOH, methanol.

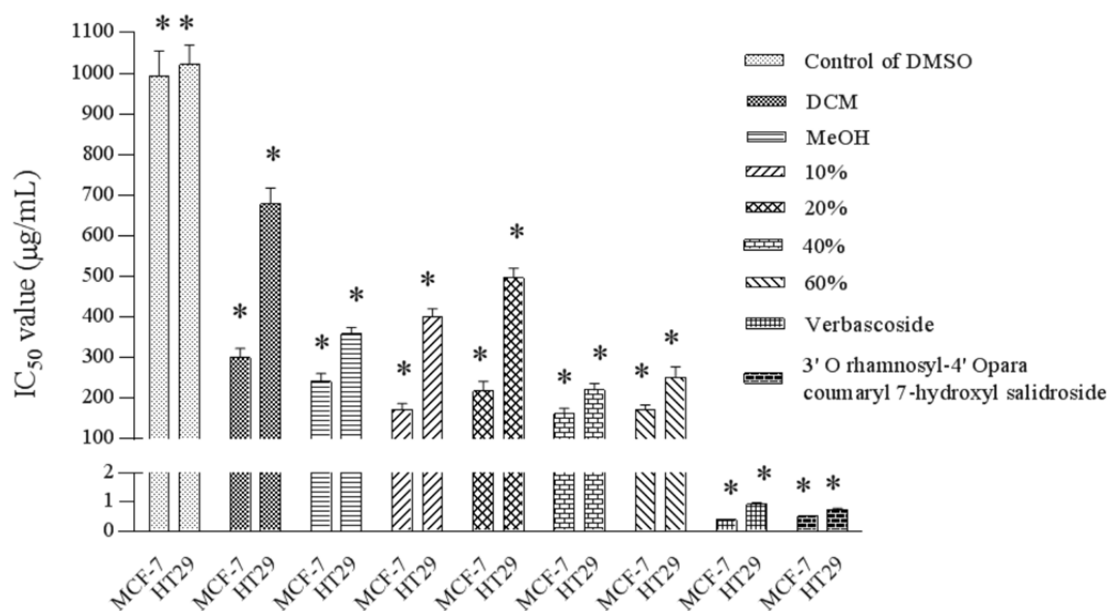


Fig. 1. Comparison of IC₅₀ values (μg/mL) of different samples with control group (DMSO) in MCF-7 and HT-29 cells. Data are presented as means ± SD. * Indicates significant differences compared with control group, $P < 0.001$.

NMR results

NMR spectroscopic data of both pure compounds are shown in Table 3 and the structure of compounds is illustrated in Fig. 2.

Antioxidant activity of *S. subaphylla* extracts and its fractions

The results of free radical scavenging activity of the extracts and Sep-Pak fractions of MeOH extract of *S. subaphylla* are shown in Table 4. In the current assay, SPE fractions of MeOH extract indicated free radical scavenging activities in a concentration-dependent manner. RC₅₀ values of 40% and 60% Sep-Pak fractions

were as following: (0.035 ± 0.004 and 0.055 ± 0.007 mg/mL, respectively).

Total flavonoids and phenolic contents

The results of TFC and TPC are presented in Table 4. The amount of flavonoids and phenolic contents in DCM and Sep-Pak fractions of MeOH extract were expressed as rutoside and gallic acid equivalents in mg/g dry samples. It is notable that the TFC and TPC contents of 40% Sep-Pak fraction were (113.70 ± 0.2 mg rutoside equivalent to 1 g of dried powdered plant material, and 154.09 ± 5.94 mg gallic acid equivalents 1 g of samples, respectively).

Table 3. Nuclear magnetic resonance spectroscopic data of compounds 1 and 2.

Position	Compound 1		Reference compound (1)		Compound 2		Reference compound (2)	
	δ_C	δ_H (J)	δ_C	δ_H (J)	δ_H (J)	δ_H (J)	δ_H (J)	δ_H (J)
1	129.21	-	131.60	-	-	-	-	-
2	113.60	6.61,s	116.40	6.65(d, 2)	6.88 (d,8.44)	7.00 (d, 8.40)	-	-
3	144.10	-	144.62	-	6.64 (d,8.34)	6.73 (d, 8.40)	-	-
4	143.56	-	146.08	-	-	-	-	-
5	115.79	6.63	117.17	6.68 (d, 8.2)	6.64 (d,8.34)	6.73 (d, 8.40)	-	-
6	119.58	6.48 (d, 7.4)	121.31	6.56 (d, 8.2)	6.88 (d,8.44)	7.00 (d, 8.40)	-	-
7	70.55	2.73	72.34	2.78	3-4*	2.99 (m)	-	-
8	34.96	3.67,3.87	36.51	3.71,3.90	3-4*	3.910,3.99 (m)	-	-
1'	102.29	4.35 (d, 7.78)	104.19	4.38 (d, 7.9)	4.44 (d,7.70)	4.41(7.8)	-	-
2'	74.60	3-4*	76.01	-	3-4*	-	-	-
3'	79.09	3-4*	81.66	-	3-4*	-	-	-
4'	69.16	4.68 (m,9.71)	70.69	-	5.10	-	-	-
5'	74.50	3-4*	76.18	-	3-4*	-	-	-
6'	60.75	-	62.49	-	4.12, 3.82	-	-	-
1''	101.24	5.01,s	102.96	5.18	5.11,s	5.064 (brs)	-	-
2''	70.39	3-4*	72.11	-	3-4*	-	-	-
3''	70.22	3-4*	72.34	-	3-4*	-	-	-
4''	71.65	3-4*	73.83	-	3-4*	-	-	-
5''	68.70	3-4*	70.69	-	3-4*	-	-	-
6'' (CH3)	18.12	0.94 (d, 6.04)	18.40	1.00 (d,6.1)	0.78 (d, 6.72)	1.00 (d,6.0)	-	-
1'''	125.55	-	127.72	-	-	-	-	-
2'''	114.74	7.02, s	115.38	7.06, d	7.92 (d,8.66)	7.52 (d,8.70)	-	-
3'''	145.52	-	146.77	-	7.34 (d,8.40)	6.86 (d,8.77)	-	-
4'''	148.44	-	149.72	-	-	-	-	-
5'''	116.21	6.71 (d, 8.13)	116.40	6.78 (d, 8.40)	7.34 (d,8.40)	6.86 (d,8.77)	-	-
6'''	121.43	6.98 (dd, 8.13)	123.18	6.95 (dd,8.40, 2.0)	7.92 (d,8.66)	7.52 (d,8.70)	-	-
7'''	115.41	6.20 (d, 15.86)	114.78	6.28 (d, 15.8)	6.30 (d, 15.90)	6.36 (d, 15.90)	-	-
8'''	147.50	7.45 (d, 15.73)	148.00	7.59 (d, 15.8)	7.47(d, 15.96)	7.69 (d, 15.90)	-	-
coo	165.72	-	168.35	-	-	-	-	-

¹H (400 MHz) and ¹³C (200 MHz) in dimethyl sulfoxide, δ in ppm, *J* in Hz; * overlapping signals in 3-4 ppm.

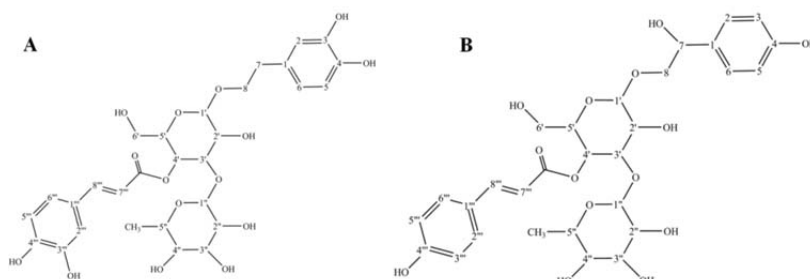
**Fig. 2.** Structures of (A) verbascoside and (B) 3' O rhamnosyl -4' O para coumaryl 7- hydroxyl solidoside

Table 4. Free radical scavenging activity, TPC and TFC of extracts, and methanolic fractions of *Scrophularia subaphylla*.

Extracts or fractions	TPC (as gallic acid equivalents) (mg/g)	TFC (mg/g)	Antioxidant activity (RC ₅₀ mg/mL)
n-hexane	2.43 ± 0.20	-	0.44 ± 0.183
DCM	56.3 ± 2.44	29.97 ± 2.11	0.65 ± 0.290
10% Sep-Pac	33.5 ± 1.00	13.38 ± 0.30	0.26 ± 0.017
20% Sep-Pac	90.4 ± 17.35	57.19 ± 0.45	0.09 ± 0.014
40% Sep-Pac	154.1 ± 5.94	113.7 ± 0.23	0.035 ± 0.004
60% Sep-Pac	116.3 ± 16.54	75.0 ± 3.40	0.055 ± 0.007
80% Sep-Pac	26.7 ± 0.13	-	0.22 ± 0.031
100% Sep-Pac	12.9 ± 0.45	-	0.311 ± 0.012

TPC, Total phenol content; TFC, total flavonoid content; DCM, dichloromethane;.

DISCUSSION

The current study was aimed to isolate the components of the methanolic extract of the aerial parts of *S. subaphylla* which had potent anti-proliferative activity. To this end, in the first step, general toxicity of all extracts against *A. salina* was evaluated. The potent extracts in BSLT were selected for further studies and subjected to MTT assay. Moreover, a bioassay-guided isolation and purification of principle components were performed. The structure elucidation of purified constituents was determined using ¹H- and ¹³C-NMR spectroscopies and also compared with former research reports (23,24). As shown in the extracts, MeOH and DCM extracts were able to inhibit the growth of *A. salina*. Hence, both extracts were selected for further evaluation against MCF-7, HT-29, and L929 as cancer and non-cancer cell lines. The cytotoxic results are indicated in Table 2 and Fig 2. The MeOH and DCM extracts considerably illustrated potent cytotoxic activity against MCF-7 and HT29 cell lines compared with control of DMSO ($P < 0.001$). Furthermore, MeOH-water Sep-Pak fractions demonstrated reduced viability of cancer cells. Subsequently, we examined the cytotoxic property of isolated pure compounds (such as acteoside and its derivative) of 40% Sep-Pak fraction. As indicated in Tables 2 and 3, two phenylpropanoid compounds were isolated through phytochemical analysis,

which illustrated inhibition effect on growth of cancer cell lines.

Verbascoside (**1**) is a famous phenylpropanoid which has been isolated and elucidated from the aerial parts and roots of many *Scrophularia* species (11,12,25). Based on our knowledge, this is the first report on verbascoside presence in the aerial parts of *S. subaphylla*.

Using RP-preparative-HPLC method, amorphous gummy solids were obtained following evaporation of pure compound solvents by vacuum under reduced pressure. The ¹H-NMR and ¹³C-NMR spectrum of compound **1** proposed the verbascoside structure for compound **1**. One group of aromatic protons belongs to the caffeic acid replacement, while others belong to the phenylethanoid structure. The structure of verbascoside is well known in many reviewed literature (23-25); our data are in good conformity with aforementioned studies. Existence of characteristic and typical peaks at δ 6.20 (*d*, 1H, $J = 15.86$ Hz) and δ 7.45 (*d*, 1H, $J = 15.73$ Hz) is the main cause for presence of phenyl propanoid structure. In addition, the presence of signal at 2.73 ppm is attributed to the CH₂ of the phenylethanoid side chain. Existence of a tri-substituted phenyl moiety indicated by the signals appearing at δ 7.02 (*bs*, 1H, H 2''), 6.98 (*d*, 1H, $J = 8.13$ Hz, H6'') and also 6.71 (*d*, 1H, $J = 8.13$ Hz, H 5'') belong to the phenylpropanoid aromatic protons. The remaining aromatic protons in $\delta = 6-7$ ppm are ascribed to 3, 4 di-hydroxy phenylethanoid moiety. The anomeric protons at 5.01 with

a small coupling and 4.35 ($J = 7.78$ Hz) were easily attributed to the H1'' and H1' rhamnose and glucose, respectively. The long coupling constant ($J = 7.78$ Hz) of the anomeric proton of glucose is in favor of a β -configuration. The methyl group of rhamnose was easily identified at $\delta = 0.94$ ppm as a doublet ($J = 6.04$ Hz). The other values for protons of glucose and rhamnose were located at $\delta = 3-4$ ppm. Some peaks were overlapped with water peak. Among the protons of glucose, H-4 glucose showed higher shift at 4.68 ppm, which proved the connection of caffeic acid substitution on C-4 glucose. ^{13}C -NMR spectrum of verbascoside was completely in agreement with published data (23-25).

Compound **2** is a derivative of compound **1**. Hence, the ^1H -NMR peaks of compound **2** indicated the identical patterns of signals observed for component **1**. Exclusive distinctions between **1** and **2** seem perspicuously as signals for aromatic protons of caffeic acid and phenyl ethyl alcohol and also for β -CH-OH of aromatic side chain of phenyl ethanoid. The rest of the signals were the same as acteoside. Concisely, ^1H -NMR data presented the two doublet signals series (1, 4 di substituted phenyl unit) at δ 7.92 (H2'', H6''), 7.34 (H3'', H5'') for phenyl propanoid and 6.88 (H2, H6), 6.64 (H3, H5) for phenyl ethanoid substitution, respectively. One proton at δ (4-5) ppm refers to β -CH-OH. As shown in Fig 2, both pure compounds illustrated potent cytotoxic activity against cancerous cell lines in comparison with control of DMSO as well as other samples ($P < 0.001$). Moreover, different studies revealed that cytotoxic mechanism of acteoside on various cancerous cells are mediated through inducing cell cycle arrest at G_0/G_1 phase and differentiation into monocyte (26). Another study by Inoue *et al.* showed the growth inhibitory effect of verbascoside by induction of apoptosis in HL-60 cell lines (27).

On the other hand, based on different reports, cytotoxic property of different species of *Scrophularia* have been evaluated thus far and it was concluded that various extracts of *S. striata*, as an example, (14-17) inhibited the viability of astrocyte cancer cells through

apoptosis mechanism. In all aforementioned reports, plants exhibited remarkable cytotoxic feature against abundant cancer cells in time and dose-dependent manner. In addition, they illustrated that potent extracts displayed dual cytotoxic activity as both cell apoptosis inducer and cell differentiation agents (16,26,27). In the present study, for more clarification of cytotoxic potency of MeOH extract, free radical scavenging activity as well as TPC and TFC of the extracts and fractions were evaluated and presented in Table 4. Fortunately, the results of free radical scavenging potency are in line with the cytotoxic activity. Sep-Pak fractions of MeOH extract showed high degree of cytotoxicity and free radical inhibition potency. It appears that not only endogenous free radical scavengers protect the body against oxidative stress and cancer, but also natural constituents as an exogenous antioxidants such as iridoids, polyphenols (phenylethanoid compounds), and flavonoids inhibit H_2O_2 -lipid peroxide free radicals which cause degenerative disease (28). Presence of various secondary metabolites in plants, which illustrates free radical scavenging and neoplastic activities, motivates researchers to isolate some anti-proliferative compounds such as phenyl propanoids against cancerous cell lines (29). In addition to the fact, cytotoxic property of the medicinal plants has been attributed to the presence of different secondary metabolites, like iridoids, polyphenols (phenylpropanoid, tannins, and flavonoids) (26). On the other hand, scientists had also verified that, as the phenolic contents of plants increase, their antioxidant activity also increases. It seems that there is a direct relationship between phenolic contents and quenching of free radicals (30). Nevertheless, polyphenol compounds are essential source of free radical scavengers, their ability to inhibit oxidizing agents that are involved in oxidative stress related diseases is different. Hence, it is very important to discover their principle function and mechanisms. In our study, the relationship between TFC and TPC as well as antioxidant capability along with cytotoxic activity of *S. subaphylla* Sep-Pak fractions of MeOH extract and also pure

compounds was evaluated. Our findings showed that, cytotoxic activity of the samples (extracts, fractions, and pure isolated compounds) against cancer cells increased with an increase in polyphenol content (TPC and TFC) of the herbal extract and excessive free radical quenching ability. Generally, the current survey recommends that the free radical scavenging and cytotoxic activity of *S. subaphylla* samples might be supportive in preventing or decelerating the improvement of diverse oxidative stress-linked diseases such as breast cancer. Undoubtedly, further approaches on verbascoside would be beneficial to recognize the mechanism of cytotoxic action. The authors of this manuscript believe this survey could provide the primary scenario for comprehensive cytotoxic consideration of this plant especially verbascoside. Hence, it is the primary description on the exploration of the cytotoxic and free radical scavenging ability of *S. subaphylla*.

CONCLUSION

In the present study two phenylpropanoids were isolated from *S. subaphylla* which inhibited the growth of cancer cells in a dose-dependent manner. Furthermore, it showed that TPC, TFC, and free radical scavenging activity of the samples were in line with each other.

ACKNOWLEDGMENT

The authors would like to express their appreciations to the Drug Applied Center as well as Monoclonal Antibody Research Center at Tabriz University of Medical Sciences, Tabriz, I.R. Iran, for their kind cooperation.

REFERENCES

1. Goodarzi S, Nateghpour M, Asgharian P, Hadjiakhoondi A, Yassa N, Tavakoli S, *et al.* Antimalarial and cytotoxic activities of roots and fruits fractions of *Astrodaucus persicus* extract. *Iran J Basic Med Sci.* 2017;20(12):1318-1323.
2. Willett W. The search for the causes of breast and colon cancer. *Nature.* 1989;338(6214):389-394.
3. Cassileth BR, Chapman CC. Alternative and complementary cancer therapies. *Cancer.* 1996;77(6):1026-1034.
4. Neidle S, Thurston DE. Chemical approaches to the discovery and development of cancer therapies. *Nat Rev Cancer.* 2005;5(4):285-296.
5. Browder T, Butterfield CE, Kråling BM, Shi B, Marshall B, O'Reilly MS, *et al.* Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res.* 2000;60(7):1878-1886.
6. Eskandani M, Abdolalizadeh J, Hamishehkar H, Nazemiyeh H, Barar J. Galbanic acid inhibits HIF-1 α expression via EGFR/HIF-1 α pathway in cancer cells. *Fitoterapia.* 2015;101:1-11.
7. Asgharian P, Heshmati Afshar F, Asnaashari S, Moghaddam SB, Ebrahimi A, Delazar A. Characterization of terpenoids in the essential oil extracted from the aerial parts of *Scrophularia subaphylla* growing in Iran. *Adv Pharm Bull.* 2015;5(4):557-561.
8. Asgharian P, Heshmati Afshar F, Asnaashari S, Bamdad Moghaddam S, Delazar A. The seasonal variations of the chemical composition of essential oil obtained from *Scrophularia frigida*. *Jundishapur J Nat Pharm Prod.* 2016;11(1):e29742-e29746.
9. Vaez H, Garjani A, Delazar A, Rameshrad M, Toutouchi NS, Nikkhah E, *et al.* Effect of methanolic extract of *Scrophularia subaphylla* on ischemia and reperfusion-induced myocardial injury. *Pharm Sci.* 2018;24(1):8-14
10. Garjani A, Vaez H, Delazar A, Rameshrad M, Heshmati Afshar F, Asgharian P. Cardioprotective effects of methanolic extract of *Scrophularia frigida* on ischemia-reperfusion-induced injuries in isolated rat heart. *Iran J Pharm Res.* 2017;16(Suppl):35-45.
11. Nikkhah E, Afshar FH, Babaei H, Delazar A, Asgharian P. Evaluation of phytochemistry and some biological activities of aerial parts and seeds of *Scrophularia umbrosa* Dumort. *Jundishapur J Nat Pharm Prod.* 2018;13(2):e65054-e65060.
12. Nikkhah E, Heshmati Afshar F, Babaei H, Asgharian P, Delazar A. Phytochemical analysis and *in-vitro* bioactivity of *Scrophularia Umbrosa* Rhizome (Scrophulariaceae). *Iran J Pharm Res.* 2018;17(2):85-94.
13. Heshmati Afshar F, Delazar A, Asnaashari S, Vaez H, Asgharian P. Screening of anti-malarial activity of different extracts obtained from three species of *Scrophularia* growing in Iran. *Iran J Pharm Res.* 2018;17(2):668-676.
14. Asgharian P, Heshmati Afshar F, Asnaashari S, Lotfipour F, Baradaran B. Evaluation of various biological activities of the aerial parts of *Scrophularia frigida* growing in Iran. *Iran J Pharm Res.* 2017;16(1):277-289.
15. Hosseini BA, Pasdaran A, Kazemi T, Shanehbandi D, Karami H, Orangi M, *et al.* Dichloromethane fractions of *Scrophularia oxysepala* extract induce apoptosis in MCF-7 human breast cancer cells. *Bosn J Basic Med Sci.* 2015;15(1):26-32.

16. Orangi M, Pasdaran A, Shanebandi D, Kazemi T, Yousefi B, Hosseini BA, et al. Cytotoxic and apoptotic activities of methanolic subfractions of *Scrophularia oxyssepala* against human breast cancer cell line. Evid-Based Complementary Altern Med. 2016;2016. Article ID 8540640.
17. Sajjadi E, Delazari Z, Aghaei M, Ghannadian M. Flavone constituents of *Phlomis bruguieri* Desf. with cytotoxic activity against MCF-7 breast cancer cells. Res Pharm Sci. 2018;13(5):422-429.
18. Tofighi Z, Asgharian P, Goodarzi S, Hadjiakhoondi A, Ostad SN, Yassa N. Potent cytotoxic flavonoids from Iranian *Securigera securidaca*. Med Chem Res. 2014;23(4):1718-1724.
19. Yari pour S, Delnavazi MR, Asgharian P, Valiyari S, Tavakoli S, Nazemiyeh H. A survey on phytochemical composition and biological activity of *Zygophyllum Fabago* from Iran. Adv Pharm Bull. 2017;7(1):109-114.
20. Hamishehkar H, Ranjdoost F, Asgharian P, Mahmoodpoor A, Sanaie S. Vitamins, are they safe? Adv Pharm Bull. 2016;6(4):467-477.
21. Panahi Kokhdan E, Sadeghi H, Ghafoori H, Sadeghi H, Danaei N, Javadian H, et al. Cytotoxic effect of methanolic extract, alkaloid and terpenoid fractions of *Stachys pilifera* against HT-29 cell line. Res Pharm Sci. 2018;13(5):404-412.
22. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med. 1982;45(1):31-34.
23. Wong IY, Dan ZD, Huang Y, Chen ZY. Antioxidative activities of phenylethanoid glycosides from *Ligustrum purpurascens*. J Agric Food Chem. 2001;49(6):3113-3119.
24. Zhang SQ, Li ZJ, Wang AB, Cai MS, Feng R. Synthesis of a phenylpropanoid glycoside, Osmanthuside B6. Carbohydr Res. 1998;308(3-4):281-285.
25. Fernández L, de Santos J, Díaz-Lanza AM, Pérez E, Villaescusa L. Quantitative determination of verbascoside in *Scrophularia scorodonia*. By high-performance liquid chromatography. Pharm biol. 2005;43(3):226-229.
26. Lee KW, Kim HJ, Lee YS, Park HJ, Choi JW, Ha J, et al. Acteoside inhibits human promyelocytic HL-60 leukemia cell proliferation via inducing cell cycle arrest at G0/G1 phase and differentiation into monocyte. Carcinogenesis. 2007;28(9):1928-1936.
27. Inoue M, Sakuma Z, Ogihara Y, Saracoglu I. Induction of apoptotic cell death in HL-60 cells by acteoside, a phenylpropanoid glycoside. Biol Pharm Bull. 1998;21(1):81-83.
28. Kanadaswami C, Lee LT, Lee PP, Hwang JJ, Ke FC, Huang YT, et al. The antitumor activities of flavonoids. In Vivo. 2005;19(5):895-909.
29. Kumazawa S, Taniguchi M, Suzuki Y, Shimura M, Kwon MS, Nakayama T. Antioxidant activity of polyphenols in carob pods. J Agric Food Chem. 2002;50(2):373-377.
30. Rietjens IM, Boersma MG, van der Woude H, Jeurissen SM, Schutte ME, Alink GM. Flavonoids and alkenylbenzenes: mechanisms of mutagenic action and carcinogenic risk. Mutat Res. 2005;574(1-2):124-138.