

***In vitro* Cytotoxic Activity of *Salsola oppositifolia* Desf. (Amaranthaceae) in a Panel of Tumour Cell Lines**

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Z. Naturforsch. **63c**, 347–354 (2008); received October 8/November 26, 2007

The aim of the present study was to evaluate for the first time the *in vitro* cytotoxic activity of fractions and isolated flavonols from *Salsola oppositifolia* Desf. (Amaranthaceae). The *n*-hexane fraction demonstrated an effective cytotoxic activity on the large lung carcinoma and amelanotic melanoma cell lines with IC₅₀ values of 19.1 µg/ml and 24.4 µg/ml, respectively. Also the dichloromethane fraction exhibited cytotoxic activity against COR-L23 (IC₅₀ 30.4 µg/ml) and C32 (IC₅₀ 33.2 µg/ml) cells, while the EtOAc fraction demonstrated a selective cytotoxic activity against MCF-7 cells (IC₅₀ 67.9 µg/ml). The major active constituents of this fraction were isorhamnetin-3-*O*-glucoside (**1**) and isorhamnetin-3-*O*-rutinoside (**2**), which showed an interesting activity against the cell line MCF-7 with IC₅₀ values of 18.2 and 25.2 µg/ml, respectively. Compound **2** exhibited a strong activity against the hormone-dependent prostate carcinoma LNCaP cell line with an IC₅₀ of 20.5 µg/ml. Constituents of *S. oppositifolia* were identified by GC-MS and NMR analyses.

Key words: *Salsola oppositifolia* Desf., Cytotoxicity, Tumour Cells, Flavonols

Introduction

In spite of the great advances in modern medicine in recent decades plants still make an important contribution to health care. Plants provide chemical diversity and bioactivity which have led to the development of hundreds of pharmaceutical drugs. Therefore, many medicinal herbs have been examined to identify new and effective anticancer compounds (Shu, 1998). Up till now, nature has been a good source for the discovery of new and potent anticancer agents such as vinblastine, vincristine, camptothecin and taxol (Nakagawa-Goto *et al.*, 2007).

Flavonoids represent one of the most important and interesting classes of biologically active naturally occurring compounds. These compounds consist of a group of polyphenolic secondary metabolites derived from 2-phenylchromane present in considerable quantities in fruits and vegetables. Flavonoids, together with some antioxidative vitamins, carotenoids and others, belong to a chemically heterogeneous group of small molecules with chemopreventive activity. Moreover, flavonoids exert specific cytotoxic activity towards cancer cells which has generated large interest in devel-

oping flavonoid-based cytostatics for anticancer therapy (Galati and O'Brien, 2004). Different mechanisms are linked to flavonoid-mediated cytotoxicity, including proteasome inhibition (Chen *et al.*, 2005), inhibition of fatty acid synthesis (Bruselmans *et al.*, 2005), topoisomerase inhibition (Constantinou *et al.*, 1995), inhibition of phosphatidylinositol 3-kinase (Agullo *et al.*, 1997), induction of cell cycle arrest (Lepley *et al.*, 1996), induction of p53 (Plaumann *et al.*, 1996) or enhanced expression of c-fos and c-myc proto-oncogenes (Chen *et al.*, 1998).

As part of our screening program that considers the search for natural products with anticancer properties (Tundis *et al.*, 2005; Loizzo *et al.*, 2005), the aim of the present investigation was to explore for the first time the potential cytotoxic activity of *Salsola oppositifolia* Desf. fractions and identified bioactive compounds. The genus *Salsola* (Amaranthaceae) comprises about 120 species and is most common in temperate and subtropical Asia, Europe, Africa and North America. Amaranthaceae is a cosmopolitan family that consists mainly of herbs, but includes some shrubs and even small trees. Many members of the family are particularly characteristic of maritime regions and localities

with saline soils. In correlation with this type of habitat, the leaves are often succulent or reduced in size, whilst, in some instances, the stem is succulent and takes on the function of carbon assimilation. Different *Salsola* species are utilized in local traditional medicine for their anticancer, antihypertensive, diuretic, emollient, purgative, anti-ulcer and anti-inflammatory properties (Fu, 1959; Hartwell, 1967–1971; Woldu and Abegaz, 1990; Al-Saleh *et al.*, 1993; Nikiforov *et al.*, 2002). In order to determine whether *S. oppositifolia* exhibits cytotoxic activity we analyzed *n*-hexane, dichloromethane, ethyl acetate and diethyl ether fractions and isolated flavonoids on renal adenocarcinoma ACHN, amelanotic melanoma C32, non-small cell lung carcinoma COR-L23, hormone-dependent prostate carcinoma LNCaP and breast adenocarcinoma MCF-7 human tumour cell lines.

Materials and Methods

General experimental procedures

Gas chromatography-mass spectrometry (GC-MS) analyses were recorded on a Hewlett-Packard 6890 gas chromatograph equipped with a SE30 non-polar capillary column (30 m × 0.25 mm i.d. × 0.25 mm film thickness) and interfaced with a Hewlett Packard 5973 mass selective detector operating in electron ionization (E.I.) mode (70 eV). Helium was used as carrier gas. The analytical conditions were as follows: oven temperature 3 min isothermal at 60 °C, then 60–280 °C at a rate of 16 °C/min, and finally 15 min isothermal at 280 °C. Injector and detector were maintained at 250 °C and 280 °C, respectively. Nuclear magnetic resonance (NMR) experiments were performed using a Bruker Avance 300 MHz spectrometer (solvent CD₃OD). The signals in the ¹H and ¹³C NMR spectra were assigned unambiguously using 2D NMR techniques. Mass spectra (MS) were performed using an electrospray ionization mass spectrometer ZAB2-SEQ. Infrared spectra were recorded on a Perkin-Elmer FT 1600 spectrometer. UV spectra were recorded on a Beckman DU 640 apparatus. TLC analyses were carried out on silica gel 60 F₂₅₄ precoated plates (VWR International, Milan, Italy). Silica gel 60 (0.040–0.063 mm, VWR International) was utilized for column chromatography (CC). Methanol, chloroform, dichloromethane, *n*-hexane, dimethylsulphoxide (DMSO), ethyl acetate, sulfuric acid, NH₄OH, diethyl ether, Dragendorff's reagent DRG, HOAc were ob-

tained from VWR International. Folin-Ciocalteu reagent was used for the determination of the total phenolics content. Roswell Park Memorial Institute (RPMI) 1640, Dulbecco's modified essential medium (DMEM), L-glutamine, penicillin/streptomycin, foetal bovine serum, sulforhodamine B (SRB), trichloroacetic acid (TCA), vinblastine sulfate, taxol and tris(hydroxymethyl)aminomethane (Sigma-Aldrich, Milan, Italy) were used for the SRB assay.

Plant material

S. oppositifolia aerial parts were collected in Sicily (2004) and authenticated by Dr. N. G. Passalacqua, Natural History Museum of Calabria and Botanical Garden of Calabria University, Italy and Dr. L. Peruzzi, Biology Department, University of Pisa, Italy. A voucher specimen (No. 12345) was deposited in the Botany Department Herbarium at the Calabria University (CLU), Italy.

Extraction and isolation

The air-dried aerial parts of *S. oppositifolia* (200 g) were powdered and exhaustively extracted with methanol for 48 h (3 × 2.5 l) through maceration at room temperature. The combined methanolic extracts were evaporated to dryness *in vacuo* (yield 22.2%). In order to separate of the chemical compounds in function of their polarity, the crude extract was dissolved in distilled water and partitioned with *n*-hexane (yield 1.1%), dichloromethane (yield 0.5%) and ethyl acetate (yield 0.6%). The aqueous residue was alkalized with NH₄OH and extracted by diethyl ether (yield 0.2%). No more alkaloids could be detected in the aqueous phase (TLC, silica gel, eluent CH₂Cl₂/MeOH 85:15 v/v, detection by Dragendorff's reagent). In order to determine the composition of the *n*-hexane, dichloromethane and diethyl ether fractions analyses were carried out using a gas chromatograph system. Instead, the ethyl acetate fraction was subjected to column chromatography over silica gel 60 (0.040–0.063 mm) using a CHCl₃/MeOH/H₂O gradient system (CHCl₃/MeOH 98:2 to CHCl₃/MeOH/H₂O 70:26:4) to yield fractions A–L. Column fractions were assayed according to their TLC profile on silica gel and detected by UV light at 254 and 365 nm, natural products reagent, polyethylene glycol reagent, and H₂SO₄ 50% v/v. Fractions E and F were further purified by silica gel column chromatography using a

CH₂Cl₂/MeOH/H₂O gradient system (CH₂Cl₂/MeOH/H₂O 90:9:1 to 30:67:3) and preparative thin layer chromatography on silica gel to afford isorhamnetin-3-*O*-glucoside (**1**, 63 mg, yield 5.12% w/w) and isorhamnetin-3-*O*-rutinoside (**2**, 87 mg, yield 7.07% w/w) (Fig. 1).

Determination of total phenolics content

Total phenolics content of the AcOEt fraction was determined by employing a method involving the Folin-Ciocalteu reagent and chlorogenic acid as a standard (Gao *et al.*, 2000). The fraction was mixed with 0.2 ml Folin-Ciocalteu reagent, 2 ml of H₂O and 1 ml of 15% Na₂CO₃, and the absorbance was measured at 765 nm (Perkin Elmer Lambda 40 UV/VIS spectrophotometer) after 2 h of incubation at room temperature. The total phenolic content was expressed as chlorogenic acid equivalents in mg/g dry material.

Cell culture and in vitro cytotoxicity assay

Cytotoxic screening models provide important preliminary data to select plants with potential anticancer compounds; therefore the SRB assay, used in this study, is commonly employed. Five cancer cell lines, renal adenocarcinoma ACHN (ATCC No. CRL-1611), hormone-dependent prostate carcinoma LNCaP (ATCC No. CRL-1740), human breast adenocarcinoma MCF-7 (ATCC No. HTB-22), amelanotic melanoma C32 (ATCC No. CRL-1585) and large cell lung carcinoma COR-L23 (ECACC No. 92031919), were used. The cytotoxic assay was performed following a published protocol (Loizzo *et al.*, 2005). In brief, the COR-L23, ACHN, C32 and LNCaP tumour cells were cultured in RPMI 1640 medium while MCF-7 cells were cultured in DMEM under a controlled humidified atmosphere (5% CO₂, 37 °C) in 96-well microtitre plates seeded in a range from 5 · 10⁴ to 15 · 10⁴ per well. After 24 h the cells were treated with the serial dilutions of samples. Each sample was initially dissolved in an amount of DMSO (0.5%) and further diluted in medium to produce different concentrations (from 1 to 100 µg/ml for fractions and from 0.25 to 50 µg/ml for isolated compounds). Eight replicates were performed for each concentration. The plates were incubated for a select exposure time of 48 h. At the end of exposure, 100 µl of ice-cold 40% TCA were added to each well, left at 4 °C for 1 h and washed five times with distilled water. The TCA-fixed cells were

stained for 30 min with 50 µl of 0.4% (w/v) SRB in 1% HOAc. The plates were washed five times with 1% HOAc and air-dried overnight. Vinblastine sulfate was used as positive control for C32, LNCaP, ACHN and COR-L23 cells while taxol was used for MCF-7 cells. The absorbance of each well was read on a Spectra Max plate reader 384 (Molecular Device, Celbio, Milan, Italy) at 564 nm.

Statistical analysis

All experiments were carried out in triplicate. Data were expressed as means ± SD. Differences were evaluated by the one-way analysis of variance (ANOVA) test completed by a multicomparison Dunnett's test. Differences were considered significant at ** *p* < 0.01. The inhibitory concentration 50% (IC₅₀) was calculated by a nonlinear regression curve with the use of Prism GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, USA). The dose-response curve was obtained by plotting the percentage of cell viability versus the concentrations.

Results and Discussion

The objective of this study was to evaluate the cytotoxic potential of *S. oppositifolia* and to analyze fractions in order to characterize them with respect to the composition. The second objective was to determine the biological activity of isolated flavonols in order to correlate their cytotoxic activity with the derived fraction.

Approximately 60% of all drugs in clinical trials against cancer are either natural products or their modified versions often displaying cytotoxic effects. Cytotoxicity is commonly used as a target for the discovery of novel anticancer compounds, and the sulforhodamine B (SRB) assay is commonly employed since it is relatively inexpensive, easy to perform and allows for a large number of samples to be processed in short time. The latter is of important advantage as different cell lines have variable susceptibility to different extracts or compounds. The cytotoxic response is dependent on the cell line tested, and obtained results cannot be applied to other cell lines (Monks *et al.*, 1991). Fractions from *S. oppositifolia* were able to inhibit the *in vitro* proliferation of five human tumour cell lines in a concentration-dependent manner (Fig. 1). All results are summarized in Table I. The EtOAc fraction displayed a cytotoxic activity

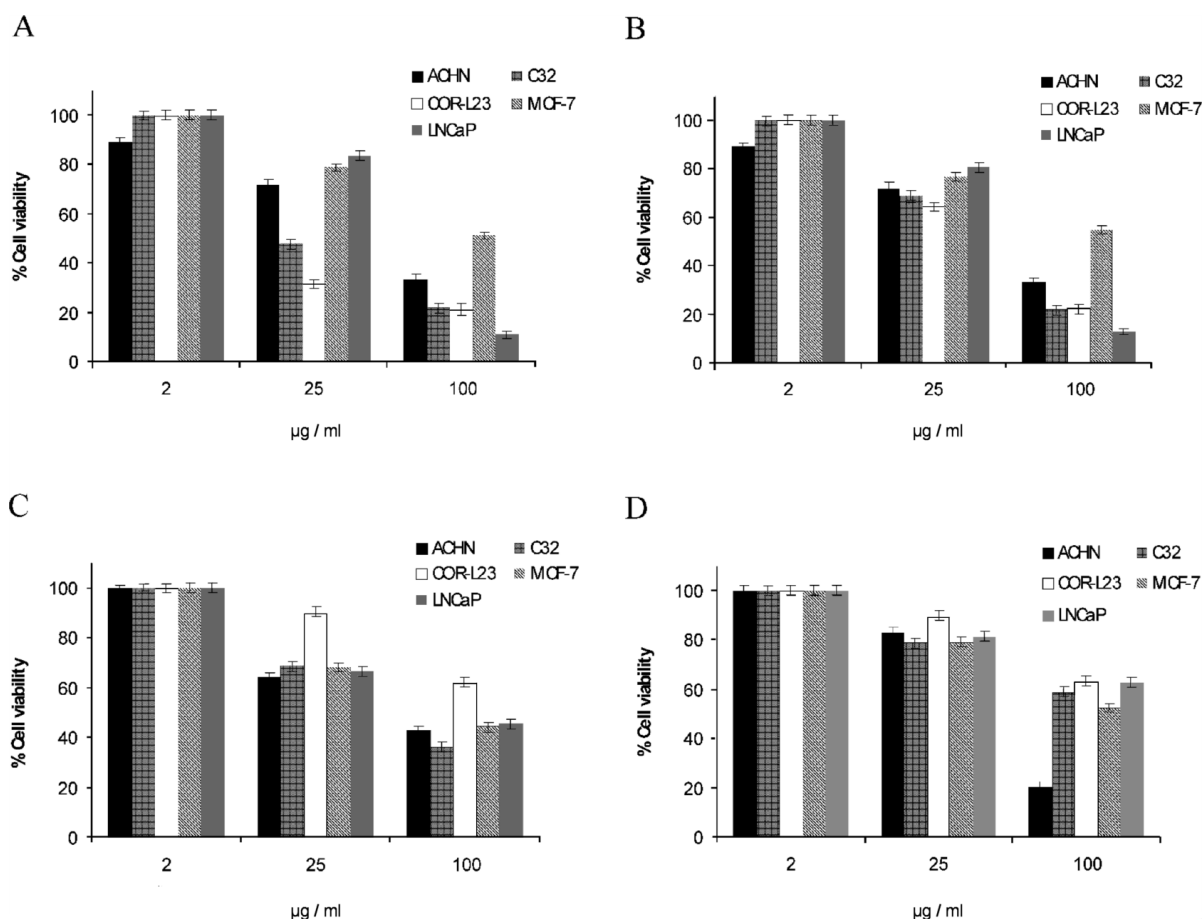
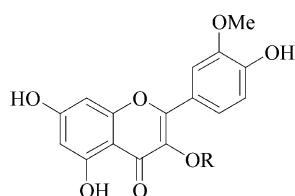


Fig. 1. Effect on tumour cell lines growth of (A) *n*-hexane, (B) dichloromethane, (C) ethyl acetate and (D) diethyl ether fractions. Values are presented as means ($n = 3$) of percentage of cell growth (%) \pm SD.



1: R = Glc
2: R = Rut

Fig. 2. Structure of isolated flavonols: isorhamnetin-3-*O*-glucoside (**1**) and isorhamnetin-3-*O*-rutinoside (**2**).

with IC_{50} values ranging from $56.4 \mu\text{g/ml}$ against C32 to $88.6 \mu\text{g/ml}$ against ACHN cells. Interestingly, it exhibited a selective cytotoxic activity against MCF-7 cells (IC_{50} $67.9 \mu\text{g/ml}$) compared to the other fractions. The ability of this fraction may

be ascribed to one or more of the polyphenols identified in this fraction. The AcOEt fraction was, in fact, investigated for the total phenolics content by the Folin-Ciocalteu reagent expressed as chlorogenic acid equivalents. It was demonstrated that this fraction possessed 62.48 mg/g of dry material. Purification of the fraction on a silica gel chromatographic column yielded two flavonols, isorhamnetin-3-*O*-glucoside (**1**) and isorhamnetin-3-*O*-rutinoside (**2**) (Fig. 2). The NMR spectra were consistent with those previously reported (Harborne and Mabry, 1982; Slimstad *et al.*, 1995). Although these flavonoids have been previously isolated and identified in different species, herein we report for the first time their isolation from *S. oppositifolia* as well as their cytotoxicity against the tumour cell lines used in this study. Our results demonstrated that compounds **1** and **2** were able

Table I. Cytotoxic activity of *S. oppositifolia* fractions.

	IC ₅₀ [μg/ml]				
	ACHN	LNCaP	COR-L23	C32	MCF-7
<i>Fraction</i>					
<i>n</i> -Hexane	43.4 ± 1.42**	45.1 ± 2.05**	19.1 ± 1.06**	24.4 ± 1.64**	> 100
Dichloromethane	40.4 ± 1.35**	41.9 ± 1.82**	30.4 ± 1.18**	33.2 ± 2.10**	> 100
Ethyl acetate	77.2 ± 2.47**	88.6 ± 2.06**	> 100	56.4 ± 1.22**	67.9 ± 1.23**
Diethyl ether	46.8 ± 2.12**	> 100	> 100	> 100	> 100
<i>Compound</i>					
Isorhamnetin-3- <i>O</i> -glc (1)	26.1 ± 1.06**	28.5 ± 1.10**	–	24.3 ± 0.96**	18.2 ± 0.55**
Isorhamnetin-3- <i>O</i> -rut (2)	27.6 ± 1.46**	20.5 ± 1.12°	–	29.2 ± 0.80**	25.2 ± 0.76**
<i>Positive control</i>					
Vinblastine sulfate	22.7 ± 0.14	29.3 ± 0.86	45.4 ± 1.05	3.0 ± 0.08	–
Taxol	–	–	–	–	0.1 ± 0.006

Vinblastine sulfate (for C32, LNCaP, ACHN and COR-L23) and taxol (for MCF-7) were used as positive control. Data are given as the mean of at least three independent experiments ± SD.

** $p < 0.01$ vs. positive control; ° $p > 0.05$ vs. positive control.

to inhibit the proliferation of MCF-7 cells with IC₅₀ values of 18.2 and 25.2 μg/ml, respectively (Table I, Fig. 3). Moreover, **1** showed a good cytotoxic activity against the renal adenocarcinoma and the hormone-dependent prostate carcinoma cells with IC₅₀ values of 26.1 and 28.5 μg/ml, respectively. Isorhamnetin-3-*O*-rutinoside (**2**) was the most active on LNCaP cells (IC₅₀ 20.50 μg/ml). Interestingly, the ability of **2** to inhibit LNCaP proliferation was comparable to the positive control vinblastine sulphate (IC₅₀ 29.3 μg/ml). Although isorhamnetin-3-*O*-glucoside (**1**) and isorhamnetin-3-*O*-rutinoside (**2**) clearly showed activity, it can be seen that the activity of the EtOAc fraction cannot be explained only in terms of its flavonols content. Table II compares the IC₅₀ value of the fraction and its flavonols content, and it can be seen that the concentration of both flavonols in all cases is below its IC₅₀ value. Other active substances may be present or other compounds may have a synergistic effect on the activity of compounds **1** and **2**. It has been reported that isorhamnetin exerted cytotoxic activity against human hepatocellular carcinoma cells, induced cell cycle arrest at G₁ phase (Teng *et al.*, 2006) and inhibited the proliferation of HL-60 and Lovo tumour cell lines (Innocenti *et al.*, 2006). Recently, the molecular mechanism that underlies the growth inhibition and apoptosis stimulatory effects of this aglycone on Eca-109 was investigated. Isorhamnetin has the ability to down-regulate bcl-2, c-myc and H-ras and to up-regulate Bax, c-fos and p53 expressions (Ma *et al.*, 2007).

Both non-polar fractions exhibited interesting cytotoxic activities against all tested tumour cell lines except against MCF-7 cells. In particular, the *n*-hexane fraction was more selective against COR-L23 compared with C32 cells with IC₅₀ values of 19.1 μg/ml and 24.4 μg/ml, respectively. A lower activity was found against ACHN and LNCaP cells (IC₅₀ values of 43.4 μg/ml and 45.1 μg/ml, respectively). In an attempt to identify putative active compounds present in this fraction, we employed a GC-MS system. Identification of the compounds was based on the comparison of the mass spectral data with Wiley 138 and Wiley 275 mass spectral libraries built from pure substances. The results are reported in Table III. Eighteen constituents, representing 97.5% of the total, were identified. Methyl palmitate (14.9%), methyl stearate (9.7%) and palmitic acid (9.8%) were the main abundant compounds in the *n*-hexane fraction.

The dichloromethane fraction showed the most interesting biological activity on COR-L23 and C32 cell growth inhibition (IC₅₀ values of 30.4 and 33.2 μg/ml, respectively). Comparable results to the *n*-hexane fraction were obtained against the renal adenocarcinoma and the hormone-dependent prostate carcinoma cells with IC₅₀ values of 40.4 μg/ml and 41.9 μg/ml, respectively. The chemical analysis revealed the presence of ten constituents, representing 75.61% of the total. Linoleic acid (18.3%), 2-monolinolenin (16.7%), linoleic acid-2-hydroxy-1-(hydroxymethyl) ethyl

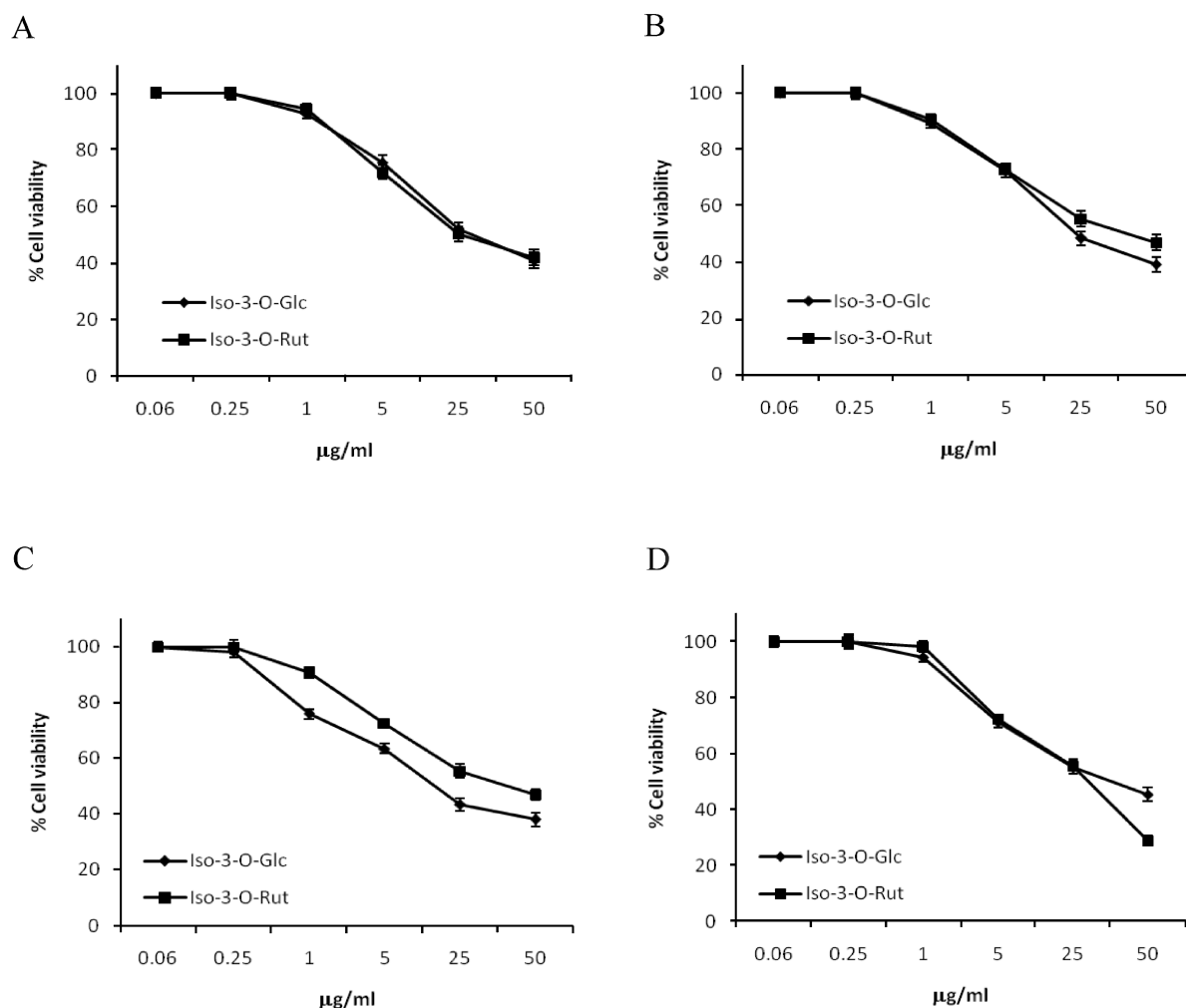


Fig. 3. Effect of isorhamnetin-3-*O*-glucoside and isorhamnetin-3-*O*-rutinoside on (A) ACHN, (B) C32, (C) MCF-7 and (D) LNCaP cell growth. Values are presented as means ($n = 3$) of percentage of cell growth (%) \pm SD.

Table II. Comparison of cytotoxicity activities of EtOAc fraction and isolated compounds.

Cell type	IC ₅₀ (EtOAc)	Corresponding concentration of (1) [µg/ml]	IC ₅₀ [µg/ml] (1)	Corresponding concentration of (2) [µg/ml]	IC ₅₀ [µg/ml] (2)
ACHN	77.2 \pm 2.47	3.95	26.1 \pm 1.06	5.46	27.6 \pm 1.46
LNCaP	88.6 \pm 2.06	4.5	28.5 \pm 1.10	6.60	20.5 \pm 1.12
C32	> 100	> 5.12	24.3 \pm 0.96	> 7.07	29.2 \pm 0.80
MCF-7	67.9 \pm 1.23	3.48	18.2 \pm 0.55	4.84	25.2 \pm 0.76

Data are given as the mean of at least three independent experiments \pm SD.

ester (12.5%) and palmitic acid (9.3%) were the main constituents.

Free fatty acids are known to play an important role in numerous biological functions, including

cell proliferation and apoptosis induction (Harada *et al.*, 2002; Hardy *et al.*, 2003). Palmitic acid showed selective cytotoxicity to human leukemic cells and induced the apoptosis in the human leu-

Table III. Major constituents and their relative abundance (%) of *S. oppositifolia* *n*-hexane and dichloromethane fractions determined by GC-MS.

Compound	<i>t_R</i> [min] ^a	Abundance (%) ^b		Method ^c
		<i>n</i> -Hexane fraction	Dichloromethane fraction	
Methyl palmitate	18.42	14.9 ± 0.13	–	GC-MS, CoI
Palmitic acid	18.75	9.8 ± 0.12	9.3 ± 0.14	GC-MS, CoI
Methyl linoleate	19.71	7.3 ± 0.16	2.9 ± 0.09	GC-MS
Methyl linolenate	19.77	8.6 ± 0.15	5.5 ± 0.10	GC-MS
Phytol	19.86	8.4 ± 0.10	–	GC-MS
Methyl stearate	19.89	9.7 ± 0.10	–	GC-MS, CoI
Linoleic acid-2-hydroxy-1-(hydroxymethyl) ethyl ester	23.95	7.0 ± 0.11	12.5 ± 0.24	GC-MS
2-Monolinolenin	24.03	5.9 ± 0.02	16.7 ± 0.19	GC-MS
β-Sitosterol	34.54	8.9 ± 0.21	–	GC-MS, CoI
Linoleic acid	20.09	–	18.3 ± 0.22	GC-MS

^a Retention time on MS SE-30 non-polar column.^b Abundance calculated as % peak area mean values, mean ± SD, *n* = 3.^c CoI, co-injection.

kemic cell line MOLT-4 at maximum concentration tested. Palmitic acid also demonstrated *in vivo* antitumour activity in mice. One molecular target of palmitic acid in tumour cells is DNA topoisomerase I, however, interestingly, it does not affect DNA topoisomerase II, suggesting that palmitic acid may be a lead compound of anticancer drugs (Harada *et al.*, 2002). Recently, the *in vitro* cytotoxic activity of fatty acids and methyl esters against various cancer cells was reported (Takeara *et al.*, 2007; Yoo *et al.*, 2007). In particular, a methyl esters mixture containing methyl palmitate and methyl stearate was investigated for mechanisms involved in the cytotoxic activity. This mixture was more active than single standards against all tested cell lines such as acute promyeloblastic leukemia HL-60, chronic myelogenic leukemia K-562, lymphoblastic leukemia CEM, and T-cell leukemia MOLT-4 (Takeara *et al.*, 2007).

On the basis of their chemical composition the interesting cytotoxic activity of the two non-polar fractions especially against COR-L23 and C32 cells may be attributable to the presence of fatty acids and methyl esters.

The diethyl ether fraction showed a selective activity against the ACHN cell line (IC₅₀ value of 46.8 µg/ml). The GC-MS analysis revealed the presence of two known tetrahydroisoquinoline alkaloids such as salsoline and salsolidine, previously isolated from *S. kali*, *S. soda*, *S. ruthenica* and *S. richteri* (Borkowski *et al.*, 1959; Kuznetsova *et al.*, 2005).

Plants are an excellent source of chemical compounds with a wide variety of biological activities, including anticancer properties. Fundamental phytochemical investigations should be encouraged, especially in view of the urgent need to discover new bioactive molecules with greater efficacy and less side effects than existing drugs.

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