PHYTOTHERAPY RESEARCH
Phytother. Res. **26**: 1023–1028 (2012)
Published online 20 December 2011 in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.3678

Antiproliferative Activity of Flavonoids: Influence of the Sequential Methoxylation State of the Flavonoid Structure

Ghazaal Moghaddam,[1] Soltan Ahmad Ebrahimi,[1]* Nahid Rahbar-Roshandel[1] and Alireza Foroumadi[2]

[1]Department of Pharmacology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[2]Department of Medicinal Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran, Iran

***Dracocephalum kotschyi* Boiss. has been used as part of an ethnobotanical remedy against many forms of human cancer in Iran. It has been demonstrated that a flavonoid named xanthomicrol from *D. kotschyi* contributes to its preferential antiproliferative activity against malignant cells. In the present study, the antiproliferative activity of its flavonoid fraction was further characterized. Using liquid–liquid extraction and a semi-preparative reversed-phase HPLC method, eight flavonoid aglycones were isolated from the aerial parts of the plant and their identities were confirmed through MS and NMR analyses as luteolin, naringenin, apigenin, isokaempferide, cirsimaritin, penduletin, xanthomicrol and calycopterin. The *in vitro* antiproliferative activity of each compound was evaluated against a panel of established normal and malignant cell lines using the MTT assay and some structure–activity relationships were observed. The hydroxyflavones (luteolin, apigenin and isokaempferide) exerted comparable antiproliferative activities against malignant and normal cells, while the methoxylated hydroxyflavones (cirsimaritin, penduletin, xanthomicrol and calycopterin) showed preferential activities against tumor cells. This activity may be of value in treating tumors as it would exert few side effects in normal tissues. Xanthomicrol selectively inhibited the growth of human gastric adenocarcinoma, while calycopterin selectively prevented human acute promyelocytic leukemia and human colon carcinoma cells proliferation. Copyright © 2011 John Wiley & Sons, Ltd.**

Keywords: Dracocephalum kotschyi; RP-HPLC; antiproliferative; flavonoids; SAR.

INTRODUCTION

Research on new anticancer agents is currently rigorously pursued, mainly due to the unacceptable toxicities associated with the conventional cancer chemotherapeutic agents. The therapeutic indices of these drugs are often small, with little difference between the dose required for their tumor preventive activity and their toxic dose in normal tissues. Therefore, agents that preferentially inhibit the proliferation of malignant cells while leaving normal cells unimpaired could, potentially, play an important role in anticancer chemotherapy (Buolamwini, 1999; Morley *et al*., 2007).

Many clinically useful drugs are either natural products or have been developed from naturally occurring compounds (Butler, 2004). Among the various chemicals isolated from natural sources, flavonoids have attracted much attention due to their broad range of pharmacological activities (Cushnie and Lamb, 2005; Kawai *et al*., 2007; Garcia-Lafuente *et al*., 2009), most important of which is their antiproliferative activity against malignant cells in *in vitro* and *in vivo* models (Kawaii *et al*., 1999; Murakami *et al*., 2000; Cardenas *et al*., 2006; Tang *et al*., 2007). Flavonoids comprise over 4000 structurally related compounds which exist in nature either as free aglycones or glycosidic conjugates and are generally classified according to their chemical structures into flavones, flavanones, flavanols, flavonols and anthocyanidins (Middleton *et al*., 2000). This diversity of structural patterns has turned flavonoids into a rich source of compounds with potential anticancer properties. From the *in vitro* point of view and at the cellular level, flavonoids have been shown to exert a broad spectrum of antiproliferative activity on different cell lines which has been attributed to this diversity in their structural patterns and the specific cell line against which they have been used (Nagao *et al*., 2002). Due to these results, it is proposed that each flavonoid compound is needed to be studied systematically against different cells in order to get an insight into its individual potency and selectivity of antiproliferative activity (Wenzel *et al*., 2000).

Dracocephalum kotschyi Boiss. (Labiatae) is a native Iranian medicinal plant which has been used in traditional medicine for years as an analgesic, antispasmodic and in combination with *Peganum harmala* L. as a remedy for many forms of human cancer especially leukemia and gastrointestinal malignancies (Sobhani *et al*., 2002). In previous research done in our laboratory, the antitumor activity of *D. kotschyi* extract was established in an *in vivo* model. Also, through bioassay-guided fractionation and using HL-60 human promyelocytic leukemia cell line, a flavonoid identified as xanthomicrol was isolated as an antiproliferative constituent present in its extract with preferential

* Correspondence to: Soltan Ahmad Ebrahimi, Pharmacology Department and Razi Institute for Drug Research, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran.
E-mail: ebrahimi@iums.ac.ir

Received 26 April 2011
Revised 06 September 2011
Accepted 15 September 2011

activity towards malignant cells (Jahaniani *et al*., 2005). Xanthomicrol has been reported to exist in *D. kotschyi* together with a number of other flavonoid compounds (Gohari *et al*., 2003; Faham *et al*., 2008) for only two of which, i.e. apigenin and luteolin, significant data exist supporting their antiproliferative activities against various cancer cell lines (Takahashi *et al*., 1998; Kawaii *et al*., 1999) and the complete antiproliferative activity profile of its other constituents remains to be elucidated.

In continuation of our work to obtain a more complete picture of the anticancer properties of *D. kotschyi*, this is a report for the first time of the isolation and structure elucidation of flavonoid aglycones, naringenin, cirsimaritin and penduletin, together with the previously reported compounds, apigenin, luteolin, isokaempferide, xanthomicrol and calycopterin (Gohari *et al*., 2003; Faham *et al*., 2008) from this plant. Also, the evaluation of the inhibitory activity of each flavonoid compound against a panel of malignant cell lines is reported. In order to determine the ability of these compounds to selectively inhibit tumor cell proliferation, their antiproliferative activities on cancer cell lines were compared with their effects on normal human cells.

MATERIALS AND METHODS

Materials. Methanol, acetonitrile, ethyl acetate, hydrochloric acid and dimethyl sulfoxide were of the analytical grade and were purchased from Merck (Merck, Germany). Purified water (Millipore, USA) was used for all the solutions and dilutions. RPMI-1640, fetal bovine serum, penicillin and streptomycin were obtained from Gibco (Paisley, UK). 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Doxorubicin was obtained from Pharmacia (Italy).

Plant material. The aerial parts of *D. kotschyi* were purchased from a herbal drug store in Isfahan province and the authenticity of the specimen was confirmed by Dr Gh. Amin, Faculty of Pharmacy, Tehran University of Medical Sciences. A voucher specimen (PMP-304) was deposited at the Herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences.

Apparatus. The semi-preparative HPLC system consisted of an SP8800 HPLC ternary pump (Spectra-Physics, USA), a manual sample injector (Rheodyne, USA), and a Uvicord SII detector (Pharmacia Biotech, Sweden). Chromatographic separation was carried out on a Nucleosil ODS column (250 × 21 mm, 10 μm) (Macherey Nagel, Germany). The analytical HPLC system consisted of a K-1001 pump (Knauer, Germany), a 2501 UV/Vis detector (Knauer, Germany), and a Teknokroma ODS2 column (150 × 4.6 mm, 5 μm) (Teknokroma, Spain). EIMS and ^{1}H-NMR spectra were recorded using an Agilent (HP) 5973 Network MSD at 70 eV and a Bruker Avance 500 DRX spectrometer, respectively.

Preparation of the extract. The aerial parts of *D. kotschyi* were powdered and 50 g of the powder was extracted overnight with 400 mL of ethyl acetate using the soxhlet method. The extract was thereafter evaporated to dryness *in vacuo* at 40 °C. The dried extract was then dissolved in chloroform and filtered through Whatman paper filter *in vacuo*. Aliquots of the filtered chloroform phase were partitioned with equal volumes of ammonia solution (pH 11) using a separating funnel. This process was repeated until the added aqueous phase became clear. The pH of the resulting aqueous phase was thereafter lowered to 2 using concentrated HCl. This aqueous phase was then extracted using ethyl acetate. The organic phase was then evaporated to dryness *in vacuo* at 40 °C and the residue was stored at −20 °C before further purification.

Isolation and purification of individual flavonoids. The dried material obtained from liquid–liquid extraction was subjected to reversed-phase HPLC using an isocratic solvent system consisting of 55% HCl 0.01 M, 25% acetonitrile, 19% methanol and 1% water. The flow rate was 7 mL/min and the column was maintained at ambient temperature. The injection volume was 1.7 mL and the detector was set at 226 nm. Data acquisition was carried out using an Advantec PCI1716 data acquisition card and an in-house developed software. Fractions were collected and pooled from different runs and each fraction was weighed after drying *in vacuo*.

Purity assessment of the isolated flavonoids. The isolated flavonoids were subjected to analytical HPLC. The mobile phase (55% HCl 0.01 M , 25% acetonitrile, 19% methanol and 1% water) was pumped at 1 mL/min and the detector was set at 220 nm.

Cell culture. Human acute promyelocytic leukemia (HL-60), human colon carcinoma (HT-29), human gastric adenocarcinoma (AGS), human osteosarcoma (SaOs-2), murine fibrosarcoma (WEHI-164) and human fetal foreskin fibroblast (HFFF-P16) cell lines were purchased from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran) and were maintained in RPMI-1640 medium supplemented with 10% (v/v) heat inactivated fetal bovine serum, 2.2 g/L sodium bicarbonate, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Cell viability assay. The MTT reduction assay was used to determine the viability of cells treated with the isolated compounds. The HL-60, HT-29 and AGS cells were plated at 5×10^3 cells per well in 96-well plates, while WEHI-164 and HFFF-P16 cells were plated at 3×10^3 and 10^4 cells per well, respectively. The isolated compounds were dissolved in DMSO and then diluted serially with RPMI-1640 just prior to use. The final concentration of DMSO in each well was kept below 0.1%. Doxorubicin was used as a positive control for each cell line. After an overnight incubation of the cells, serial concentrations of the prepared compounds were added to the plates. The cells were then incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 72 h. The medium in each well was replaced with 200 μL fresh culture medium. 20 μL of MTT solution in deionized water (5 mg/mL) was added to each well and the cells were incubated under the same conditions for 3 h. The medium was aspirated thereafter and the resulting formazan precipitate in each well was dissolved in 100 μL of DMSO. The absorbance of the solution in each well was measured at 570 nm using a

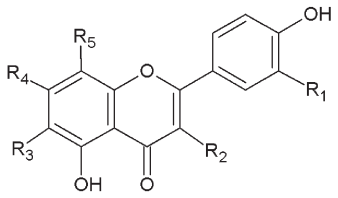
Dynex MRX microplate reader. The IC_{50} value reported for each compound was the concentration of the compound that resulted in a 50% inhibition of cell growth.

RESULTS

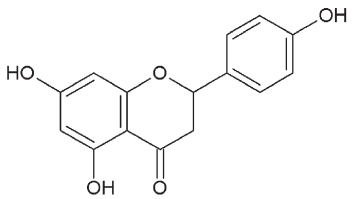
Isolation and identification of flavonoids

Eleven fractions were collected after semi-preparative HPLC purification of the residual extract from the liquid–liquid extraction. Corresponding fractions from ten chromatographic runs were pooled, dried and analysed by MS and NMR spectroscopy. Fractions 5, 7 and 10 could not be fully identified, due to the insufficient quantities recovered, yet they were shown to possess the flavonoid backbone. The results of the MS and NMR analyses revealed the identities of fractions 1, 2, 3, 4, 6, 8, 9 and 11 after comparison with previously published data as luteolin (**1**), naringenin (**2**), apigenin (**3**), isokaempferide (**4**), cirsimaritin (**6**), penduletin (**8**), xanthomicrol (**9**) and calycopterin (**11**), respectively (Rodriguez *et al*., 1972; Parmar *et al*., 1987; Akkal *et al*., 2003; Wollenweber *et al*., 2003; Pistelli *et al*., 2005; Kurkin and Lamrini, 2007) (Fig. 1).

The amounts of the eight identified compounds in the dried extract were calculated based on the isolation scheme and the results are summarized in Table 1. Analytical HPLC was used to assess the purity of the isolated compounds and all the isolated fractions appeared to be essentially pure, assuming similar molar extinction coefficients at 220 nm.



Flavonoid	R_1	R_2	R_3	R_4	R_5
Luteolin (**1**)	OH	H	H	OH	H
Apigenin (**3**)	H	H	H	OH	H
Isokaempferide (**4**)	H	OCH_3	H	OH	H
Cirsimaritin (**6**)	H	H	OCH_3	OCH_3	H
Penduletin (**8**)	H	OCH_3	OCH_3	OCH_3	H
Xanthomicrol (**9**)	H	H	OCH_3	OCH_3	OCH_3
Calycopterin (**11**)	H	OCH_3	OCH_3	OCH_3	OCH_3



Naringenin (2)

Figure 1. The chemical structures of flavonoid compounds isolated from *D. kotschyi*.

Table 1. The amounts of the eight flavonoids recovered from *D. kotschyi* extract

Flavonoid	Amount (μg/g)
Luteolin (**1**)	80 ± 0.01
Naringenin (**2**)	10 ± 0.003
Apigenin (**3**)	100 ± 0.01
Isokaempferide (**4**)	30 ± 0.009
Cirsimaritin (**6**)	20 ± 0.003
Penduletin (**8**)	20 ± 0.005
Xanthomicrol (**9**)	90 ± 0.01
Calycopterin (**11**)	60 ± 0.01
Total	0.05% (w/w) of dry sample

Results represent mean values ± SD of three independent experiments.

In vitro antiproliferative activities of identified flavonoids

The antiproliferative activities of the identified flavonoids together with doxorubicin were evaluated using different cell lines, i.e. AGS, HT-29, HL60, SaOs-2, WEHI-164 and HFFF-P16 at different concentrations for 72 h using the MTT assay. The IC_{50} values against each cell line are presented in Table 2. Among the isolated flavonoids, naringenin (**2**), did not show significant antiproliferative activity against any of the malignant cell lines within the concentration range used (3.125–50 μg/mL) except for HT-29 against which an IC_{50} of 36 μg/mL was obtained. However, all the other compounds showed growth-inhibitory activity against the cell lines used within the same concentration range. The growth-inhibitory activity of doxorubicin against the tested cell lines was much higher than the tested flavonoids, although its IC_{50} against HT-29 was higher than the other cell lines.

Among the identified compounds, luteolin (**1**) showed the highest activity with IC_{50} values less than 15 μg/mL against all the cell lines, followed by apigenin (**3**) and isokaempferide (**4**), which showed slightly less activity against SaOs-2 and HL-60 cell lines, respectively. Cirsimaritin (**6**) showed moderate inhibitory activity against AGS and HT-29 cell lines, while its overall activity against SaOs-2 and WEHI-164 was weak. Penduletin (**8**) showed antiproliferative activities against all the malignant cell lines, while it proved to be slightly less active against WEHI-164. Although xanthomicrol (**9**) showed weak antiproliferative activity against HL-60, SaOs-2, HT-29 and WEHI-164 malignant cell lines, it is notable that it strongly inhibited the growth of the AGS cell line with an IC_{50} value of 4.5 μg/mL which was smaller than the other tested compounds on any of the tested cell lines. A similar activity was detected in calycopterin (**11**) which showed strong antiproliferative activity against HL-60 and HT-29 cells with IC_{50} values equal to 6 and 9 μg/mL, respectively.

In order to give an indication of the preferential effects of the test compounds on malignant versus non-malignant cell lines, an antiproliferative index was calculated for each compound as the IC_{50} of test compound against HFFF-P16, divided by its IC_{50} against individual cancer cell lines and the results are presented in Fig. 2.

Table 2. Antiproliferative activities of the isolated flavonoids from *D. kotschyi*

Flavonoid	IC_{50} (μg/mL)					
	AGS	HT-29	HL-60	SaOs-2	WEHI-164	HFFF-P16
Luteolin (**1**)	9.2 ± 0.6	9.8 ± 1.0	6.2 ± 0.7	12.5 ± 1.4	13.6 ± 0.9	11.3 ± 1.0
Naringenin (**2**)	> 50	36.5 ± 2.4	> 50	> 50	> 50	40.6 ± 0.8
Apigenin (**3**)	5.1 ± 0.8	8.2 ± 0.7	10.8 ± 0.9	28.8 ± 1.1	6.4 ± 1.2	15.6 ± 0.9
Isokaempferide (**4**)	10.5 ± 1.0	12.3 ± 0.7	35.3 ± 1.6	12.4 ± 0.9	17.5 ± 2.2	12.8 ± 1.2
Cirsimaritin (**6**)	14.4 ± 0.6	13.1 ± 1.2	NA	38.5 ± 1.0	40.7 ± 1.9	51.3 ± 1.7
Penduletin (**8**)	9.7 ± 0.7	11.9 ± 0.9	7.5 ± 0.8	18.3 ± 1.0	23.5 ± 1.5	39.5 ± 0.9
Xanthomicrol (**9**)	4.5 ± 1.0	42.6 ± 1.1	38.5 ± 1.0	40.6 ± 2.1	32.8 ± 1.7	55.9 ± 1.4
Calycopterin (**11**)	> 50	9.3 ± 1.4	6.2 ± 0.8	> 50	> 50	60.1 ± 1.5
Doxorubicin	0.02 ± 0.01	0.35 ± 0.03	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.04 ± 0.01

IC_{50} is defined as the required flavonoid concentration (μg/mL) to cause a 50% inhibition of cell growth. Results represent mean values ± SE of three independent experiments.
NA, not available

DISCUSSION

D. kotschyi is a medicinal plant that is used in the clinic as a constituent of an ethnobotanical anticancer remedy in Iran (Sobhani *et al.*, 2002). Xanthomicrol was previously isolated from *D. kotschyi* and its preferential antiproliferative activity against a number of cancerous cell lines compared with non-malignant cells were reported (Jahaniani *et al.*, 2005). As cell proliferation involves complex combinations of biochemical processes and different flavonoids might influence these processes in different ways, it is proposed that each flavonoid compound must be studied systematically in order to gain insight into its individual potency and selectivity of antiproliferative activity (Wenzel *et al.*, 2000). Thus, the present work tried to characterize the antiproliferative activity profile of the flavonoid constituents of *D. kotschyi* in more detail, using an *in vitro* cell proliferation inhibition test.

The presence of phenolic functional groups in flavonoids was utilized to implement a relatively selective extraction protocol to separate these compounds from the other constituents of the extract prior to their final purification using high performance liquid chromatography. As HPLC has proved to be one of the most useful techniques for the separation of flavonoids in plant extracts (Vande Casteele *et al.*, 1982; Abad-Garcia *et al.*, 2007), a semi-preparative reversed-phase HPLC method was used for the separation of the flavonoid constituents of *D. kotschyi* in quantities required for further pharmacological studies. Using the described elution system, 11 compounds were successfully isolated, eight of which were completely identified using MS and NMR analyses. Except for naringenin (**2**) which has a flavanone backbone, all the other identified compounds in this study belong to the flavone subgroup of flavonoids. Luteolin (**1**), apigenin (**3**), isokaempferide (**4**), xanthomicrol (**9**) and calycopterin (**11**) had been reported in this plant before (Gohari *et al.*, 2003), while the existence of naringenin (**2**), cirsimaritin (**6**) and penduletin (**8**) in *D. kotschyi* are being reported for the first time through this work.

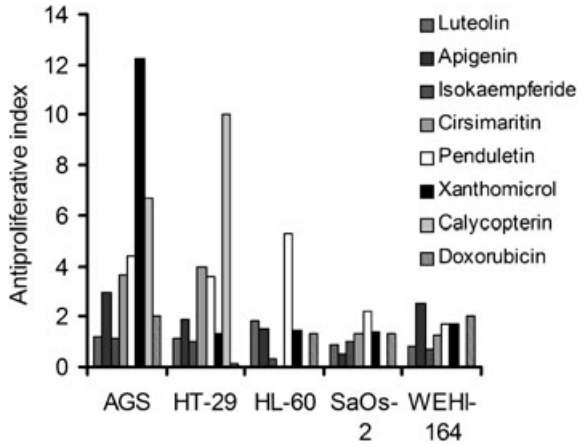


Figure 2. The antiproliferative indices of doxorubicin and the flavone compounds isolated from *D. kotschyi* on a panel of cell lines. The graph displays the ratio of the IC_{50} value of each flavonoid compound for HFFF-P16 and the IC_{50} value of it against a particular malignant cell line.

Results from the previous reports have demonstrated that the growth-inhibitory activity of one flavonoid against different cancer cell lines might not always be the same, indicating the differences in the sensitivity of the malignant cell lines under study (Beutler *et al.*, 1998; Takahashi *et al.*, 1998; Pouget *et al.*, 2001). The identified flavonoids were closely related in their chemical structures and some structure–activity relationships were established using malignant cell lines. The only flavanone, naringenin (**2**), did not show any significant antiproliferative activity against the malignant cell lines within the tested concentration range, except for HT-29 against which a low activity was seen. Unlike naringenin (**2**), its corresponding flavone, apigenin (**3**), showed effective inhibitory activity against all the malignant cell lines tested. Different groups had previously reported the antiproliferative activity of these two compounds against HT-29 (Manthey and Guthrie, 2002) and HL-60 (Takahashi *et al.*, 1998) cell lines with comparably similar results to the data presented here and had underlined the greater inhibitory activity of apigenin to naringenin, which is attributed to the presence of the double bond between C_2 and C_3 (Kawaii *et al.*, 1999). An antiproliferative activity of apigenin is demonstrated here as well against cell lines (AGS, SaOs-2 and WEHI-164) previously not studied.

From the structural point of view, the identified flavones in this report could be divided into hydroxyflavones (luteolin (**1**), apigenin (**3**) and isokaempferide (**4**)) and methoxylated hydroxyflavones (cirsimaritin (**6**), penduletin (**8**), xanthomicrol (**9**) and calycopterin (**11**)) which all possess the 5,4′-dihydroxyflavone backbone (Fig. 1). While the two closely related hydroxylated-flavones, i.e. luteolin (**1**) and apigenin (**3**), proved to be effective antiproliferative agents against all the malignant cell lines, they showed slightly different potencies of activity, suggesting an effect of the hydroxyl substitution at $C_{3'}$ of 5,7-dihydroxyflavones on their activities. Although the absence of this substitution in apigenin **(3)** did not affect the potency against HT-29, it caused a decrease in potency against SaOs-2 and HL-60 and an increase in potency against AGS and WEHI-164. Luteolin (**1**) has been demonstrated to be widely distributed in plant kingdom and its effective antiproliferative properties have been shown previously against various cancer cell lines including human HL-60 leukemia (Takahashi *et al*., 1998), AGS human gastric adenocarcinoma (Wu *et al*., 2008), MDA-MB-435 and MCF-7 breast cancer, HT-29 colon carcinoma, DU-145 prostate cancer, SK-MEL5 melanoma and DMS-114 lung cancer cell lines (Manthey and Guthrie, 2002). The observed antiproliferative activity of luteolin in this study was consistent with the previously reported results on AGS (Wu *et al*., 2008), HT-29 (Manthey and Guthrie, 2002) and HL-60 (Takahashi *et al*., 1998; Manthey and Guthrie, 2002) cell lines.

All the malignant cell lines were shown to be almost equally sensitive to the growth preventive activity of isokaempferide (**4**) in this study, except for HL-60 against which the compound showed weak inhibitory activity (Table 2). The influence of the methoxy substituent of isokaempferide at C_3 on the activity against the malignant cell lines was examined by comparison of the activities of isokaempferide (**4**) with its counterpart, apigenin (**3**). This comparison showed that the introduction of a methoxy group at C_3 caused a decrease in the activity against AGS, HT-29, HL-60 and WEHI-164 cell lines, while it enhanced its activity against SaOs-2.

The methoxylated hydroxyflavones in this study differed both in the number and substitution patterns of the methoxy groups. From the IC_{50} values of penduletin (**8**) and xanthomicrol (**9**), which possess the same number of methoxy substituents, it is evident that the substitution patterns of the methoxy groups may influence the antiproliferative activity of these agents (Table 2). While penduletin (**8**) showed effective antiproliferative activities against all the malignant cell lines, xanthomicrol (**9**) proved to have a selective antiproliferative activity against AGS with the most potent effect in this study. The inhibitory activities of the trimethoxylated-hydroxyflavones (penduletin (**8**) and xanthomicrol (**9**)) were compared with those of cirsimaritin (**6**) and calycopterin (**11**), respectively, and some structure–activity relationships were identified. Cirsimaritin (**6**) as the only dimethoxylated-hydroxyflavone showed moderate inhibitory activity against AGS and HT-29 while its overall activity against SaOs-2 and WEHI-164 was weak (Table 2). On the other hand, calycopterin (**11**) as the only tetramethoxylated-hydroxyflavone, was selectively active against HL-60 and HT-29 cell lines.

Comparative analysis of the antiproliferative activities of cirsimaritin (**6**), penduletin (**8**), xanthomicrol (**9**) and calycopterin (**11**) revealed that the absence of a methoxy substituent at C_8 in the presence of a methoxy group at C_3 resulted in an enhancement of the activity against AGS, HT-29, SaOs-2 and WEHI-164 cell lines while its presence resulted in selectively high antiproliferative activities against HL-60 and HT-29. Also, the presence of a methoxy substituent at C_8 in the absence of a methoxy at C_3 resulted in a selectively high antiproliferative activity against AGS.

Xanthomicrol was isolated from *D. kotschyi* leaf extract and an IC_{50} value of 0.88 μg/mL was reported for this flavone against HL-60 cells (Jahaniani *et al*., 2005). In the present work, although xanthomicrol showed activity against HL-60 cells, its IC_{50} value was considerably higher (38.5 μg/mL). However, the IC_{50} value obtained for doxorubicin, used as a positive control in both works, are very similar suggesting more or less similar experimental conditions. The results of preliminary experiments on the combinatorial effects of these flavonoids carried out in our laboratory, suggested that there could be an additive or synergistic relationship for the antiproliferative effects of xanthomicrol and calycopterin. In the presence of 3.2 μg/mL calycopterin and 6.2 μg/mL xanthomicrol more than 50% of HL-60 cells died (data unpublished). As Jahaniani *et al*. (2005) did not report the isolation of calycopterin from *D. kotschyi* leaf extract and as calycopterin and xanthomicrol are structurally very similar, it is possible that the isolated xanthomicrol in the former study contained significant amounts of calycopterin leading to such a small IC_{50} value against HL-60 cells.

Although *D. kotschyi* has been used in combination with *P. harmala* against many forms of human cancer in Iran, it has been reported to be especially active against leukemia and gastrointestinal malignancies (Sobhani *et al*., 2002). In view of the results obtained thus far (Table 2), the most sensitive cell lines towards the isolated flavonoids in this study were AGS, HL-60 and HT-29 which are gastric adenocarcinoma, promyelocytic leukemia and colon carcinoma cells, respectively. Based on the fact that the present *in vitro* data did not negate these claims, more comprehensive studies are needed to validate these clinical observations.

As shown in the results (Table 2), the antiproliferative activities of the hydroxyflavones, i.e. luteolin (**1**), apigenin (**3**) and isokaempferide (**4**), against HFFF-P16 cells proved to be negligibly different from each other and no meaningful structure–activity relationship could be deduced. This result was comparably reflected as well in the tested methoxylated hydroxyflavones, i.e. cirsimaritin (**6**), penduletin (**8**), xanthomicrol (**9**) and calycopterin (**11**). However, a distinctive difference between the IC_{50} values of the hydroxyflavones and methoxylated hydroxyflavones was observed. In order to better explore these results, the antiproliferative index of each flavonoid compound was calculated. As is evident from Fig. 2, the antiproliferative indices for doxorubicin, luteolin (**1**), apigenin (**3**) and isokaempferide (**4**) were similar in the tested cell lines, suggesting the similarity of their exerted antiproliferative activities on both malignant and normal cells. This means that the HFFF-P16 normal cells are as vulnerable as the malignant cells to the growth preventive activity of these compounds and that their application as anticancer agents might not be advantageous. However, the antiproliferative indices for cirsimaritin (**6**), penduletin (**8**), xanthomicrol

(**9**) and calycopterin (**11**) showed their preferential antiproliferative activity towards two, three, one and two of the malignant cell lines, respectively, compared with the normal cells. From these results, it could be concluded that although the hydroxyflavones proved to be highly effective against the panel of malignant cells in this study, they produced similar damage to normal cells which might lead to side effects and toxicities. This is in contrast to the tested methoxylated hydroxyflavones that strongly inhibit the growth of malignant cells selectively and preferentially. On the basis of the above findings it could be concluded that xanthomicrol and calycopterin may be candidates for further research as anticancer agents.

Acknowledgements

This work was supported by Grant no. 687 from Tehran University of Medical Sciences. We would like to thank Dr Gh Amin, Head of the Herbarium at the faculty of Pharmacy, for his help with identification of the plant.

Conflict of Interest

The authors have declared that there is no conflict of interest.

REFERENCES

Abad-Garcia B, Berrueta LA, Lopez-Marquez DM, Crespo-Ferrer I, Gallo B, Vicente F. 2007. Optimization and validation of a methodology based on solvent extraction and liquid chromatography for the simultaneous determination of several polyphenolic families in fruit juices. *J Chromatogr A* **1154**: 87–96.

Akkal S, Benayache F, Bentamene A, Medjroubi K, Seguin E, Tillequin F. 2003. Flavonoid aglycones from *Centaurea napifolia*. *Chem Nat Comp* **39**: 219–220.

Beutler JA, Hamel E, Vlietinck AJ, *et al*. 1998. Structure-activity requirements for flavone cytotoxicity and binding to tubulin. *J Med Chem* **41**: 2333–2338.

Buolamwini JK. 1999. Novel anticancer drug discovery. *Curr Opin Chem Biol* **3**: 500–509.

Butler MS. 2004. The role of natural product chemistry in drug discovery. *J Nat Prod* **67**: 2141–2153.

Cardenas M, Marder M, Blank VC, Roguin LP. 2006. Antitumor activity of some natural flavonoids and synthetic derivatives on various human and murine cancer cell lines. *Bioorg Med Chem* **14**: 2966–2971.

Cushnie TP, Lamb AJ. 2005. Antimicrobial activity of flavonoids. *Int J Antimicrob Agents* **26**: 343–356.

Faham N, Javidnia K, Bahmani M, Amirghofran Z. 2008. Calycopterin, an immunoinhibitory compound from the extract of *Dracocephalum kotschyi*. *Phytother Res* **22**: 1154–1158.

Garcia-Lafuente A, Guillamon E, Villares A, Rostagno MA, Martinez JA. 2009. Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. *Inflamm Res* **58**: 537–552.

Gohari AR, Saeidnia S, Matsuo K, *et al*. 2003. Flavonoid constituents of *Dracocephalum kotschyi* growing in Iran and their trypanocidal activity. *J Nat Med* **57**: 250–252.

Jahaniani F, Ebrahimi SA, Rahbar-Roshandel N, Mahmoudian M. 2005. Xanthomicrol is the main cytotoxic component of *Dracocephalum kotschyii* and a potential anti-cancer agent. *Phytochemistry* **66**: 1581–1592.

Kawai M, Hirano T, Higa S, *et al*. 2007. Flavonoids and related compounds as anti-allergic substances. *Allergol Int* **56**: 113–123.

Kawaii S, Tomono Y, Katase E, Ogawa K, Yano M. 1999. Antiproliferative activity of flavonoids on several cancer cell lines. *Biosci Biotechnol Biochem* **63**: 896–899.

Kurkin VA, Lamrini M. 2007. Flavonoids of *Lavandula spica* flowers. *Chem Nat Comp* **43**: 702–703.

Manthey JA, Guthrie N. 2002. Antiproliferative activities of citrus flavonoids against six human cancer cell lines. *J Agric Food Chem* **50**: 5837–5843.

Middleton E Jr, Kandaswami C, Theoharides TC. 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev* **52**: 673–751.

Morley KL, Ferguson PJ, Koropatnick J. 2007. Tangeretin and nobiletin induce G1 cell cycle arrest but not apoptosis in human breast and colon cancer cells. *Cancer Lett* **251**: 168–178.

Murakami A, Nakamura Y, Torikai K, *et al*. 2000. Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. *Cancer Res* **60**: 5059–5066.

Nagao T, Abe F, Kinjo J, Okabe H. 2002. Antiproliferative constituents in plants 10. Flavones from the leaves of *Lantana montevidensis* Briq. and consideration of structure-activity relationship. *Biol Pharm Bull* **25**: 875–879.

Parmar VS, Jain R, Simonsen O, Boll PM. 1987. Isolation of candirone: a novel pentaoxygenation pattern in a naturally occurring 2-phenyl-4h-1-benzopyran-4-one from *Tephrosia candida*. *Tetrahedron* **43**: 4241–4247.

Pistelli L, Noccioli C, Giachi I, *et al*. 2005. Lupane-triterpenes from *Bupleurum flavum*. *Nat Prod Res* **19**: 783–788.

Pouget C, Lauthier F, Simon A, *et al*. 2001. Flavonoids: structural requirements for antiproliferative activity on breast cancer cells. *Bioorg Med Chem Lett* **11**: 3095–3097.

Rodriguez E, Vander Velde G, Mabry TJ, Subramanian SS, Nair AGR. 1972. The structure of calycopterin. *Phytochemistry* **11**: 2311–2312.

Sobhani AM, Ebrahimi SA, Mahmoudian M. 2002. An *in vitro* evaluation of human DNA topoisomerase I inhibition by *Peganum harmala* L. seeds extract and its beta-carboline alkaloids. *J Pharm Pharm Sci* **5**: 19–23.

Takahashi T, Kobori M, Shinmoto H, Tsushida T. 1998. Structure-activity relationships of flavonoids and the induction of granulocytic- or monocytic-differentiation in HL60 human myeloid leukemia cells. *Biosci Biotechnol Biochem* **62**: 2199–2204.

Tang M, Ogawa K, Asamoto M, *et al*. 2007. Protective effects of citrus nobiletin and auraptene in transgenic rats developing adenocarcinoma of the prostate (TRAP) and human prostate carcinoma cells. *Cancer Sci* **98**: 471–477.

Vande Casteele K, Geiger H, Van Sumere CF. 1982. Separation of flavonoids by reversed-phase high-performance liquid chromatography. *J Chromatogr A* **240**: 81–94.

Wenzel U, Kuntz S, Brendel MD, Daniel H. 2000. Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. *Cancer Res* **60**: 3823–3831.

Wollenweber E, Dorr M, Rivera D, Roitman JN. 2003. Externally accumulated flavonoids in three Mediterranean *Ononis* species. *Z Naturforsch C* **58**: 771–775.

Wu B, Zhang Q, Shen W, Zhu J. 2008. Anti-proliferative and chemosensitizing effects of luteolin on human gastric cancer AGS cell line. *Mol Cell Biochem* **313**: 125–132.

