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Antiproliferative and Antioxidant Effects of Three *Hypericum* Species of Turkish Origin: *H. perforatum*, *H. montbretii* and *H. origanifolium*

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ABSTRACT

Hypericum species have been used in traditional medicine for various purposes including neoplastic diseases. In the present study, antiproliferative and antioxidant activities of extracts of *Hypericum perforatum*, *H. montbretii* and *H. origanifolium* were investigated. Antiproliferative effects of *Hypericum* extracts were examined by using MTT method on A549 and HeLa cell lines. The β-Carotene-linolenic acid system and the Rancimat method were used to evaluate antioxidant activities, while the DPPH method was employed for testing free radical scavenging activities of the extracts. Total phenolic compounds, flavonoids and flavonols were also measured, whereas major constituents like hypericin, hyperforin, quercetin, quercitrin, isoquercitrin, hyperoside, rutin and chlorogenic acid were determined by HPLC-DAD analyses. The findings indicated that flower extracts of *H. montbretii* and *H. origanifolium* have higher antioxidant and antiradical activities than the extract from aerial part of *H. perforatum*. While flower extract of *H. origanifolium* has no effect on MTT measurement of A549 cells, leaf extract of this plant at 250 µg/ml concentration exhibits only a weak effect on these cells. Leaf and flower extracts of the same plant possess a similar activity with *H. perforatum* extract on MTT measurement of HeLa cells. As measured with MTT assay, flower extracts of *H. montbretii* at the concentrations of 100 and 250 µg/ml exhibit an antiproliferative effects on A549 cells, which is in similar magnitude with *H. perforatum* extract. Leaf extract of *H. montbretii* seems to be ineffective on the proliferation of A549 cells, whereas it exhibit only a week antiproliferative effect on HeLa cells at 250 µg/ml concentration. Results suggested that *Hypericum* species tested have antiproliferative and apoptotic effects, which seems to be related to their antioxidant activities.

Keywords: A549 cells, cancer, HeLa cells, *Hypericum* species, free radicals, proliferation

INTRODUCTION

The main purpose of this study was to investigate antiproferative/cytotoxic effects of extracts from three *Hypericum* species of Turkish origin (*H. montbretii* Spach., *H. origanifolium* Willd., *H. perforatum* L.) and to evaluate a possible correlation with their antioxidant and free radical scavenging activities. For this purpose, official species, *H. perforatum*, or St.-John's Wort, whose antiproliferative and antioxidant activities have previously been reported, was used for comparison of the observed effects.

Being possible sources of pharmaceutical industry, St.-John's Wort and other *Hypericum* species have been used as a traditional medicine for treatments of various diseases, most of which have been confirmed in experimental studies (Duke 1985; Öztürk and Öztürk 2008). For instance, choleretic and hepatoprotective effects of *H. perforatum*, but not *H. calycinum*, have been reported confirming its folkloristic use in the treatment of jaundice and other liver diseases (Öztürk *et al.* 1992; Herekman-Demir *et al.* 2001). Analgesic activities of *H. perforatum* and *H. calycinum*, but not those of *H. hyssopifolium* ssp. *elongatum* var. *elongatum* have been demonstrated in various pain models (Öztürk *et al*. 1996a; Öztürk 1997; Öztürk and Öztürk 2001). Antidepressant activities of *H. perforatum* and *H. calycinum* have also been observed in experimental models of depression (Öztürk *et al*. 1996a; Öztürk 1997). In accordance with these observations, experimental studies have indicated that extracts prepared from *H. perforatum* may be effective both for the treatment of alcohol-dependence (Perfumi *et al.* 1999; Rezvani *et al*. 1999) and for the withdrawal symptoms of dependences to alcohol (Coskun *et al*. 2007), caffeine (Uzbay *et al.* 2007) and nicotine (Catania *et al.* 2003; Uzbay *et al.* 2005, 2006). The wound-healing activity of St.-John's Wort has been demonstrated using cultured chicken embryonic fibroblast cells as an experimental model (Korkmaz *et al*. 2001; Öztürk *et al*. 2007).

Ethnomedical surveys have indicated that St.-John's Wort have been used as a folk remedy for its antitumoral effects (Duke 1985). Its ethnomedical uses against tumoral diseases have been confirmed in various experimental studies. Anticancer activities of *H. perforatum* have been described mainly with photodynamic therapy (Agostinis *et al.* 2001, 2002; Piette *et al*. 2003). There are, however, experimental studies, which have demonstrated cytotoxic and antiproliferative effects of various *Hypericum* species, including *H. annulatum* (Vijayan *et al.* 2003), *H. empetrifolium* (Itogawa 1988), *H. erectum* (Chapuis *et al.* 1988), *H. mysorense*, *H. patulum*. Cytotoxic and antiproliferative effects of *Hypericum* species seem to be related to hypericin and hypocrellin contents (Ali *et al.* 2001; Piette *et al*. 2003) and antioxidant capacities (Couladis *et al*. 2002). They also seem to be due to apoptotic cell deaths rather than necrotic ones (Ali *et al.* 2001).

There are a number of *Hypericum* species whose antioxidant activities have been continually reported in various systems (Herold *et al.* 2003; Conforti *et al.* 2007; Galati *et al.* 2008; Sagratini *et al.* 2008). According to current con-

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cepts, antioxidant activity seems to be important for cancer chemoprevention and may be important for cancer therapy (Khan *et al.* 2008; Weaver *et al.* 2008).

MATERIALS AND METHODS

Plant materials and preparation of plant extracts

H. origanifolium Willd. (HO), *H. montbretii* Spach. (HM), and *H. perforatum* L. (HP) were collected around Eskişehir, Turkey. *H. origanifolium* Willd.: Sivrihisar, Tekören village, 1100 m, June 20, 2003 (Herbarium specimen deposited: OUFE 10334), *H. montbretii* Spach.: Kalabak village, 1300 m, June 22, 2003 (Herbarium specimen deposited: OUFE 10332) and *H. perforatum* L.: Türkmen Mountains, upper parts of Kalabak, 1300 m, June 22, 2003 (Herbarium specimen deposited: OUFE 10337). The plants were identified according to the Flora of Turkey and the East Aegean Islands (Robson 1967, 1988; Dönmez 2000).

Extraction procedures for the experiments of antioxidant and free-radical scavenging activities were applied as proposed elsewhere (Tsao and Den 2004). Dried flowers and leaves of *Hypericum* species were ground and extracted with petroleum ether in a Soxhlet apparatus. Fat-free air dried material was extracted with methanol: water (70: 30, v/v) in a 40°C water bath for 30 min four times. The methanol extract (ME) was concentrated to dryness in vacuum and aqueous solution was lyophilized. Second extracts were prepared as follows: fat-free air dried material was extracted with methanol: water (70: 30, v/v) in a 40°C water bath for 30 min four times and it was concentrated in vacuum and aqueous phase was extracted with ethyl acetate at room temperature. Then ethyl acetate extract (EAE) was concentrated to dryness in vacuum and the aqueous solution (AQE) was lyophilized.

Extraction procedures for the experiments of cytotoxicity and antiproliferative activities were applied as follows: 15 g of dried flowers and leaves of HO and HM were ground and extracted with 150 ml of ethanol-water (50:50, v/v) mixture at 40°C (water bath, 60 min, four times). The same procedure was used for aerial parts of *H. perforatum*. The extract was then concentrated to dryness under vacuum and aqueous solution was lyophilized. All fractions obtained were weighed to determine the percent yields of soluble constituents.

Drugs and chemicals used

The chemicals gallic acid (GA), protocathechuic acid (protoCA), *p*-hydroxy benzoic acid (p-hydBA), caffeic acid (CA), chlorogenic acid (ChA), syringic acid (SA), p-coumaric acid (p-COU), ferulic acid (FA), *o*-coumaric acid (o-COU), *trans*-cinnamic acid (tr-CIN), propyl paraben, rutin, and Folin-Ciocalteu phenol reagent were provided by Sigma Co. (St. Louis, MO, USA), 2,2-diphenyl-1-picryhydrazyl radical (DPPH˙) was from Aldrich Chemical Co. (Milwaukee, WI, USA). Crude olive oil was kindly provided by Üstün Co. (Balıkesir, Turkey). For HPLC analyses of the extracts, quercetin, hypericin, hyperforin, chlorogenic acid, rutin and quercitrin were supplied by Sigma Chemical Co. (Milan, Italy), while hyperoside and iso-quercitrin were supplied from Applied Biosystem (Milan, Italy). Chemicals and reagents for cell culture including dimethylsulfoxide (DMSO), Dulbecco's Modification Eagles Medium, Foetal bovine serum (FBS), Hank's Balanced Salt Solution, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), penicillin-streptomycin solution and typsin-EDTA solutions were purchased from Sigma (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

Determination of total phenolics, flavonoids and flavonols

Total phenolics were determined by spectrophotometrically with the method of Folin Ciocalteu (1928). This method gives a general measurement of phenolic content, as it is not completely specific for phenolic compounds and not all phenolic compounds exhibit the same level of activity in the assay. Total phenolics in *Hypericum* extracts were expressed as a equivalent of gallic acid.

Flavonoid contents were determined by the $AlCl_3$ method as a species of rutin as it was proposed in a previous study (Miliauskas *et al.* 2004). One ml of plant extract in methanol (10 g/l) was mixed with 1 ml of aluminum chloride in ethanol (20 g/l) and diluted with ethanol to 25 ml. To 0.5 ml of each sample or standard rutin solution, 0.5 ml aluminum chloride solution was added and the absorption at 415 nm was read after 60 min at room temperature. Blank samples were prepared from 1 ml plant extract and 1 drop acetic acid, and diluted to 25 ml. The flavonoid contents were found by comparing the absorbance values of the extracts with those of the standard rutin solutions which were prepared as a stock solution of 0.05 g rutin. All determinations were carried out in duplicate. The amount of flavonoids in plant extracts as rutin equivalents (RE) was calculated by the following formula (Miliauskas *et al.* 2004):

$$X = (A \bullet m_o \bullet 10) / (A_o \bullet m)$$

where: X = flavonoid content, mg/g plant extract as RE; A = the absorption of plant extract solution; A_o = the absorption of standard rutin solution; m = the weight of plant extract, g; m_o = the weight of rutin in the solution, g.

$AlCl_3$+Na-acetate method was used to determine total flavonol contents of extracts as a species of rutin (Miliauskas *et al.* 2004). The calibration curve of rutin was constructed by mixing 2 ml of rutin solution having concentrations in the range of 0.5-0.017 mg/ml with 2 ml (20 g/l) aluminum chloride and 6 ml (50 g/l) sodium acetate. The absorption at 440 nm was read after 2.5 h at room temperature. The same procedure was applied to plant extracts similar to rutin solution. The experiments were always duplicated. The content of flavonols, in rutin equivalents (RE), was calculated by the following formula:

$$X = C \bullet V/m$$

where: X = flavonol content, mg/g plant extract as RE; C = the concentration of rutin solution, established from the calibration curve, mg/ml; V, m = the volume and the weight of plant extract, ml, g.

DPPH method for antiradical activity

Free radical scavenging effects of the fractions on DPPH˙ were estimated according to the method of (Sánchez-Moreno *et al.* 1998) with some modifications. The reaction mixture was left at room temperature for 30 min in dark; absorbance of the resulting solution was then measured spectrophotometrically at 517 nm. Antiradical activities of *Hypericum* extracts were compared to that of BHT (butylated hydroxy toluene), a synthetic antioxidant and antiradical compound. The scavenging activity was plotted against concentration and the concentration that showed 50% DPPH scavenging activity (IC_{50}) was calculated following the logarithmic procedures.

Rancimat method for antioxidant activity

Antioxidant activities of extracts obtained from *Hypericum* species were also measured by the Rancimat Method (A743 Rancimat apparatus, Metrohm AG, SW) at the concentration of 1% (Exarchou *et al.* 2002). A flow of air (20 L/h) was bubbled through the oil heated at 110°C, and the volatile compounds were collected in cold water, increasing the water conductivity. The conductivity was monitored continuously until a sudden rise signified the end of the induction period. Each sample was dispersed in 3 g of olive oil rich in linoleic acid (65% of fatty acids) at the concentration of 1%. Olive oil without antioxidant as the control was run similarly. The test was applied in triplicate. Antioxidant activities of *Hypericum* extracts were compared to that of BHT. Induction index was calculated by following equation:

Induction Index (II) = Induction time of sample / Induction time of control

As observed from the formula, a higher induction index indicates higher antioxidant activity (Esquivel *et al.* 1999).

β-Carotene-linolenic acid system for antioxidant activity

Antioxidant activity of extracts of *Hypericum* species was determined according to β-carotene bleaching methods (Yu *et al.* 2006). β-carotene (10 mg) was dissolved in 3 ml of chloroform. The β-carotene solution, dissolved in chloroform, was added to 40 mg linoleic acid and 400 mg Tween 80. Chloroform was removed with a rotary evaporator at 50°C. Oxygenated distilled water (100 ml) was added to the β-carotene emulsion and mixed well. Antioxidant extract (0.2 ml) was mixed with 3 ml β-carotene emulsion was monitored spectrophotometrically at 470 nm after 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180 min incubation at 50°C. The same procedure was repeated with the positive control BHT and a blank. Distilled water was used instead of antioxidant extract as a control treatment. Antioxidative capacities of extracts were compared with that of BHT at the same concentration and blank consisting of MeOH (0.2 ml).

Experiment to determine cytotoxic and antiproliferative activities

1. Preparation of cell lines

A549 non-small cell lung adenocarcinoma cells and HeLa cervix adenocarcinoma cells (JCRB cell bank, Osaka, Japan) were cultured in Dulbecco's Modification Eagles Medium (DMEM) containing 10% FBS and 1% penicilline-streptomycine solution. Stock cultures (containing approximately 2 × 105 cells/ml), maintained in 25-cm^2 polystyrene flasks (Corning Costar, Cambridge, MA, USA), were grown to confluence, while the sterility of cultures was regularly checked by an inverted microscope (Olympus, Model CK-2-BIC-1, Tokyo, Japan). When the cells attained the confluence, flasks containing cultured cells were transferred into a laminar flow cabinet (Holten, Model HV2448, Allerod, Denmark). The medium was, then, discarded and 2X trypsin-EDTA solution in Hank's Balanced Salt Solution was added at the amount of 2 ml per 25 cm^2. Trypsinized cells were incubated in 95% O_2+5% CO_2 at 37°C for 5-10 min. 4 ml of DMEM with FBS (10%) was added into the flasks, and then, flasks were filled with DMEM up to a half volume. After shaking gently, cell suspensions of 0.1 ml (containing approximately 25,000 cells) were transferred into 96-wells plates and plates were incubated for 24 h for the adhesion of cells in the wells. After the incubation, plates were decanted and fresh medium solution containing plant extracts at various concentrations were added into the wells. A control group and a vehicle group containing 1.0% DMSO were also established using 8 wells for each group in the plate. The following extracts were added into wells at concentrations of 1, 10, 50, 100, 250 µg/ml: Extract prepared from aerial part of *Hypericum perforatum* (HPE); Extracts prepared from flowers and leaves of *Hypericum montbretii* (HMFE and HMLE, respectively) and *Hypericum origanifolium* (HOFE and HOLE, respectively) consisting 8 wells for each experimental group. After that, plates were incubated in CO_2 incubator for 24-, 48-, 72- and 96-h measurements of the MTT assay.

2. MTT method for antiproliferative activity

Cell proliferation/cytotoxicity was tested by using MTT assay with A549 and HeLa Cells, which is a colorimetric test based on the formation of complex between mitochondrial succinate dehydrogenase enzyme and tetrazolium salts (Carmichael *et al.* 1987; Zhang *et al.* 2004). As a stock solution, MTT was dissolved as 5 mg/ml in phosphate buffer saline (PBS). It was filtered through membrane filter with 0.22 µm of pore size and stocked at -20°C. Giving a final concentration of 500 µg/ml in cell medium, MTT working solution was prepared from stock solution with a dilution of 1:10 in culture medium. At the end of incubation periods of 24, 48, 72 and 96 h, MTT working solution was added to each well (100 µl/well) and plates were incubated for 4 h at 37°C. After 4 h, media were removed and cells were lysed with 100 µl DMSO. Cells were incubated for a further 10 min at the room temperature shaking gently. Absorbances were determined at 540 nm using a microtiter plate reader (Bio-Tek, Winooski, VT, USA).

Table 1 Linear gradient program for HPLC analysis.

Time (min)	Solvent A (%) [a]	Solvent B (%) [b]	Solvent C (%) [c]
0	85	15	0
10	85	15	0
30	65	35	0
45	10	90	0
60	10	90	0
61	0	0	100
70	0	0	100
75	0	100	0
105	0	100	0
110	85	15	0
130	85	15	0

[a] Solvent A = water and phosphoric acid, pH 2.7
[b] Solvent B = 90% acetonitrile, 10% methanol
[c] Solvent C = 10% ethyl acetate, 90% mix solvent A and B (10%A, 90%B)

3. HPLC-DAD analysis of extracts

A Hewlett Packard (Palo Alto, CA, USA) HP-1100 series, equipped with a binary solvent pump, an autosampler, with the volume injection set to 20 µl, and a diode-array detector (DAD) coupled with an HPLC/DAD ChemStation (Rev. A. 06. 03) was used. Separation was performed on a LUNA C18 (150 × 4.6 mm) protected by a Security guard cartridge C18 (4 × 2 mm I.D.), both from Phenomenex USA (distributed by Chemtek Analytica, Bologna, Italy). The monitored wavelengths were 210 nm for phenolic compounds, 270 nm for hyperforin, 590 nm for hypericin. The adopted chromatographic method is reported in **Table 1**. The sample concentration was 3 mg/ml in methanol and the flow rate was 1 ml/min. The method used for components identification was the comparison of their retention times with respect to those of standards, chromatographed under the same conditions. In addition, UV spectra of both samples and standards were compared using the DAD. Moreover, the confirmation of the identified compounds was obtained injecting standard and sample solutions in a HPLC-MS equipped with an ESI interface in negative ionization mode using the same chromatographic conditions (formic acid was used instead of phosphoric acid at the same pH).

Analyses of data and statistics

All the data given, except of cell culture experiments and HPLC analyses, represented the mean of n determinations ± standard deviation (SD). The data from cell culture experiments were given in average values ± standard error of mean (SEM). Linear regression analyses were applied to evaluate calibration curves. To evaluate significance levels between differences of results obtained from cell culture experiments, One-way ANOVA with Tukey as a post-hoc method was applied by using SPSS. For other group of experiments, statistical significances relative to control were determined by Student's *t*-test using SPSS software. Significance level was accepted as $p<0.05$ (n=8 for each experimental group).

RESULTS AND DISCUSSION

Total phenols, flavonoid and flavonol contents of the extracts

Table 2 shows us brief results of *Hypericum* species employed in this study comprising the extraction yields, total phenols, flavonoid and flavonol contents for the extracts recovered with solvents of different polarities. The extraction yield as % plant material range varies from 7.27 for HOF-EAE to 39.63 for HPF-ME. Methanol extracts of all species showed that higher yield was obtained by comparing to the others. This might be due to the presence of more polar compounds in the methanol extracts of plants, as methanol is a solvent with higher polarity. The amounts of total phenols in the extracts were investigated in different solvent polarities. Total phenolic content among all samples varied between 104 and 451 mg/g as gallic acid equivalents. Significantly, highest results were found in ethyl acetate extracts in the order of increase HOF-EAE < HPF-EAE <

Table 2 The extraction yields, contents of the total phenols, flavonoids and flavonols in a various extracts of *H. montbretii* (HM), *H. origanifolium* (HO) and reference plant *H. perforatum* (HP).

Extracts	Extraction yield (%)[1]	Total phenolic compounds (mg/g ± SD)	Total flavonoids (mg/g ± SD)	Total flavonols (mg/g ± SD)
HOF-ME	29.4	278.63 ± 1.25	16.30 ± 0.37	2.58 ± 0.05
HOF-EAE	7.3	302.67 ± 1.25	50.04 ± 0.81	6.92 ± 0.83
HOF-AQE	21.5	154.52 ± 1.49	2.13 ± 0.36	0.19 ± 0.01
HOL-ME	34.9	172.09 ± 0.72	12.01 ± 0.60	2.02 ± 0.17
HOL-EAE	7.5	451.33 ± 4.81	54.77 ± 0.63	7.15 ± 0.03
HOL-AQE	23.5	104.91 ± 5.78	1.16 ± 0.45	0.14 ± 0.02
HMF-ME	24.8	199.19 ± 1.86	19.30 ± 0.31	3.31 ± 0.18
HMF-EAE	15.5	442.41 ± 6.16	55.30 ± 0.65	7.32 ± 0.11
HMF-AQE	28.2	192.22 ± 1.63	1.87 ± 0.30	0.41 ± 0.04
HML-ME	25.8	336.91 ± 0.59	10.48 ± 0.23	0.96 ± 0.07
HML-EAE	7.3	394.30 ± 0.53	16.00 ± 0.55	1.90 ± 0.01
HML-AQE	23.0	216.03 ± 0.82	2.86 ± 0.21	0.24 ± 0.01
HPF-ME	39.6	319.34 ± 2.46	10.33 ± 0.00	2.51 ± 0.03
HPF-EAE	12.2	385.98 ± 1.23	25.37 ± 0.60	5.75 ± 0.04
HPF-AQE	27.9	257.78 ± 1.82	4.59 ± 0.15	0.78 ± 0.15
HPL-ME	35.7	328.70 ± 0.99	12.72 ± 0.09	2.82 ± 0.31
HPL-EAE	7.8	443.43 ± 2.32	38.48 ± 0.54	7.49 ± 0.23
HPL-AQE	25.9	271.91 ± 3.13	6.67 ± 0.41	1.51 ± 0.02

[1] %, w/w on dry weight basis, Results are represented as means ± standard deviation (n=3). L, leaves; F, flowers; ME, methanol extract; EAE, ethylacetate extract; AQE, water extracts.

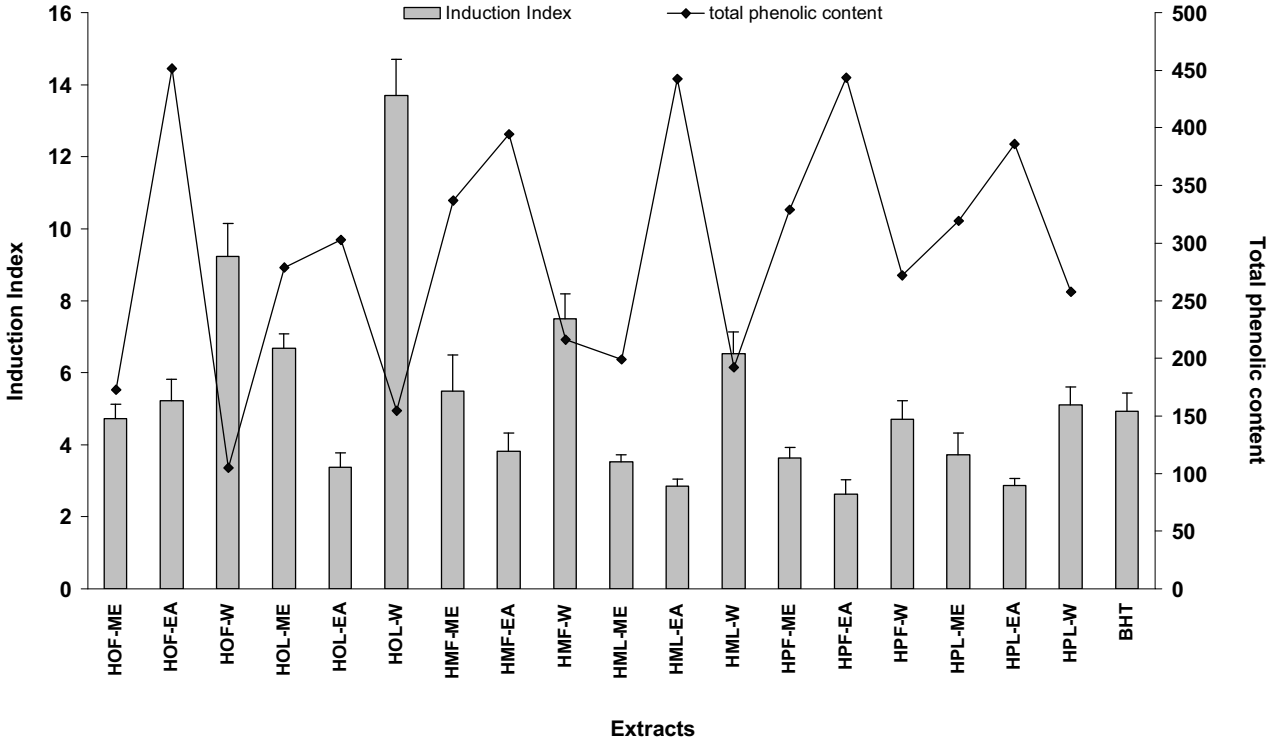

Fig. 1 Comparisons of radical-scavenging activities (IC_{50}), as measured DPPH method and total phenolic contents of studied *Hypericum* extracts. Vertical bars indicate standard error of mean. n=3 for each experimental group.

HML-EAE < HMF-EAE < HPL-EAE < HOL-EAE. It is easily observed in **Table 2** that water extracts have the lowest content of total phenols. The content of flavonoids, as rutin equivalents in mg/g of plant extract varied from 1.16 to 55.33 mg/g. The highest results of flavonoids were found in the ethyl acetate extracts, in turn, increasingly HML-EAE < HPF-EAE < HPL-EAE < HOF-EAE < HOL-EAE < HMF-EAE. Water extracts also had the lowest content of total flavonoids, similar to the results those of total phenolic contents of extracts.

The concentrations of flavonols are expressed in rutin equivalent in mg/g of plant extract and they were varied from 0.14 to 7.49 mg/g. The highest results of flavonols were also found in the ethyl acetate extracts as mentioned above with the order of HML-EAE < HPF-EAE < HOF-EAE < HOL-EAE < HMF-EAE < HPL-EAE. As expected theoretically from solvent polarity, these results are in general agreement.

DPPH experiments

There are many radical ions in the nature and they are scavenged by some compounds with different chemical reactions. Relatively stable organic radical, DPPH is one of them and it has been widely used for the determination of the antioxidant activity of pure compounds as well as the different plant extracts (Brand-Williams *et al.* 1995). One parameter that has been introduced recently for the interpretation of the results from the DPPH method is the efficient concentration or EC_{50} value, otherwise called inhibitory concentration, IC_{50} value. This is defined as the concentration of substrate that causes 50% loss of the DPPH activity (Molyneux 2004). The higher antioxidant activity corresponds to the lower value of IC_{50}.

Radical-scavenging activities of each extracts were measured employing different concentrations (9.6×10^{-4}, 1.8×10^{-3} and 3.6×10^{-3} mg/ml) and the results were pre-

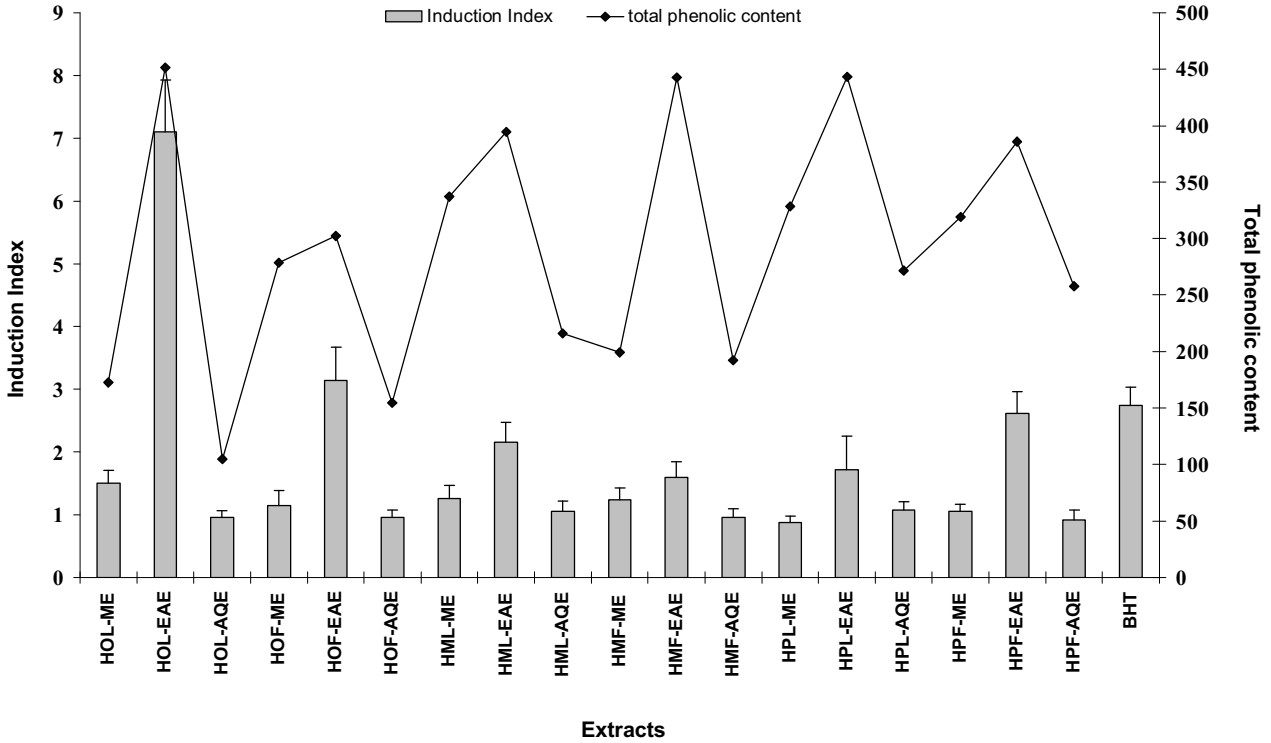

Fig. 2 Comparisons of Induction Index values as measured with Rancimat method and total phenolic contents of studied *Hypericum* extracts. Vertical bars indicate standard error of mean. n=3 for each experimental group.

sented as IC_{50} (**Fig. 1**). The highest radical-scavenging activity was exhibited by the ethyl acetate extracts. The lowest activity was observed in water extracts. Leaves and flowers of *H. montbretii* and leaves of *H. origanifolium* were the most active at the tested concentrations exhibiting an activity comparable to that of the positive control BHT, but all extracts, with the exception of leaves of *H. montbretii*, showed weak activity with respect to those of leaves and flowers of *H. perforatum* as reference plant.

Since there is no previous study reporting the chemical contents and antioxidant activity of *H montbretii* and *H. origanifolium*, the results obtained here can be only compared with those of *H. perforatum* known for its antioxidant activity (Hunt *et al.* 2001; Benedi *et al.* 2004; Silva *et al.* 2005; Conforti *et al.* 2007) and those of synthetic antioxidant BHT. Generally, leaves of the plants carry higher antioxidant activity as phenolic compounds when compared to flowers. Significantly the lowest IC_{50} data were found in ethyl acetate fractions, in turn, HOF-EAE < BHT < HMF-EAE < HOL-EAE < HML-EAE < HPF-EAE < HPL-EAE. Water (Aqueous) extracts had the lowest antiradical activities. The total phenolic compounds show mainly correlation with those results of radical scavenging activity. These results and their comparisons are demonstrated in **Fig. 1**.

Rancimat experiments

The Rancimat method is commonly used to evaluate the antioxidative properties of various natural and synthetic antioxidants and is based on the increase of electrical conductivity due to the formation of volatile compounds as a result of lipid oxidation. The extracts obtained from *Hypericum* species exhibited different effects on retarding the olive oil oxidation. The highest induction index (II) data were found in ethyl acetate extracts, in turn, HOL-EAE > HOF-EAE > BHT > HPF-EAE > HML-EAE > HPL-EAE > HMF-EAE. On the contrary, methanol and water extracts of *Hypericum* species did not inhibit the oxidation of olive oil at the studied concentrations.

Among the *Hypericum* species, extracts of *H. origamifolium* were found to have extremely potent antioxidant activity in olive oil. Its induction index value at the studied concentration was about three fold active than that of BHT (synthetic antioxidant) and *H. perforatum* (reference plant). Induction index of *H. montbretii* extracts was also approximate to that of BHT and *H. perforatum*. The total phenolic compounds show mainly correlation with those results of antioxidant activity. These results and their comparisons are demonstrated in **Fig. 2**.

The strong antioxidant activities of plant materials are believed to be a result of phenolic compounds (flavonoid, flavonol, phenolic acid, etc.) in their chemical structures (Salvador *et al.* 2001). In general, extracts or fractions with higher radical scavenging and antioxidant activities showed a higher phenolic content, and some good correlations were found with these parameters.

β-Carotene-linolenic acid experiments

In β-carotene/linoleic acid co-oxidation assay, the degree of lipid peroxidation is measured. The inhibition percentages of all the extracts of *Hypericum* species are presented in **Fig. 3.** According to **Fig. 3**, all the extracts inhibited the oxidation of linoleic acid in a statistically same (p<0.05) degree. The highest antioxidant activity values were shown by ethyl acetate extracts. Hierarchy of the EAE extracts were HOF < HOL < HPL < BHT < HPF < HML < HMF. Antioxidant activity of ME extracts is more than that of AQE extracts. Hierarchy of the ME extracts were HML < HOL < HPF < HPL < BHT < HMF < HOF. As shown in **Fig. 3**, the antioxidant activity of three *Hypericum* species flowers is also more than that of leaves. In addition, EA extracts of flowers was found to be as active as the positive control BHT.

HPLC analyses of *Hypericum* extracts

Along with extract yields, results of quantitative HPLC analyses of *Hypericum* extracts are summarized in **Table 3**. As expected, all constituents investigated, except for hyperforin in the extract of *H. montbretii* leaves, were found in all extracts, although their amounts varied considerably due to different species and plant parts analyzed. It is interesting to note that hyperforin was not detected in the extracts prepared from *H. montbretii* leaves. Several papers have previously reported the analysis of *H. perforatum* extracts. However, most of them have focused only on some indivi-

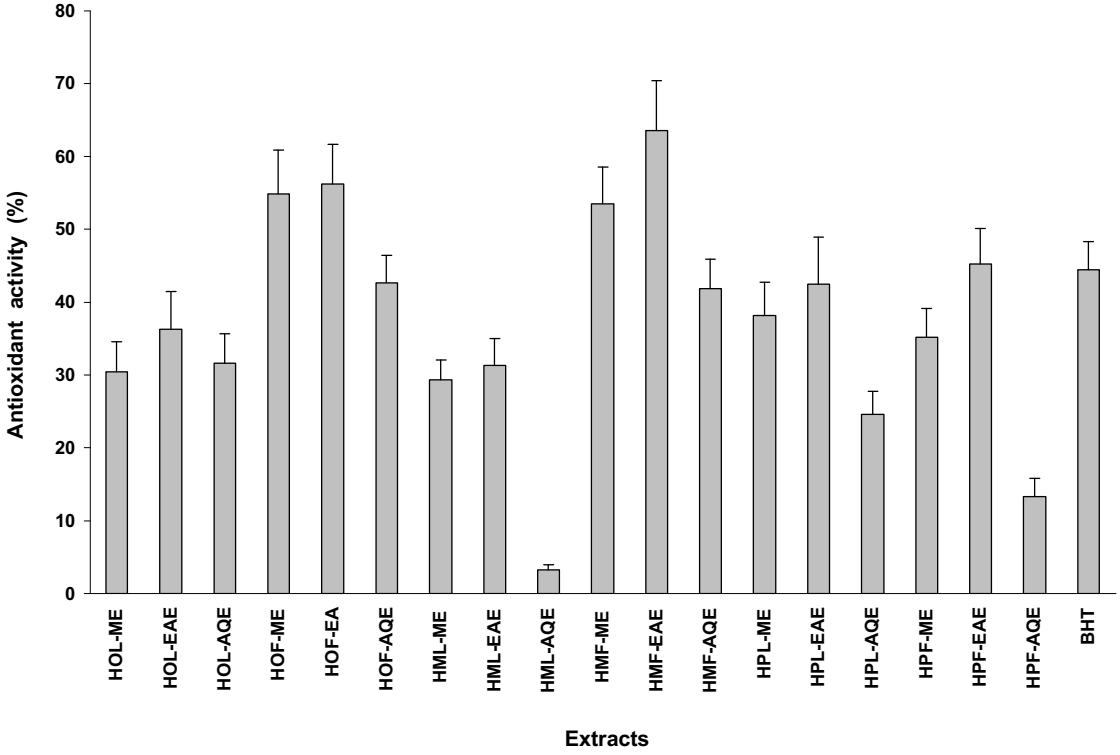

Fig. 3 The antioxidant activities in β-carotene-linoleic acid system of studied *Hypericum* extracts. Vertical bars indicate standard error of mean. n=3 for each experimental group.

Table 3 Constituents of extracts prepared from aerial parts of *H. perforatum* (HPE), leaves and flowers of *H. origanifolium* (HOLE and HOFE, respectively) and *H. montbretii* (HMLE and HMFE, respectively).

Extract contents	HPE	*H. origanifolium* extracts		*H. montbretii* extracts	
(ppm)		HOLE	HMLE	HMLE	HMFE
Yield (%)[1]	2.18	1.90	1.30	2.12	2.33
Chlorogenic acid	247.73	260.69	49	3661.43	918.67
Rutin	891.82	2150.69	1519	1131.71	3321.33
Hyperoside	198.18	14.14	19.45	3.80	26.22
Isoquercitrin	24.77	19.36	29.06	13.31	13.00
Quercitrin	297.27	1042.76	784	42.87	494.67
Quercetin	148.64	27.31	0.6	3.24	22.82
Hyperforin	222.95	9.2	2.91	N.D.[2]	212
Hypericin	55.66	10.37	10.93	1.96	45.73

[1] w/w on dry weight basis, L, leaves; F, flowers.
[2] N.D.: Not detectable, as its amount is under the limit of detection (LOD) of the method

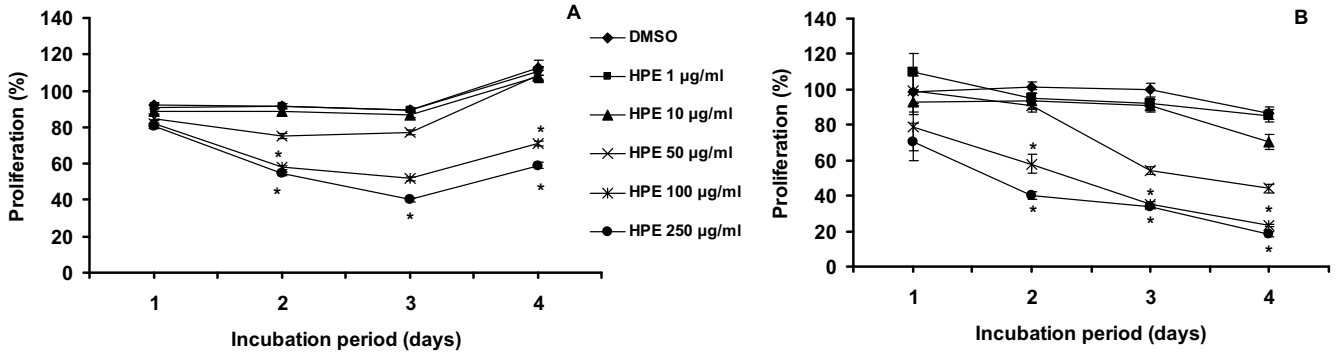

Fig. 4 Antiproliferative action of extract of *Hypericum perforatum* on A549 (A) and HeLa (B) cells as measured MTT method. HPE refers to extract prepared from aerial parts of *Hypericum perforatum*). *Denotes statistically significant inhibition of proliferation relative to DMSO controls. $p<0.05$; n=8 for each experimental group; Vertical bars indicate standard error of mean.

dual compounds, such as the hypericins or the phloroglucinols; LC–MS studies have also been done (Fuzzati *et al*. 2001; Tolonen *et al.* 2002; Silva *et al.* 2005). In this work, we fully analyzed the extracts obtained from *H montbretii* and *H. origanifolium* flowers and leaves by HPLC–DAD and the major compounds were identified and characterized. Phenolic compounds were identified by their UV spectra.

MTT experiments with cultured cells

Exhibiting similar action profiles, all extract tested in MTT assay showed antiproliferative/cytotoxic effects on A549 and HeLa cells. Extract prepared from aerial parts of *H. perforatum* with a concentration of 250 µg/ml exhibited a maximum antiproliferative effect against A549 and HeLa cells on the 3rd day and 4th of incubation, respectively (**Fig. 4**). Extracts prepared from *H. montbretii* leaves and flowers

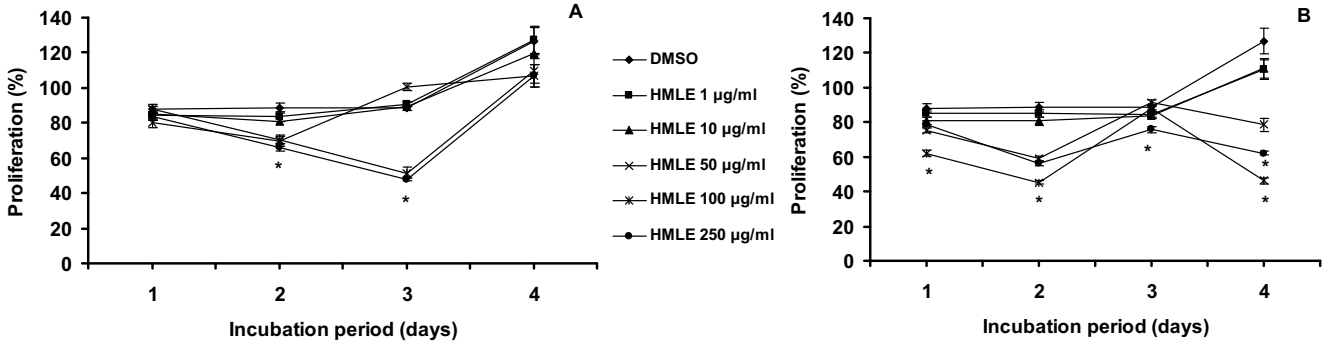

Fig. 5 Antiproliferative effects of extract prepared from leaves (A) and flowers (B) of *Hypericum montbretii* on A549 cell lines. HMLE, HMFE refer to extracts prepared *H. montbretii* leaves and flowers, respectively. Vertical bars indicate standard error of mean. $p<0.05$ relative to DMSO controls; n=8 for each experimental group.

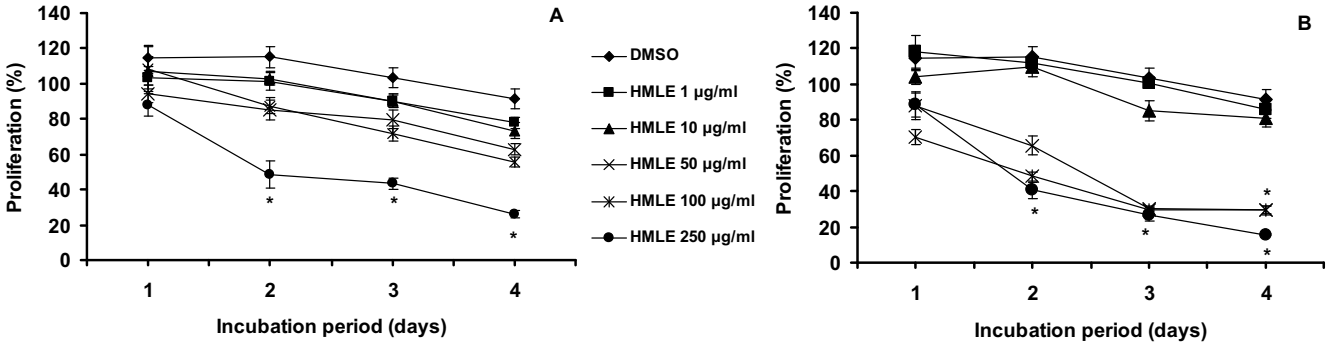

Fig. 6 Antiproliferative effects of extract prepared from leaves (A) and flowers (B) of *Hypericum montbretii* on HeLa cell lines. HMLE, HMFE refer to extracts prepared *H. montbretii* leaves and flowers, respectively. Vertical bars indicate standard error of mean. $p<0.05$ relative to DMSO control; n=8 for each experimental group.

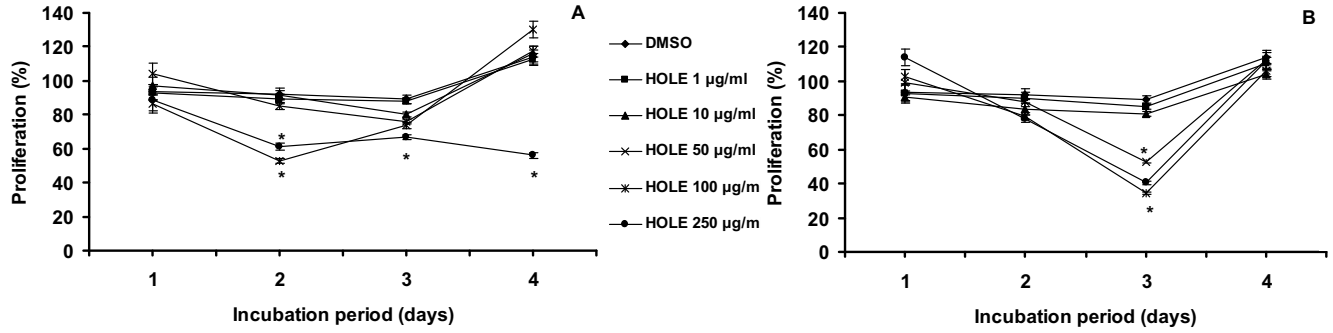

Fig. 7 Antiproliferative effects of extract prepared from leaves (A) and flowers (B) of *Hypericum origanifolium* on A549 cell lines. HMLE, HMFE refer to extracts prepared *H. montbretii* leaves and flowers, respectively. Vertical bars indicate standard error of mean. $p<0.05$ relative to DMSO controls; n=8 for each experimental group.

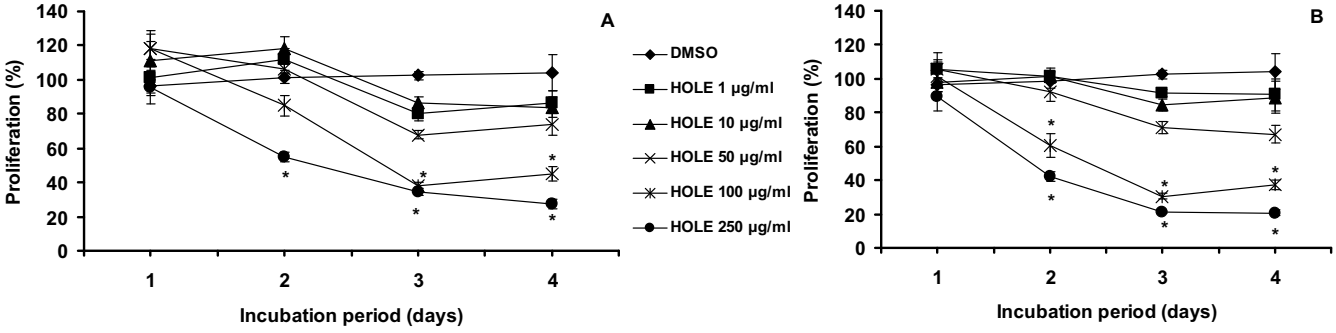

Fig. 8 Antiproliferative effects of extract prepared from leaves (A) and flowers (B) of *Hypericum origanifolium* on HeLa cell lines. HMLE, HMFE refer to extracts prepared *H. montbretii* leaves and flowers, respectively. Vertical bars indicate standard error of mean. $p<0.05$ relative to DMSO controls; n=8 for each experimental group.

with concentrations of 100 µg/ml exerted maximum antiproliferative effects against A549 cells on the 3rd and 4th days of incubations, respectively, while they were maximally effective at 250 µg/ml against HeLa cells on the 4th day of incubation (**Figs. 5** and **6**). In A549 cells, maximum antiproliferative effects of extracts prepared from *H. origanifolium* leaves and flowers occurred with 100 and 250 µg/ml on the 3rd and 4th days of incubations, respectively, while they were maximally effective at 250 µg/ml against HeLa cells on the 4th day of incubation (**Figs. 7** and **8**). From these findings it may be concluded that *Hypericum* extracts contain different amounts of antiproliferative com-

pounds, which have distinct mechanism of action on cell proliferation. As measured in HPLC analyses, all extracts seem to have a significant amount of chlorogenic acid, which is a potent antioxidant (Hsu *et al.* 2006; Takahama *et al.* 2007) and antiproliferative (Hsu *et al.* 2006; Granado-Serrano *et al.* 2007) compound. The antiproliferative effect of chlorogenic acid may be due to both its apoptotic and antioxidant actions (Hsu *et al.* 2006; Granado-Serrano *et al.* 2007; Takahama *et al.* 2007), being related to the inhibition of matrix metalloproteinase-9 enzyme (Jin *et al.* 2005). *Hypericum* extracts also contain significant amounts of rutin and its aglycone quercetin, which have been reported to have antiproliferative actions (Roseghini *et al.* 2007; Tanigawa *et al.* 2008). According to the literature data, quercitrin, isoquercitrin and hyperoside seem to have questionable antiproliferative effects.

CONCLUSION

The present study demonstrated for the first time that extracts prepared from *Hypericum montbretii* and *Hypericum origanifolium* leaves and flowers are effective against cancer cells, as indicated in cultured A549 lung cancer and HeLa cervix adenocarcinoma cells. The inhibitory effects of *H. montbretii* and *H. origanifolium* extracts on the proliferation of cancer cells seems to be closely related to their antioxidant and antiradical activities, which were also described for the first time in the present study, and phenolic compounds and naphtodianthrones of three *Hypericum* species as antioxidant and antiradical constituents are important in their antiproliferative activities. Another interesting issue raised with this study was the fact that all extracts exhibited antiproliferative/cytotoxic activities in a different profile of action and at different concentrations. It seems quite possible that these differences in antiproliferative action are due to phytochemical differences of the extracts tested. Obviously, additional studies will be required both for further characterization of antiproliferative effects and for the isolation of responsible compounds.

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