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Original Research Article

Antiproliferative Activity of Some Medicinal Plants on Human Breast and Hepatocellular Carcinoma Cell Lines and their Phenolic Contents

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Abstract

Purpose: *To determine the phenolic composition and antiproliferative activity of 16 different extracts (hexane, dichloromethane, methanol and water) obtained from Bellis perennis, Convolvulus galaticus, Trifolium pannonicum and Lysimachia vulgaris on human breast cancer (MCF-7) and human hepatocellular carcinoma (HepG2/C3A) cell lines.*
Methods: *The aerial parts of the plants were successively extracted with hexane, dichloromethane, methanol and water using a Soxhlet apparatus. The phenolic content of the plants were determined by plants by high performance liquid chromatography (HPLC) while their antiproliferative activity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, a yellow tetrazole (MTT) assay.*
Results: *Among the tested extracts, the methanol extract of B. perennis showed the best anti-proliferative activity against MCF-7 cell line with IC_{50} (inhibiting 50 % of cell growth) value of 71.6 μg/mL. Furthermore, the dichloromethane extract of C. galaticus showed the best anti-proliferative activity against HepG2/C3A cell line with IC_{50} of 57.3 μg/mL. The HPLC data for the plant extracts showed the presence of the following phenolic compounds: gallic acid monohydrate, caffeic acid, rutin hydrate, luteolin-7-O-β-D glucoside, kaempferol, myricetin, quercetin, coumarin and apigenin.*
Conclusion: *The findings of this study indicate that there is some justification for the use of B. perennis and C. galaticus as traditional anticancer medicinal herbs.*

Keywords: *Bellis perennis, Convolvulus galaticus, Trifolium pannonicum subsp. elongatum, Lysimachia vulgaris, MCF-7, HepG2/C3A, Phenolics, Breast cancer, Antiproliferative*

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INTRODUCTION

Bellis perennis L. (common daisy) is a herbaceous perennial herb in the family Asteraceae [1]. Common daisy is known as a traditional wound herb [2,3] and it had been used for the treatment of bruises, broken bones [4], sore throat [5], headache [6], common cold [7], rheumatism, inflammation and infections of the upper respiratory tract in traditional medicine [8]. The main constituents are saponins [9,10], essential oils [11], phenolics [12,13]. Antibacterial [11], antifungal [11,14], antioxidant [8,11], wound healing activity [2], anxiolytic properties [15], in vitro regeneration protocol [13], antitumor activity [16] and cytotoxic activity against HL-60 human promyelocytic leukemia cells [17] of *B. perennis* has also been investigated.

Convolvulus galaticus Rostan ex Choisy (Grizzle bindweed) is an endemic, perennial herb in the family Convolvulaceae. Leaves and roots of *C. galaticus* have been used as chalogogue, laxative, antihelmintic and strongly purgative in traditional phytotherapy. Furthermore, its flowers are used in the relief of toothache [18,19]. Antibacterial [20,21], antitumor [21] and anticancer activities [22] of *C. galaticus* have been reported.

Trifolium pannonicum Jacq. subsp. elongatum (Willd.) Zoh. (Hungarian clover) is an endemic perennial plant belonging to Fabaceae family. *Trifolium* spp. has been used in folk medicine for the treatment of skin conditions [21]. *T. pannonicum* contains triterpene saponins in the seeds and flavonoid glycosides in the aerial parts [23]. Antibacterial [20,21] and antioxidant [23] activities of *T. pannonicum* have been reported.

Lysimachia vulgaris L. (yellow loosestrife) is a rhizomatous perennial herb in the family Primulaceae [24]. It has been used in the treatment of fever, ulcers, diarrhea, wounds and as an analgesic, expectorant and anti-inflammatory agent since ancient time [19]. It is a convenient plant for phytopurification of wastewater [25]. It contains flavonoids, sterols, phenolic acids and tannins [26,27]. Podolak *et al* [27] reported that a benzoquinone pigment and triterpene saponosides from underground parts of yellow loosestrife had a cytotoxic activity in vitro against several cancer cell lines (human and mouse melanoma cells) and also inhibited the growth of *Candida albicans* strains.

The present study aims, for the first time to the best of our knowledge, to evaluate the anti-proliferative properties of *B. perennis, C. galaticus, T. pannonicum* subsp. *elongatum* and *L. vulgaris* extracts (hexane, DCM, MeOH and water) against human hepotocellular carcinoma (HepG2/C3A) cell and human breast cancer (MCF-7) cell lines.

EXPERIMENTAL

Plant materials and preparation of extracts

B. perennis, C. galaticus, and *T. pannonicum* Jacq. subsp. *elongatum* aerial parts were collected from Abant Izzet Baysal University Campus, Bolu/Turkey in 2013. L. vulgaris was collected from Abant Lake, Bolu/Turkey in 2013. Identification of the species was made by Arzu Ucar Turker using “Flora of Turkey and the East Aegean Islands” [24,28-29] and voucher specimens (Table 1) were deposited at the Abant Izzet Baysal University (AIBU) Herbarium, Bolu, Turkey.

The air-dried (1 week) powdered plant parts of *B. perennis, C. galaticus, T. pannonicum* and *L. vulgaris* were successively extracted with hexane (at 65-70 ^{o}C), DCM (at 55-60 ^{o}C), MeOH (at 60 ^{o}C) and water (at 80 ^{o}C) to achieve extraction of both non-polar compounds to polar compounds, using a Soxhlet apparatus for 24 h. The extracts were filtered and extraction solvents (hexane, DCM and MeOH) were evaporated under low pressure at a temperature not higher than 45 ^{o}C using rotary evaporator. Aqueous extracts were evaporated using a lyophilizator at -65 ^{o}C. Plant materials, brief extraction procedure, designation of studied extracts and yields are shown in Table 1.

Human cancer cell lines and culture conditions

Human breast adenocarcinoma (MCF-7) and human hepatocellular carcinoma (HepG2/C3A) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were grown in Dulbecco’s minimum essential medium (DMEM) with Earle’s salts (Mediatech Cellgro, Herndon, USA). Culture medium was supplemented with 10 % fetal bovine serum (FBS; Hyclone, Logan, USA), a solution of vitamins, sodium pyruvate and non-essential amino acids (all at 1:100 v/v dilution of supplied solutions), penicillin (100 I.U./mL) and streptomycin (100 µg/ mL) (Mediatech Cellgro, VA). Cells were cultured at 37 °C in a humidified environment containing 5 % CO_2.

Cell viability assay

Exponentially growing cells were plated in 96-well microplates (Costar , Corning Inc.) at a density of 10 × 10^3 cells per well in 100 µL of culture medium and were allowed to adhere for 16 h before treatment. Increasing concentrations of each extract in DMSO (Sigma-Aldrich) were then added (100 µL per well) and the cells were incubated for 24 h. The final concentration of DMSO in the culture medium was maintained at 0.5 % (v/v) to avoid solvent toxicity. Cytotoxicity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, a yellow tetrazole (MTT) assay [30] on an automated 96-well Multiskan FC micro plate photometer reader (Thermo Fisher Scientific Inc.) at 570 nm. The proliferation test is based on the color reaction of mitochondrial dehydrogenase in living cells by MTT. The culture medium was removed and replaced with 90 µL of fresh culture medium. Ten

microlitres of sterile filtered MTT solution (5 mg/mL) in phosphate buffered saline (PBS, pH 7.4) were added to each well, reaching a final concentration of 0.5 mg MTT/ ml which was then incubated at 37 ˚C in 5 % CO_2 for 4 h. After 4 h, 100 µl/well of DMSO were added to all samples for dissolving the formazan that is the final product of MTT reaction and were allowed to incubate at 37 °C, in a 5 % CO_2 humidified incubator for a night. After incubation, absorbance of formazan was measured spectrophotometrically in a Multiskan FC microplate photometer reader at 570 nm. Each experiment was carried out three times in triplicate. The relative cell viability (%) relative to control wells containing cell culture medium without samples was calculated as:

Relative cell viability = 100 × A570 nm (sample) / A570 nm (control)

HPLC analysis of phenolic compounds

The DCM and methanolic extracts were analyzed using a HPLC system (VWR-Hitachi LaChrom Elite®) equipped with a Hitachi L-2455 Diode-Array Detector (DAD), Hitachi L-2130 Pump, Hitachi L-2200 Autosampler. Chromatographic separation was achieved using Hitachi column oven L-2300 and Venusil XBP C18 column (Bonna-Agela Technologies, particle size 5 µm, 4.6 x 250 mm). Flow rate was 1 ml/min with 25 ºC oven and injection volume was 20 µL. All solvents were HPLC grade (Merck) and mobile phase was composed of solvent A (acetronitrile) and solvent B (0.1 % acetic acid). A gradient elution was performed. Mobile phases and ultrapure water (SG Labostar) were filtered through a 0.45 µm hydrophilic polypropylene membrane filter (47 mm) (Pall Corporation) prior to HPLC injection. Spectra data were recorded from to 200 to 400 nm during the entire run. The chromatograms were obtained at 280 nm.

Sample preparation for HPLC analysis

Dried DCM and MeOH extracts (100 mg) were dissolved in 1 ml ACETRONITRILE. All standards (gallic acid monohydrate, caffeic acid, rutin hydrate, luteolin-7-O-β-D glucoside, kaempferol, myricetin, quercetin, coumarin and apigenin) were prepared at 1 mg/ml in ACETRONITRILE and mixed together to make five different concentrations (1, 5, 10, 20, 40, 60, 80 and 100 mg/L) for the preparation of standard curve. All extracts and standards were filtered through a 0.2-µm GHP Acrodisc (25 mm) (Pall Corporation) into 2-mL HPLC vials. Procedures were repeated 3 times for each sample tested.

Statistical analysis

All data were analyzed by analysis of variance (ANOVA) with the last factor as a within subject or repeated design using SPSS version 15 (SPSS Inc., Chicago, IL, USA). Values were considered statistically significant at $p \leq 0.05$. The data are presented as mean ± standard error (SE) after back transforming from ANOVA results.

RESULTS

The cytotoxic effects of the 16 crude extracts (hexane, dichloromethane, methanol and water) of *B. perennis, C. galaticus, T. pannonicum* and *L. vulgaris* at various concentrations were evaluated with *in vitro* cytotoxicity assay against MCF-7 and HepG2/C3A cell lines (Table 1). Percentage of the cell viability was measured by MTT assay (Figures 1 and 2). A plant extract is usually regarded as interesting for in vitro cytotoxic activity if IC_{50} < 100 µg/ml [31].

The cytotoxic effects of all extracts against MCF-7 and HepG2/C3A cells were shown in Table 1. Among the tested extracts, the methanolic extract of *B. perennis* showed the best antiproliferative activity against MCF-7 cell line with IC_{50} value of 71.6 µg/mL. The aqueous extracts of *B. perennis* showed a moderate antiproliferative activity against MCF-7 cells, with the IC_{50} value of 147.6 µg/mL. Meanwhile, DCM extract of *C. galaticus* showed also a moderate anti-proliferative activity against MCF-7 cell line, with the IC_{50} value of 172.4 µg/mL (Table 1).

Among the tested extracts, the DCM extract of *C. galaticus* showed the best anti-proliferative activity against HepG2/C3A cell line with IC_{50} value of 57.3 µg/mL. Furthermore, MeOH extracts of *B. perennis* and *C. galaticus* showed high anti-proliferative activity against HepG2/C3A, with IC_{50} value of 73.9 and 75.4 µg/ml, respectively.

Other tested plant extracts did not show any antiproliferative activity against MCF-7 and HepG2/C3A cell lines at all concentrations tested (Table 1). The shape of dose-response curves indicated a significant inhibition of cell growth in a dose-dependent manner (Figures 1 and 2).

In the present study, quantification of the chosen phenolics in *B. perennis, C. galaticus, T. pannonicum* and *L. vulgaris* was performed using the HPLC technique.

Table 1: *In vitro* anti-proliferative effect of studied plants on MCF-7 and HepG2/C3A cell lines

Family and Plants Species	Collection Number	Parts Used	Solvents	Designation	Yield (%)*	IC50 (µg/mL)	
						MCF-7	HepG2/C3A
Asteraceae							
Bellis perennis L.	AUT-1909	Flower	Hexane	BH	3.9	> 200	> 200
			DCM	BD	2.5	> 200	184.7 ± 1.2
			MeOH	BM	29.9	71.6 ± 2.0	73.9 ± 3.0
			Water	BW	21.8	147.6 ± 4.0	154.2 ± 0.7
Convolvulaceae							
Convolvulus galaticus Rostan ex Choisy	AUT-2013	Aerial	Hexane	CH	1.7	> 200	166.9 ± 0.2
			DCM	CD	5.2	172.4 ± 0.5	57.3 ± 0.9
			MeOH	CM	11.7	> 200	75.4 ± 1.5
			Water	CW	5.7	> 200	> 200
Fabaceae							
Trifolium pannonicum Jacq. subsp. *elongatum* (Willd.) Zohary	AUT-2014	Aerial	Hexane	TH	0.9	> 200	> 200
			DCM	TD	1.5	> 200	> 200
			MeOH	TM	7.1	> 200	> 200
			Water	TW	10.9	> 200	> 200
Primulaceae							
Lysimachia vulgaris L.	AUT-2008	Aerial	Hexane	LH	2.1	> 200	> 200
			DCM	LD	3.4	> 200	> 200
			MeOH	LM	10.3	> 200	> 200
			Water	LW	2.9	> 200	> 200

Note: *Mean values (±standard deviation) for triplicate assays. IC_{50}: Concentration of extract that cause 50% inhibition of cell proliferation.*

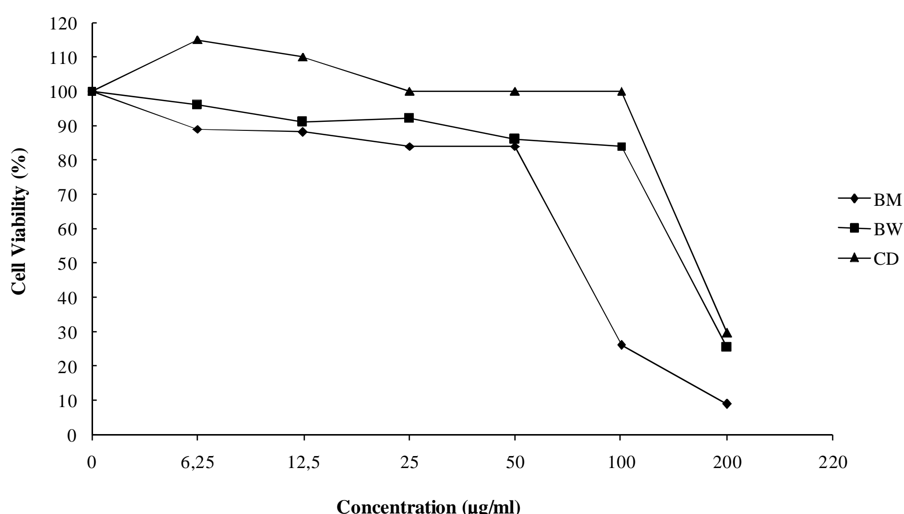

Fig. 1: Dose-response curves of the effect of BM, BW and CD extracts on cell growth in MCF-7 cells. Cells were treated with various concentrations (6.25, 12.5, 25, 50, 100 and 200 µg/mL) of extracts, after 24 h of exposure. Antiproliferative effects were measured by MTT assay. Results were expressed as mean ± SD (n = 3)

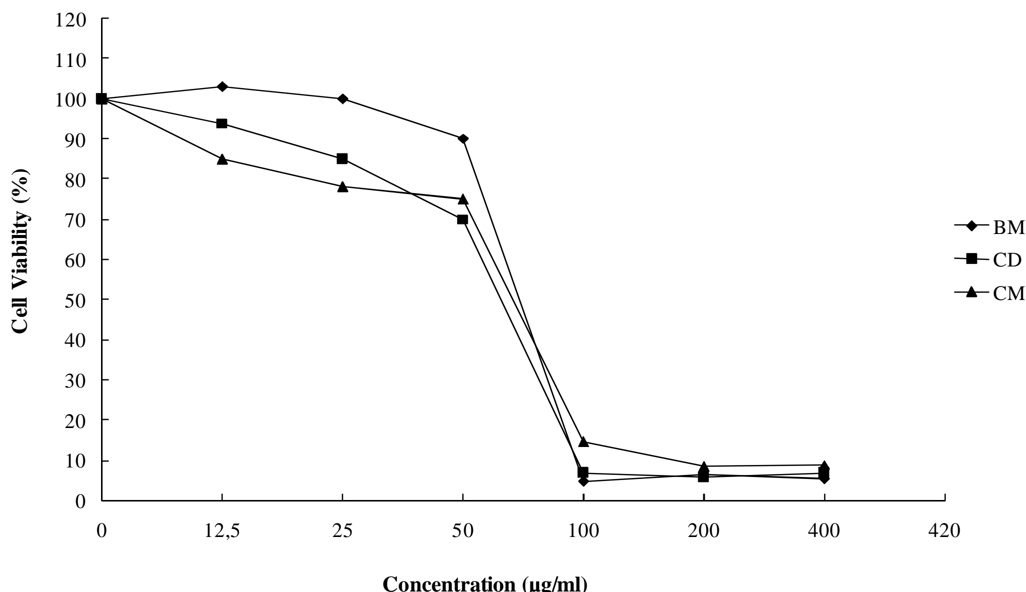

Fig. 2: Dose-response curves of the effects of BM, CD and CM extracts on cell growth in HepG2/C3A cells. Cells were treated with various concentrations (12.5, 25, 50, 100, 200 and 400 µg/mL) of extracts, after 24 h of exposure. Antiproliferative effects were measured by MTT assay. Results were expressed as mean ± SD for three independent determinations

The MeOH extract of *L. vulgaris* contained the highest total phenolic compounds (40632.4 µg/g dry extract) (Table 2) while the DCM extract of *C. galaticus* contained the lowest total phenolic compounds (339.36 µg/g dry extract). Considerable amounts of quercetin (12517.02 µg/g dry extract) and apigenin (512.54 µg/g dry extract) were found in *B. perennis* extracts. The most dominant were rutin hydrate and luteolin-7-O-β-D glucoside in *C. galaticus, T. pannonicum* and *L. vulgaris* extracts (Table 2).

DISCUSSION

A high number of new natural drugs derived from plant secondary metabolites have been used in the treatment and/or prevention of cancer [32]. Since 1990, there has been a 22 % increase in cancer incidence and mortality, with over 10 million new cases [32]. Important progress has been made in cancer chemotherapy, a considerable portion of which can be attributed to plant-derived drugs [32]. *B. perennis* has been used as a medicinal herb against cancer, breast cancer and uterine cancer [34]. In the literature, some saponins isolated from the root of B. perennis were found to be cytotoxic against only human promyelocytic leukemia (HL-60) cells [17]. However, no anticancer activity has been shown for flower extracts of *B. perennis*. Although there are some studies indicating the biological activities of *B. perennis* flower extracts, there is no study on the anticancer activity of this species. We therefore aimed to evaluate the anticancer activity of *B. perennis* by MTT assay on human breast adenocarcinoma (MCF-7) and human hepatocellular carcinoma (HepG2/C3A).

Generally, methanol extracts were more active on MCF-7 cell line. Methanol extracts of plant materials may contain active components such as tannins, polyphenols, polyacetylenes, flavonol, terpenoids, and flavonoids [35]. In the present study, TLC plates also showed that methanolic extracts contained more phenolic and saponin compounds qualitatively than other tested extracts of *B. perennis* (data not shown). Natural products have been shown to be an excellent and reliable source for the development of new drugs [36]. Saponins are well known compounds in B. perennis and they have anticancer activity against human promyelocytic leukemia (HL-60) cells [17]. In the present study, TLC results showed that MeOH extracts of flowers of *B. perennis* have more phenolic compounds and saponins than other extracts, (data not shown). Thus, in our present study, the high levels of anticancer activity of MeOH extracts of *B. perennis* may be attributed to this high saponin and phenolic contents. Extracts of *B. perennis* flowers have anticancer activity against the human breast adenocarcinoma (MCF-7) and human hepatocellular carcinoma (HepG2/C3A) cell lines. Altogether, these results support the traditional use of *B. perennis* in the treatment of cancer.

Table 2: Content of selected phenolics in examined *B. perennis*, *C. galaticus*, *T. pannonicum* and *L. vulgaris* extracts. Values are means ± SD (n = 3)

STANDART COMPOUNDS			PLANT EXTRACTS[b] (µg/g dry extract)							
Name	**Peak number[a]**	**RT (min)**	**BD**	**BM**	**CD**	**CM**	**TD**	**TM**	**LD**	**LM**
Gallic acid monohydrate	1	4.7	10.88±0.1	11.64±0.05	38.12±0.03	133.01±0.01	8.4±0.02	80.5±0.04	41.64±0.02	139.0±0.1
Caffeic acid	2	10.65	47.06±0.07	95.82±0.1	34.0±0.01	306.0±0.01	4.0±0.01	189.0±0.01	83.0±0.02	75.0±0.01
Rutin hydrate	3	11.92	244.62±0.01	3239.12±0.02	47.67±0.01	2772.4±0.05	6.73±0.01	1285.4±0.5	2613.0±0.4	29024.0±0.7
Luteolin-7-O-β-D glucoside	4	12.52	63.64±0.07	-	8.29±0.02	1003.4±0.2	20.4±0.02	20959.4±0.9	244.7±0.1	8930.0±0.3
Kaempferol	5	12.7	147.8±0.1	3241.94±0.01	4.16±0.03	521.1±0.1	37.63±0.01	73.5±0.01	287.0±0.01	437.0±0.02
Myricetin	6	14.1	67.42±0.05	86.04±0.01	68.21±0.01	≤ 0.001	≤ 0.001	4680.1±0.7	145.0±0.02	605.0±0.1
Quercetin	7	15.76	340.52±0.02	12517.02±0.01	28.73±0.02	329.5±0.02	85.85±0.2	3407.9±0.2	1153.0±0.6	958.0±0.05
Coumarin	8	16.68	1.34±0.01	-	51.37±0.01	91.0±0.1	282.2±0.01	≤ 0.001	≤ 0.001	≤ 0.001
Apigenin	9	17.05	512.54±0.02	353.7±0.05	58.8±0.5	112.8±0.01	≤ 0.001	318.3±0.09	≤ 0.001	453.1±0.02
Total Phenolics			1435.82	19545.38	339.36	5136.0	445.06	30993.8	4566.54	40632.4

Although Tokgun *et al* [22] reported the strongest anti-proliferative activity in methanolic extract of *C. galaticus* against MCF-7 cell line at low concentration (0.32 mu g/mL), we found that the methanolic extract did not have a notable anti-proliferative activity against MCF-7 cell line. But DCM extract of *C. galaticus* had a moderate anti-proliferative activity against MCF-7 cell line in our study. The differences in these two studies may have arisen from plant extraction procedure. Furthermore, we are the first to report that DCM and methanol extracts of *C. galaticus* have antiproliferative activity against HepG2/C3A cell lines.

Podolak *et al* [27] showed the anticancer activity of *L. vulgaris* on human and mouse melanoma cells, however, we could not find the same activity against MCF-7 and HepG2/C3A cancer cells in our present study. The results of all studies indicated that different parts of plants had different levels of biological activity and phenolic compounds depending on the type of solvent used in the extraction procedure. The most important human diseases such as cancer, neurodegenerative and cardiovascular diseases are the result of free radicals.

Phenolics are that antioxidant compounds that are widely found in natural environments and inhibit free radical formation [37]. Because of this reason, we investigated the nine phenolic compositions (gallic acid monohydrate, caffeic acid, rutin hydrate, luteolin-7-O-β-D glucoside, kaempferol, myricetin, quercetin, coumarin and apigenin) of tested extracts by HPLC analysis. The phenolic profiles of *C. galaticus* and *T. pannonicum* were detected for the first time and all tested standart phenolic compounds were found in both species. Toth *et al* [37] showed that polyphenol composition of three Lysimachia species. They similarly detected caffeic acid, chlorogenic acid, myricetin, isorhamnetin, quercetin and kaempferol in *L. vulgaris*. However, gallic acid, rutin hydrate, luteolin-7-O-β-D glucoside and apigenin in *L. vulgaris* were demonstrated for the first time with our present study. It has been previously shown that phenolic constituents of *B. perennis* include flavonoids [13,38], anthocyanins [39] and phenolic acids (caffeic, ferulic, sinapic, p-coumaric, and salicylic acids) [40].

The following flavonoids were described in daisy flowers: quercetin, apigenin, kaempferol and isorhamnetin [38]. In the present study, some phenolic compounds such as coumarin, luteolin-7-O-β-D glucoside, myricetin and rutin hydrate were determined in flowers of *B. perennis* for the first time by HPLC analysis.

CONCLUSION

Our findings demonstrated that methanol extract of *B. perennis* flowers has anti-proliferative effect both on human breast cancer (MCF-7) and human hepatocellular carcinoma (HepG2/C3A) cancer cells. In addition, the methanolic and DCM extracts of *C. galaticus* have anti-proliferative effects on HepG2/C3A cell line. With this study, *B. perennis* and *C. galaticus* gained scientific justification as anticancer medicinal herbs. Anticancer activities of *C. galaticus* against HepG2/C3A and *B. perennis* against MCF-7 and HepG2/C3A cancer cell lines were revealed for the first time. Furthermore, anticancer activities of aerial parts of *L. vulgaris* and *T. pannonicum* on human breast cancer (MCF-7) and human hepatocellular carcinoma (HepG2/C3A) were evaluated for the first time. Unfortunately, these plant extracts were not active to selected cancer cell lines in this study. Future studies should be focused on fractionation of the active plant extracts to identify active components with anticancer activity.

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