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Qualitative HPLC-DAD-ESI-TOF-MS analysis, cytotoxic and apoptotic effects of Croatian endemic *Centaurea ragusina* L. aqueous extracts

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Abstract

Centaurea ragusina L.*,* an endemic Croatian plant species, revealed a good cytotoxic activity of aqueous extracts on human bladder (T24) and human glioblastoma (A1235) cancer cell lines. The chemical constituents were tentatively identified using high performance liquid chromatography HPLC-DAD-ESI-TOF-MS in negative ionization mode. The main compounds of herba extract were sesquiterpene lactones: solstitialin A-3, 13 diacetate and epoxyrepdiolide; organic acid: quinic acid. The main compounds of flower extract were organic acids: quinic acid, citric acid and malic acid; sesquiterpene lactone: cynaropicrin; phenolic compounds: chlorogenic acid and phenylpropanoid: syringin. The aqueous extracts of *C. ragusina* were investigated for correlation of their effects on

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human bladder (T24) and human glioblastoma (A1235) cancer cell lines using the MTT assay. Although both extracts showed significant dose- and time-dependent cytotoxic activity against both cancer cell lines, the flower extract exhibited slightly higher activity. In order to determine type of cell death induced by treatment, cell lines were exposed subsequently to a treatment with both flower and herba aqueous extracts. The majority of the cells died by induced apoptosis treatment. Flower aqueous extract (26.25 %), compared to a leaf aqueous extract (22.15 %) showed slightly higher percentage of an apoptosis in T24 cells, when compared to a non-treated cells (0.04 %).

Keywords: *Centaurea ragusina*; cytotoxic; apoptotic; MTT; sesquiterpene lactone

Introduction

Plants natural products, over a long historical period, have been used as chemo-protective agents in cancer treatment [1, 2]. Natural products may not necessarily be the final drug compound, but may serve as sources of novel structures for a final anticancer medicine. Over the time period of more than 70 years, 48.6 % of 175 small molecules, used in cancer treatment, are either natural products or directly derived from natural products [1]. Some of the current anticancer drugs derived from plant extracts belong to a group of terpenes [3]. *Centaurea* species are rich in terpene compounds, mainly sesquiterpenes, and are a good potential source of cytotoxic compounds that may serve as an anticancer drug [4, 5]. *Asteraceae* species are well known in many bioactive compounds, although there are still many species where the biological activity of their extracts has not yet been investigated [6, 7].

Centaurea genus is one of the largest in the *Asteraceae* family, comprising around 400-700 species, distributed mainly in the Mediterranean area [8]. In Croatia, there are more than 80 *Centaurea* species with 27 endemics [9]. *C. ragusina*, Dubrovnik's cornflower or dusty miller, is an endemic Croatian plant species growing in a very small geographic area, in the arid and salt-affected gaps of the vertical cliffs along the Adriatic coast and Croatian islands.

The species which belong to *Centaurea* genus possess a valuable source of bioactive compounds and have been used in ethno-medicines throughout Europe, especially in the Mediterranean region [7, 10-14]. The wild growing *Centaurea* plants have been traditionally used for treatment of various diseases due to their antimicrobial, antifungal, anti-inflammatory, antidiabetic, antioxidant, antiviral, antitumor and other properties [10-12, 15].

Cytotoxic properties of the *Centaurea* plant extracts are usually related to their chemical composition. The majorities of compounds in aqueous extracts (AE) belong, but are not limited to the groups of sesquiterpene lactones (SQLs), acetylenes, phenolics, flavonoids, glycosides, lignans, coumarines and others [16-21]. Sesquiterpene lactones, phenols, flavonoids and lignans are usually regarded as a group of compounds responsible for diverse biological activity of *Centaurea* plant extracts [15, 16, 22].

The majority of the *Centaurea* species have not yet been investigated on the phytochemical profile and biological activity of their extracts [23].

Previous research on *C. ragusina* species studied phytochemical composition of volatile compounds and its efficient antimicrobial effects [24] as well as total polyphenol content and antioxidant activity of plant specimens cultivated *in vitro* and collected in natural habitats [25].

The medicinal importance and diverse biological activity of *Centaurea* genus species motivated us to study the biological activity of *C. ragusina* AE. To our best knowledge, no data on phytochemical studies of *C. ragusina* AE have been available to date. This study aims to evaluate the cytotoxic activity and phytochemical profile of AE obtained from endemic Croatian species *C. ragusina*.

Results and discussion

Phytochemical profile of the aqueous extracts of *C. ragusina*

C. ragusina herba and flower AE phytochemical profile was studied using HPLC-DAD-ESI-TOF-MS system. Results are presented as chromatographs (Fig. 1) and list of determined chemical compounds (Table 1.). The detected compounds were all tentatively identified by comparison of their spectroscopic data with data reported in the literature and by correlation with previous literature reports.

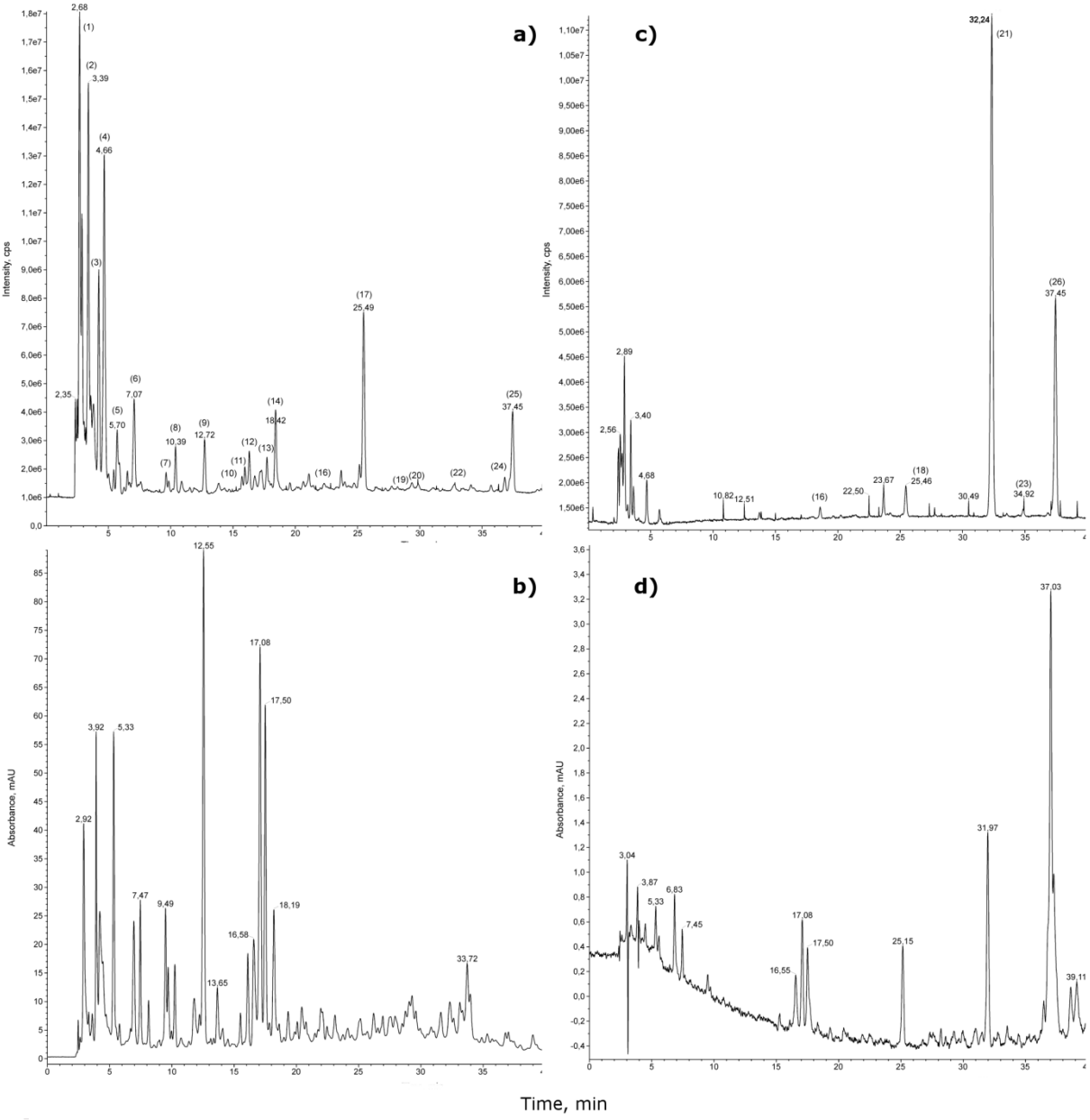

Figure 1: The TIC chromatogram of negative mode (a) and HPLC-DAD chromatogram of *Centaurea ragusina* L. flower aqueous extract (b); the TIC chromatogram of negative mode (c) and HPLC-DAD chromatogram of *Centaurea ragusina* L. herba aqueous extract (d)

Table 1. Tentative assignment of chemical components found in herba and flower of *Centaurea ragusina* L. aqueous extracts by using HPLC-DAD-ESI-TOF-MS in negative ionization mode.

	Proposed compound (peak number)	Rt (min)	[M–H]- m/z	Molecular formula	Spectral methods used in identification	Reference	*Centaurea Ragusina* L. (herba extract)	*Centaurea Ragusina* L. (flower extract)
OA	Quinic acid **(1)**	2.89	191.0559	$C_7H_{12}O_6$	HRMS	[26, 27] (a)	+	+
OA	Malic acid **(2)**	3.40	133.0140	$C_4H_6O_5$	HRMS	[26, 27] (a)	+	+
OA	Malonic acid **(3)**	3.62	103.0038	$C_3H_4O_4$	HRMS	[26]c	+	+
OA	Citric acid **(4)**	4.68	191.0194	$C_6H_8O_7$	HRMS, UV	[26, 27] (a)	+	+
OA	Succinic acid **(5)**	5.70	117.0191	$C_4H_6O_4$	HRMS	[26, 27] (a)	+	+
P	Mucic acid dimethyl ester 2-O-gallate **(6)**		389.0719	$C_{15}H_{18}O_{12}$	HRMS	[28, 29] (c)	-	+
P	Hydroxybenzoic acid hexoside **(7)**	9.64	299.0775	$C_{13}H_{16}O_8$	HRMS, UV	[26](c)	-	+
	Unknown (8)	10.39	368.0984	$C_{16}H_{19}NO_9$	HRMS		-	+
P	Protocatechuic acid **(9)**	12.73	153.0189	$C_7H_6O_4$	HRMS, UV	[30, 31] (a)	-	+
P	Glycosyringic acid **(10)**	13.84	359.0983	$C_{15}H_{20}O_{10}$	HRMS, UV	[26, 31] (a)	-	+
	Unknown (11)	16.31	376.1761	$C_{20}H_{27}NO_6$	HRMS		-	+
P	4-hydroxybenzoic acid **(12**)	17.31	137.0235	$C_7H_6O_3$	HRMS	[32, 33] (a)	-	+
PPG	Syringin **(13)**	17.73	371.1420	$C_{17}H_{24}O_9$	HRMS	[18, 34] (a)	-	+
P	Chlorogenic acid **(14)**	18.42	353.0873	$C_{16}H_{18}O_9$	HRMS, UV	[18] (a)	-	+

SQL	Solstitialin **(15)**	18.58	279.1239	$C_{15}H_{20}O_5$	HRMS, UV	[35] (a)	+	-
P	5-O-p-coumaroylquinic acid **(16)**	22.31	337.0926	$C_{16}H_{18}O_8$	HRMS, UV	[26, 36] (a)	-	+
SQL	Deacylcynaropicrin **(17)**	25.46	262.1205	$C_{15}H_{18}O_4$	HRMS, UV	[37] (b)	+	-
	Unknown **(18)**	25.49.	460.1967	$C_{24}H_{31}NO_8$	HRMS		-	+
PG	Luteolin-3,7-di-O-glucoside **(19**)	27.25	609.1453	$C_{27}H_{30}O_{16}$	HRMS, UV	[38, 39] (a)	-	+
PG	Patulitrin **(20)**	29.07	493.0979	$C_{22}H_{22}O_{13}$	HRMS, UV	[39] (a)	-	+
SQL	Solstitialin A-3, 13 diacetate **(21)**	32.34	363.1438	$C_{19}H_{24}O_7$	HRMS, UV	[35] (a)	+	-
PG	Kaempferol 3-methyl ether **(22)**	32.75	477.1038	$C_{22}H_{22}O_{12}$	HRMS, UV	[17] (a)	-	+
SQL	Elemacarmanin **(23)**	34.92	365.1608	$C_{19}H_{26}O_7$	HRMS	[19, 40] (a)	+	-
SQL	Onopordopicrin **(24)**	36.82	347.1573	$C_{19}H_{24}O_6$	HRMS	[19] (a)	-	+
SQL	Cynaropicrin; sauprin **(25)**	37.45	345.1416	$C_{19}H_{22}O_6$	HRMS	[19] (a) [37] (b)	-	+
SQL	Epoxyrepdiolide **(26)**	37.45	345.1355	$C_{19}H_{22}O_6$	HRMS, UV	[19, 35] (a)	+	-

HPLC-DAD-ESI-TOF-MS: High performance liquid chromatography-diode array detector-electrospray ionization-time of flight-mass spectrometry; $[M–H]^-$ *m/z*-deprotonated ion mass-to-charge ratio.

Identification was supported by comparison of their spectroscopic data with those reported in the literature: [(a)] known constituents of other *Centaurea* species; [(b)] compound already reported as constituents of *C. ragusina*; [(c)] not reported in the genus *Centaurea* before

Compound class: OA -organic acid, SQL-sesquiterpene lactone, PPG-phenylpropane glucosides, P-phenolic, PG- flavonoid glycoside; peak numbers and retention times (RT) refer to HPLC chromatograms in Figure 1.

The main compounds in herba AE were SQLs: solstitialin A-3, 13 diacetate and epoxyrepdiolide; quinic acid as organic acid. The main compounds in flower AE were organic acids: quinic acid, citric acid, malic acid; SQLs cynaropicrin; phenolic compounds chlorogenic acid and phenylpropanoid syringin.

Herba and flower AE have different chemical compositions. The main difference is that phenolic compounds are not found in herba AE. Flower AE contains three flavonoid compounds: luteolin-3,7-di-O glucoside, patulitrin and kaempferol 3-methyl ether and two SQLs, cynaropicrin and onopordopicrin, which are absent in the herba AE.

Chemical composition of herba AE contains SQLs solstitialin A-3, 13 diacetate and epoxyrepdiolide, together with three SQLs identified in herba AE only: solstitialin, deacylcynaropicrin, elemacramanin. It is important to say that SQLs are a typical group of compounds present in the *Asteraceae* family [41].

Among detected chemical compounds in flower extract three unknown nitrogen containing compounds were found. This may imply the presence of alkaloid compounds. There are some known alkaloid compounds detected in *Centaurea* species, however there is no data on the presence of nitrogen containing compounds in *Centaurea* species detected in the present study [36].

Diversity of phytochemicals in *Centaurea* species is connected with environmental conditions and germination stage as is the common case with the plants. It is also connected with a plant response to an environment and natural enemies [42]. The specific phytochemicals present in plants may have implications for the conservation of the species. In the context of *Centaurea ragusina*, Croatian endemic species this may be considered as well.

Anti-proliferative activity of *C. ragusina* aqueous extracts

Cytotoxic activity of herba and flower AE of *C. ragusina* was tested on human glioblastoma (A1235) and human bladder cancer (T24) cell lines after 4 h, 24 h, 48 h and 72 h treatment, using the MTT assay.

Both *C. ragusina* flower and herba AE showed a dose- and time-dependent cytotoxic activity against both tested cell lines. The flower AE showed better cytotoxic activity on both cell lines using all concentrations.

After 24 h treatment with both, flower and herba AE, in concentrations of 2 g/L and 1 g/L, cells viability decreased below 15 % and 25 % respectively, compared to non-treated cells (100% of viability, cell survival =1, Fig. 2).

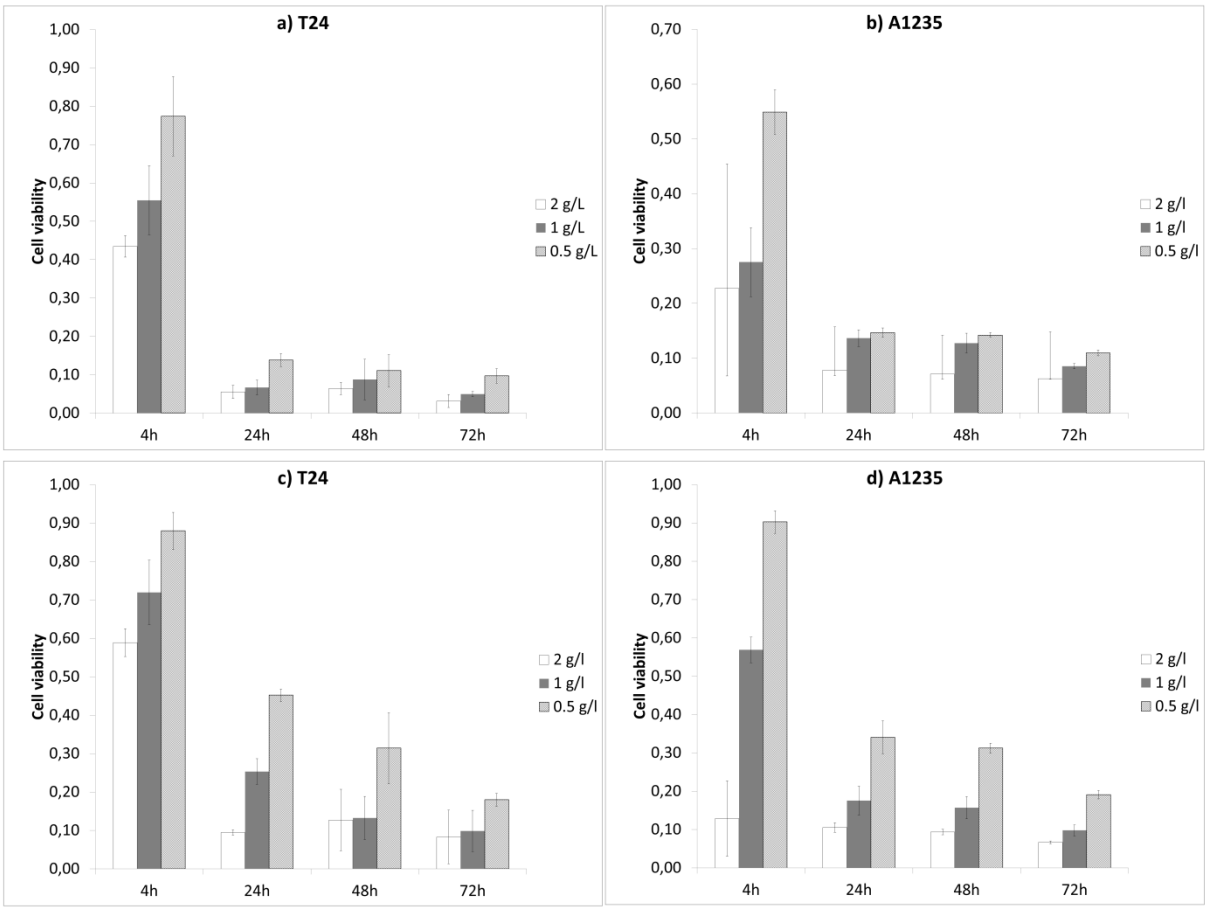

Figure 2. Dose-time response effect of *Centaurea ragusina* L. flower aqueous extract on human bladder (a) and human glioblastoma (b) cancer cell lines and herba aqueous extract on human bladder (c) and human glioblastoma (d) cancer cell lines using the MTT assay.

The flower AE expressed a significant cytotoxic activity toward both cell lines with very small difference in concentration-dependent activity after 24 h, 48 h, and 72 h treatment.

The results indicate that, after 72 h treatment, both flower and herba AE significantly inhibit cell proliferation in a concentration-dependent manner. The flower AE had a higher effect compared to herba AE. At concentration of 0.5 and 1.0 g/L, it inhibited the cell growth by 65.5 ± 2.4 % ($P < 0.01$) and 93.1 ± 0.4 % ($P < 0.01$), respectively.

The extracts induced cell death of T24 cells occurs mainly by apoptosis

Type of the cell death was determined using the 48 h treatment with 1 g/L of the both, flower and herba AE; of T24 bladder cancer cells [43, 44].

The majority of the cells died by treatment induced apoptosis (Fig. 3). Flower extract (26.25 %) compared to a leaf extract (22.15 %) showed slightly higher percentage of an apoptosis in T24 cells compared to non-treated cells (0.04 %).

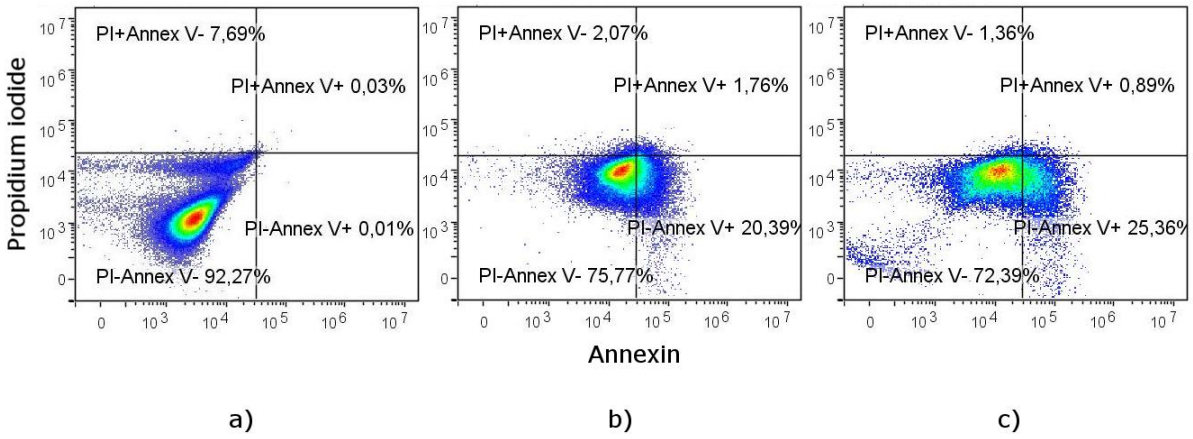

Figure 3: Apoptosis after treatment with 1 mg/mL *Centaurea ragusina* L. flower (b) and herba aqueous (c) extract in duration of 48 h in T24 cell lines in comparison to nontreated cells (a).

Previous studies of the genus *Centaurea* revealed that aqueous extracts of some *Centaurea* species show cytotoxic activity on human cell lines [15, 22, 45-55]. Sesquiterpene lactones, a group of chemical compounds typical for *Asteraceae* family, are well known on their wide spectrum of biological activities including cytotoxic activities [41, 56]. A common feature of SQLs that result in their strong biological activity is the presence of the α-methylene-γ-lactone ring, a *β*-unsaturated cyclopentenone ring or *α*-epoxycyclopentenone system. The mode of action of SQLs includes quick reaction with thiols such as cysteine by rapid Michael-type addition. This way SQLs inhibit tumor growth by controlling cell division reacting with thiol group in key enzymes in this process. Other mode of reaction in tumor inhibition includes inhibition of sulphydryl enzymes, phosphofructokinases and glycogen synthase activities [41, 57]. Sesquiterpene lactones also react with DNA polymerases, thymidylate synthase as well as ribosomal polymerase and mitochondrial energy processes. [41]. Sesquiterpene lactones are generally known to impair the activity of the NF-*κ*B transcription factor [56]. In the case of activity of *Centaurea omphalotricha* sesquiterpene lactones on the growth of the human leukemia cell lines, study revealed that the presence of exocyclic, conjugated double bond is essential for the cytotoxic activity against HL-60 and U937 cells determined by MTT test [46].

Syringin, phenylpropanoid glycoside, showed diverse pharmacological properties including cytotoxicity [58] against MCF-7, HeLa and Du145 cancer cell line. It showed no apoptotic effect in cell cycle analysis but DNA fragmentation study revealed necrosis as cytotoxic effect [59]. Cynaropicrin has been reported to possess immunomodulatory effects on cytokine release, nitric oxide production and immunosuppressive effects [22]. Cynaropicrin and deacylcynaropicrin also showed cytostatic activity on HeLa cells [4, 41, 55]. There is no data on cytotoxic effect of solstitialin A-3, 13 diacetate, whereas it is known that solstitialin monoacetate has a high cytotoxic activity [54]. Elemacarmanin, isolated from *Saussurea deltoideam,* showed no cytotoxic activity against three cancer cell lines A549, Hela and SMMC-7721 [60]. Onopordopicrin showed cytotoxic activity on the human epidermoid carcinoma cell line KB [61].

A number of epidemiological studies have been conducted to prove the protective effect of flavonoids against cancer, while some of the flavonoids, found also in *Centaurea* species, were connected with good cytotoxic activity [47, 62, 63]. The inhibition of tumor promotion by flavonoids may involve molecular mechanism of pro-oxidant process via inhibiting xanthine oxidase, COX or LOX55

[63]. Luteolin has been shown to penetrate into human skin, making it also a candidate for the prevention and treatment of skin cancer [64]. Some studies showed that luteolin and kaempferol derivatives exhibit cytotoxic activity [62, 63]. Patulitrin showed growth inhibitory effect against HeLa cells [65].

Conclusions

Centaurea ragusina flower and herba AE showed good cytotoxic activity against human bladder (T24) and human glioblastomma (A1235) cancer cell lines. Apparent cytotoxic activity of tested *C. ragusina* AE can be connected with chemical composition of both tested extracts. Some of the chemical compounds detected in the studies AE are already known on their cytotoxic properties. *Centaurea ragusina* AE represents a valuable source of sesquiterpene lactones and flavonoids, which anticancer activity has been reported in numerous studies. Hence, the preliminary cytotoxic activity results deserve further investigation in order to elucidate the specific compounds, or their combinations, responsible for the cytotoxic activity.

Experimental section

Plant material and preparation of aqueous extracts

The plant material of *C. ragusina* endemic species was collected in July in coastal habitats of island of Vis, Croatia. Plant was identified by a botanist and voucher specimens were deposited at the Department of Biochemistry, Faculty of Chemistry and Technology, Split, Croatia, under a name Cent_ragusina_Vis. Plant material was air dried at a room temperature. An AE from flower and herba dried plant material were prepared separately. Plant material of 15 g was dissolved in 0.15 L of hot distilled water. Tea solution was filtered after 30 min and AE was subjected to water evaporation by a low vacuum using the rotary evaporator. Dried AE was dissolved in distilled water and kept in a fridge at a temperature of -20 °C for further assays.

Determination of *C. ragusina* extracts chemical composition

The high-resolution liquid chromatography/photo-diode array/electrospray ionization/time of flight mass spectra (HPLC/PDA/ESI/TOF MS) was performed on a HPLC instrument (Agilent 1100 Series) equipped with an auto-sampler, using a LiCrospher 100 RP18e analytical column (5 µm particle size, 4.0 mm×250 mm) and a PDA detector (DAD) coupled with a 6210 TOF LC/MS system (Agilent Technologies).

Crude extracts of endemic *C. ragusina* were dissolved in methanol to an approximate concentration of 5 g/L. The HPLC/PDA/ESI/TOF MS analyses were performed under the following conditions: the mobile phase consisted of water containing 0.2 % formic acid (A) and acetonitrile (B). A gradient program was used as follows: 0–0.5 min, 4 % B; 0.5–40 min, 4–50 % – 95 % B; 40–45 min, 50–60 % B; 45–60 min, 60–95 % B; 60–68 min, 95 % B; 68–70 min, 95–5 % B, 70–80 min, 4% B. The flow rate of the mobile phase was 0.8 mL/min, the column temperature was kept at 30 °C and the injection volume was 10 µL. UV spectral data from all detected peaks were accumulated in the range of 190–450 nm and chromatograms were recorded at 260 nm. MS data were collected by applying the following parameters: ionization, negative ESI capillary voltage 4000 V, gas temperature 350 °C, drying gas 12 L/min, nebulizer pressure 45 psi*, fragmentor voltage 70 V, mass range 100–1500 m/z. MassHunter Workstation software was used for data acquisition and processing.

Exact mass measurements of pseudomolecular ions of the analytes was performed with a time-of-flight (TOF) mass spectrometer operating in the negative polarity mode, which enabled the determination of the molecular formula of most of the constituents. Chemical compounds in extracts have been tentatively identified by comparison of their spectroscopic data with literature records.

Biological activity

Human cancer cell lines

T24 bladder cancer cell line and A1235 glioblastoma cell line were grown at 37 °C in a humidified incubator and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM, EuroClone, Milano, Italy) containing 10% fetal bovine serum and 1% antibiotics (Penicillin Streptomycin, EuroClone, Milano, Italy).

Cytotoxic activity assay

Cell viability was determined using the tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; MTT Sigma-Aldrich) in reduction MTT assay [66]. An equal number of cells was seeded into the wells of 96-well plate and allowed to attach overnight. Cells were afterward treated with flower and herba AE (100 µL), in triplicate concentrations of 0.5 g/L; 1 g/L; 2 g/L, in growing media, for 4 h, 24 h, 48 h and 72 h. Following treatment, cells were incubated with 0.5 g/L MTT in growing media for 1 h after media was removed cells were treated with DMSO (Sigma-Aldrich). Absorbance was measured at 570 nm (signal) and 690 nm (background).

Flow cytometric analysis

An equal number of cells were seeded in 6-well plates and treated 100 µL with 1 g/L flower and herba AE analyzed for apoptosis after 48 h treatment. After this treatment the cells were treated with trypsin, washed with PBS (phosphate buffer saline); suspended in 100 µL of the binding buffer containing 5 µL Annexin-V-FITC and/or 5 µL of propidium iodide (PI) (Annexin-V-FITC Apoptosis Detection Kit I, BD Biosciences, San Jose, CA, USA) and incubated for 15 min at room temperature in the dark. The amount of apoptotic cells were analyzed using the flow cytometry (BD Accuri C6, BD Biosciences) and FlowLogic Software. The viability of cells was expressed in percent (%) as the ratio of the absorbance of cells treated with AE and the absorbance of non-treated cells multiplied with 100. Results were presented as mean value ± SD.

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