ORIGINAL ARTICLE

RESEARCH REGARDING OBTAINING HERBAL EXTRACTS WITH ANTITUMOUR ACTIVITY. NOTE II. PHYTOCHEMICAL ANALYSIS, ANTIOXIDANT ACTIVITY AND CYTOTOXIC EFFECTS OF *CHELIDONIUM MAJUS* L., *MEDICAGO SATIVA* L. AND *BERBERIS VULGARIS* L. DRY EXTRACTS

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Abstract

The aim of our research was the evaluation of the antioxidant activity and cytotoxic properties, together with the phytochemical characterization of selective dry extracts from greater celandine (*Chelidonium majus* L.), alfalfa (*Medicago sativa* L.) aerial parts and common barberry (*Berberis vulgaris* L.) bark that might be used to produce a phytomedicine with antitumour activity. Dry extracts (obtained in 50% ethanol) were characterized by quantification of flavones, phenolcarboxylic acids, total polyphenols and berberine, by means of spectrophotometric and HPLC methods. The scavenger activity upon DPPH (2,2-diphenyl-1-picrylhydrazyl), $ABTS^{\cdot+}$ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) free radicals and ferric reducing power assays were used for the antioxidant capacity evaluation. Our results pointed out that common barberry dry extract has the highest total phenolic content (14.95 g expressed as tannic acid/100 g dry extract), phenolcarboxylic acids (3.64 g expressed as chlorogenic acid/100 g dry extract) and berberine (7.94 g/100 g dry extract). Alfalfa and greater celandine dry extracts have similar flavones content (1.46 g expressed as rutin/100 g dry extract and 1.52 g respectively). Common barberry dry extract showed the best antioxidant activity, irrespective of the method used (EC_{50} = 0.28 mg/mL - reducing power assay, EC_{50} = 60.04 mg/mL - DPPH assay and EC_{50} = 54.08 mg/mL - $ABTS^{\cdot+}$ assay). Cytotoxic properties against *Daphnia magna* decreased as follows: common barberry dry extract > greater celandine dry extract > alfalfa dry extract. Our results offer encouraging premises for the formulation of a phytomedicine with potential antitumour activity.

Rezumat

Obiectivele lucrării au constat în obţinerea, caracterizarea fitochimică, stabilirea profilelor antioxidant şi citotoxic ale unor extracte uscate, selective, din părţi aeriene de rostopască (*Chelidonium majus* L.), lucernă (*Medicago sativa* L.) şi scoarţă de dracilă (*Berberis vulgaris* L.), în perspectiva dezvoltării unui fitopreparat cu potenţial efect antitumoral. Extractele uscate (obţinute în etanol 50%) au fost caracterizate prin determinarea conţinutului de polifenoli totali, flavone, acizi fenolcarboxilici şi berberină, utilizând metode spectrofotometrice şi HPLC. Activitatea antioxidantă *in vitro* a fost evaluată pe baza capacităţii de scavenger a radicalilor liberi DPPH (2,2-difenil-1-picrilhidrazil), $ABTS^{\cdot+}$ (acid 2,2'-azino-bis(3-etilbenzotiazolin-6-sulfonic) şi prin reducerea fierului. Extractul de dracilă are cel mai mare conţinut de polifenoli totali (14,95 g exprimaţi în acid tanic/100 g extract), acizi fenolcarboxilici (3,64 g exprimaţi în acid clorogenic/100 g extract) şi berberină (7,94 g/100 g extract). Extractele obţinute din lucernă şi rostopască au un conţinut asemănător de flavone (1,46 g exprimaţi în rutozidă/100 g extract şi respectiv 1,52 g exprimaţi în rutozidă/100 g extract). Cea mai bună activitate antioxidantă a fost determinată pentru extractul din dracilă, independent de metoda utilizată (EC_{50} = 0,28 mg/mL – metoda reducerii ferului, EC_{50} = 60,04 mg/mL – metoda DPPH şi EC_{50} = 54,08 mg/mL– metoda $ABTS^{\cdot+}$). Citotoxicitatea asupra nevertebratului *Daphnia magna* scade în ordinea: extract uscat din dracilă > extract uscat din rostopască > extract uscat din lucernă. Rezultatele obţinute oferă premise încurajatoare pentru obţinerea unui fitopreparat cu potenţială acţiune antitumorală.

Keywords: berberine, chlorogenic acid, polyphenols, cytotoxic effect, antioxidant, antitumour

Introduction

Cancer is a multifactorial disease, with high incidence and mortality. It is well known that chemotherapy is responsible for numerous side effects (immunosuppression, cardiotoxicity, peripheral neuropathy). Resistance to chemotherapy is the major cause of failure in cancer treatment, one of the main mechanisms being the overexpression of a drug efflux pump ATP dependent (P-glycoprotein) [12]. The role of plants natural compounds in chemoprevention represents an area of great interest [6]. According to scientific literature, lignans (podophyllotoxin and derivates), taxanes (from *Taxus* sp.), indole alkaloids of *Vinca rosea* L., isoquinoline alkaloids (camptothecin from *Campthoteca acuminata* L.) and lectins from *Viscum album* L. have antitumour properties [5].

Among indigenous herbal products, aerial parts of greater celandine (*Chelidonium majus* L.) are a source of isoquinoline alkaloids (chelidonine, chelerythrine, sanguinarine), with cytotoxic properties upon B16 (melanoma), HepG2 (liver cancer) cell lines and Ehrhlich ascites carcinoma. Malignant cells death is the consequence of caspases 3, 9 activation, inhibition of cell division, activation of pro-apoptotic factors such as Bax/Bak proteins and increased glutaminase activity [1, 15, 19]. Common barberry bark (*Berberis vulgaris* L.) is also a source of isoquinoline alkaloids (berberine, berbamine). Berberine has anti-tumour effects in oesophageal carcinoma cells [18], hepatocellular carcinoma [22], colorectal cancer [11], melanoma B16F10 [10], MCF-7 (breast cancer) [14] and HeLa (human cervical carcinoma) [8]. Berberine induces apoptosis through caspases activation, increasing levels of cytoplasmatic cytochrome C, decreasing levels of BcL2 protein [10, 11, 18, 22], up-regulation of p53 and p27 proteins [14] and epigenetic modulation by affecting the histone code [20, 24].

Alfalfa aerial parts (*Medicago sativa* L.) are a source of phytoestrogens (genistein, daidzein, coumestrol), flavones (derivates of apigenin, luteolin and millepurpan, medicarpin), saponins (medicagenic acid, soyasapogenol), phenolcarboxylic acids (gentisic acid, salicylic acid), chalcones (flavokavin B, isoliquiritigenin), amino acids (L-canavanine) and polysaccharides [2, 9, 17, 23]. According to Gatouillat G. *et al.* millepurpan and medicarpin induce apoptosis and overcome multidrug resistance in leukaemia P388 cells [2].

Taking into consideration the scientific data, the aim of our research was the evaluation of the antioxidant activity and cytotoxic properties of selective dry extracts from greater celandine/alfalfa aerial parts and common barberry bark that might be used for obtaining a phytomedicine with antitumour activity.

Materials and Methods

The aerial parts of *Chelidonium majus* L. (greater celendine), *Medicago sativa* L. (alfalfa) and the bark of *Berberis vulgaris* L. (common barberry) were harvested in 2015, from Hunedoara district, Romania (ecological crop). Dried herbal products have been used for further analysis. Voucher specimens were deposited in the official Herbarium of the Pharmacognosy, Phytochemistry and Phytotherapy Department, Faculty of Pharmacy, Bucharest.

Reagents and solvents. All reagents and solvents were purchased from Roth. (Germany), unless otherwise stated.

The research was carried out in several steps: **1**. obtaining the selective dry extracts and extraction yield determination; **2.** phytochemical characterization of dry extracts by means of spectrophotometric and HPLC methods; **3.** evaluation of dry extracts antioxidant activity *in vitro*; **4.** assessment of dry extracts cytotoxic properties using *Daphnia magna* bioassay.

Obtaining the selective dry extracts and extraction yield determination: 300 g of greater celandine, alfalfa aerial parts and common barberry bark were heated twice with 50% ethanol under a reflux condenser for 30 min. using 1:10 herbal product/ solvent ratio for the first extraction and 1:5 for the second one. After cooling, the combined filtrates were concentrated using a rotary evaporator (Buchi R 210 - 215) to remove the solvent and then freeze-dried (using a Christ Alpha 1-2/B Braun, Biotech International lyophylizator). The dry extracts were encoded as follows: **CE** (greater celandine dry extract), **ME** (alfalfa dry extract) and **BE** (common barberry dry extract).

The *extraction yield* was expressed as the percentage of the total mass of the dry extract (Mext, without the water content) with respect to the mass of the raw material (Mpv) loaded onto to the flask for solvent extraction:

$$Y\% = (Mext / Mpv) \times 100$$ [3, 26].

Preparation of stock solutions for spectrophotometric, HPLC and antioxidant assays: 0.100 g CE, ME and BE dry extracts were dissolved in 100 mL 50% ethanol.

Preparation of stock solutions for cytotoxic properties evaluation using Daphnia magna bioassay: 50 mg CE, ME, BE dry extracts were dissolved in 10 mL of 5% DMSO (dimethyl sulfoxide) solution.

Spectrophotometric assays. The flavones (F) (expressed as rutin equivalents), phenolcarboxylic acids (PAC) (expressed as chlorogenic acid equivalents) and total phenolic content (TP) (expressed as tannic acid equivalents) were determined according to our previously described methods [3].

HPLC analysis was carried out using a previously published external standard method with gradient elution and UV detection [3].

The standards (caffeic acid, chlorogenic acid, ferulic acid, rutin, quercetin and berberine) were identified based on their retention time and UV spectra (Figure 1, Table I). Calibration curves for caffeic acid (17.20 - 172 µg/mL, R^2 = 0.99, n = 6), chlorogenic acid (37 - 370 µg/mL, R^2 = 0.989, n = 7), rutin (4.21 - 201 µg/mL, R^2 = 0.99, n = 6) and berberine (1.5 - 50 µg/mL, R^2 = 0.995, n = 6) showed good linearity.

Table I

Retention time of standards

Compound	Retention time (Rt) (min)
Chlorogenic acid	7.12
Caffeic acid	7.96
Ferulic acid	13.14
Rutin	15.19
Quercetin	18.87
Berberine	18.44

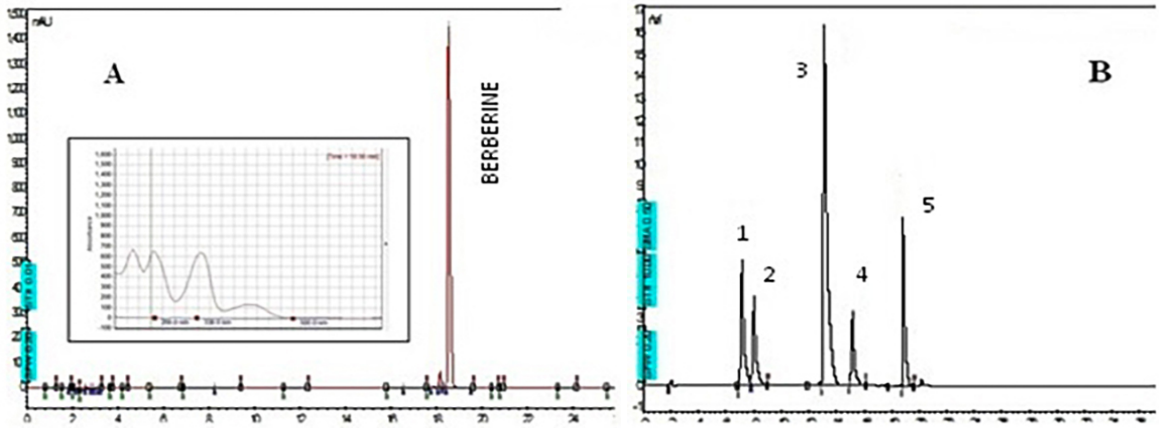


Figure 1.

Results of HPLC analysis: A) chromatogram and UV- spectrum for berberine; B) chromatogram for phenolic compounds, 1- chlorogenic acid; 2- caffeic acid, 3- ferulic acid, 4- rutin, 5- quercetin

Antioxidant capacity of dry extracts. The scavenger activity upon DPPH (2,2-diphenyl-1-picrylhydrazyl), $ABTS^{\cdot+}$ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) free radicals and ferric reducing power assays were conducted according to our previously published work [3].

The concentration range was 5.80 - 116.00 mg/mL (for DPPH assay), 22.40 - 244 mg/mL (for $ABTS^{\cdot+}$ assay) and 0.25 - 1.30 mg/mL (for ferric reducing power method). The results were expressed as EC_{50} (mg/mL), that represents the concentration of dry extracts test solutions that inhibited 50% of the free radicals activity [(for DPPH and $ABTS^{\cdot+}$ method)/ concentration of dry extracts test solutions providing 0.5 of absorbance (for ferric reducing power assay)].

Assessment of dry extracts cytotoxic properties. The cytotoxicity was determined using *Daphnia magna* bioassay, according to our previous published work [3]. The concentration range was 20 - 1500 µg dry extracts/mL. Berberine was used as a positive control (0.34 - 340 µg/mL). The cytotoxic activity is presented as lethality percentages after 24 h of incubation with both dry extracts or positive control.

Statistical analysis was performed using Microsoft Excel 2010 software (Microsoft Corp., USA) and GraphPad Prism v. 5.0. (GraphPad Software, USA).

Results and Discussion

Hydroalcoholic dry extracts were obtained using 50% ethanol as solvent. The solvent choice was based on herbal products quality assessment, according to our previous work [4]. After freeze-drying we have obtained 45 g of CE, 34 g of ME and 50 g of BE dry extracts. The highest extraction yield was found for common barberry dry extract (Figure 2).

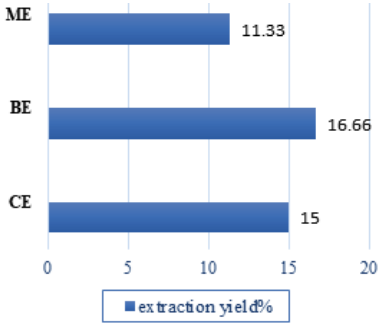


Figure 2.

Extraction yield for analysed dry extracts

CE – greater celandine dry extract, BE – common barberry dry extract, ME – alfalfa dry extract

The spectrophotometric results (Figure 3) pointed out that common barberry dry extract has the highest total phenolic (14.95 g expressed as tannic acid/100 g dry extract), and phenolcarboxylic acids (3.64 g expressed as chlorogenic acid/100 g dry extract) content. We have found similar results regarding alfalfa and greater celandine dry extracts flavones content (1.46 g expressed as rutin/100 g dry extract and 1.52 g respectively).

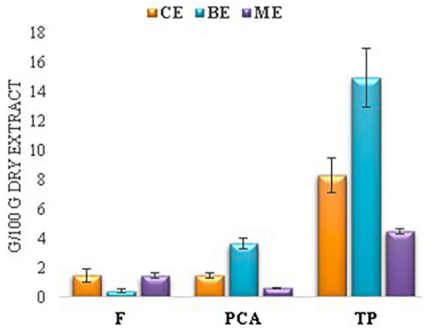


Figure 3.

Spectrophotometric results for dry extracts phenolic compounds analysis; results are mean ± SD of three independent experiments

F – flavones; PCA – phenolcarboxlic acids; TP – total phenoliccontent; CE – greater celandine dry extract; BE – common barberry dry extract; ME – alfalfa dry extract

Retention times (Rt) and UV spectra confirmed the presence of berberine in all analysed extracts (Figure 4), according to the findings of other authors [25]. According to scientific literature, berberine content of common barberry dry extracts vary widely, depending on plant's organ (roots 7.85 - 9.83 g%, twigs 0.82 - 1.53 g%) [7, 16]. Our results (7.94 g% berberine) falls within these limits (Table II). Common barberry dry extract has a higher content of berberine, compared to other author's results, probably due to different solvent/extraction methods. According to our HPLC results, caffeic and chlorogenic acids were also identified in CE and BE dry extracts [13]. Moreover CE dry extract has a higher content of caffeic acid (0.2 g%) (Table II) compared to Park J.H. *et al.* results that found only 0.073 g% [13].

Table II

HPLC results for analysed dry extracts

Dry extract	Berberine (g%)	Chlorogenic acid (g%)	Caffeic acid (g%)
CE	0.259	-	0.201
BE	7.942	0.084	-
ME	+	+	-

CE – greater celandine dry extract; BE – common barberry dry extract; ME – alfalfa dry extract; + – compound present but not quantified; - – not determined

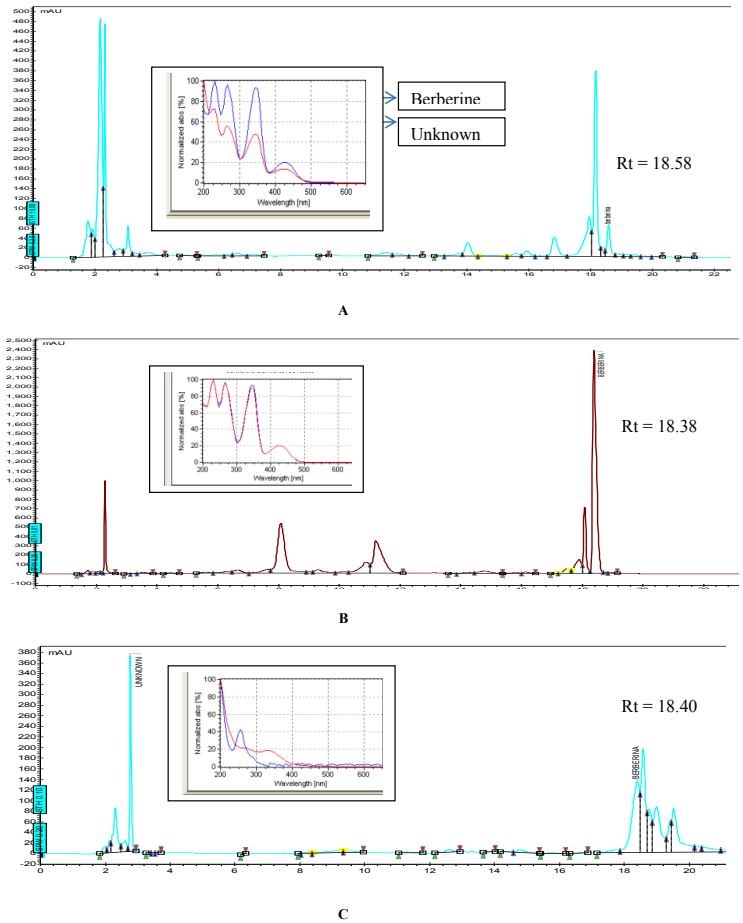


Figure 4.

HPLC chromatograms and UV-spectrum for: A) greater celandine dry extract; B) common barberry dry extract; C) alfalfa dry extract

The antioxidant activity was determined by means of validated methods [21]. As shown in Table III, all dry extracts show scavenging activity towards free radicals (DPPH and $ABTS^{\cdot+}$) and ferric reducing properties. The highest antioxidant activity was found for BE, followed by CE and ME dry extracts. The antioxidant capacity seems to be correlated with our spectrophotometric results, since the highest phenolic content was also found for common barberry dry extract.

Table III

EC_{50} (mg/mL) values for the analysed extracts

Dry extract	METHOD		
	Ferric reduction power	DPPH	$ABTS^{\cdot+}$
CE	0.47 ± 0.01	168 ± 0.0	144.73 ± 3.77
BE	0.28 ± 0.051	60.04 ± 0.06	54.08 ± 0.28
ME	2.02 ± 1.08	727.07 ± 34.04	214.05 ± 9.17

CE – greater celandine dry extract; BE – common barberry dry extract; ME – alfalfa dry extract. Results are mean ± SD of three independent experiments.

Daphnia magna bioassay (Table IV, Figure 5) revealed that common barberry and greater celandine dry extracts have high cytotoxic properties, but lower compared to berberine, used as positive control.

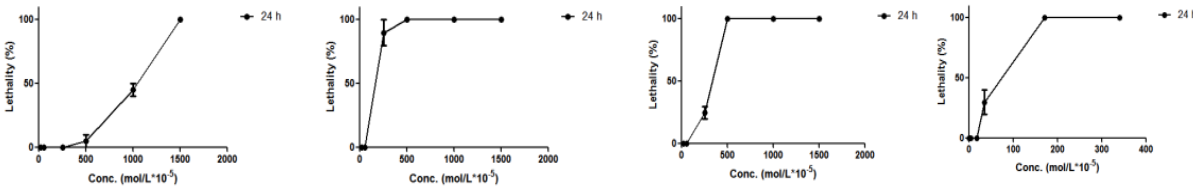
Berberine induced lethality only at high concentrations (340 µg/mL and 170 µg/mL). R^2 coefficients are high (> 0.99), thus showing a good correlation between the concentration and the biological effect.

Table IV

Cytotoxicity of dry extracts and berberine on *Daphnia magna*

Dry extract	Time of determination	LC_{50} (µg/mL)	IC 95% (µg/mL)	r^2
ME	24 h	1008	ND	0.9910
BE	24 h	201.3	ND	0.9922
CE	24 h	258.1	ND	0.9981
Berberine hydrochloride	24 h	34.93	ND	0.9918

CE – greater celandine dry extract; BE – common barberry dry extract; ME – alfalfa dry extract; LC_{50} – median lethal dose; IC 95% – 95 % confidence interval of LC_{50}.



A **B** **C** **D**

Figure 5.

Lethality on *Daphnia magna vs.* concentration after 24 h incubation

A – alfalfa dry extract; B – common barberry dry extract; C – greater celandine dry extract; D – berberine

Conclusions

The presents study states the obtaining of standardized dry extracts (from greater celandine/alfalfa aerial parts and common barberry bark), their phyto-chemical analysis, evaluation of antioxidant activity and cytotoxic properties upon *Daphnia magna*.

Common barberry dry extract has the highest total phenolic content along with phenolcarboxylic acids and berberine. The antioxidant activity and cytotoxic properties decrease as follows: common barberry dry extract > greater celandine dry extract > alfalfa dry extract.

Future research is needed in order to establish the exact pharmaco-toxicological profile of the extracts, for obtaining a phytomedicine with potential anti-tumour activity.

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