ORIGINAL ARTICLE

A COMPARATIVE STUDY ON THE BIOLOGIC ACTIVITY OF *CENTAUREA CYANUS VERSUS CALENDULA OFFICINALIS*

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Abstract

Considering that the therapeutic activity of *Centaurea cyanus* is less known, in the present study we aimed to evaluate its biological activity compared to *Calendula officinalis*. The alcoholic extracts from the two vegetal products were evaluated in terms of chemical composition, the concentrations being expressed in total polyphenols (gallic acid) and total flavonoids (quercetin). The studies carried out have shown the protective effect of the plant extracts against free radicals (superoxide dismutase - SOD-like activity *in vivo*), their antioxidant capacity (DPPH, FRAP, CUPRAC methods) and cytotoxicity assays. *Centaurea cyanus* possesses superior biological activities *versus Calendula officinalis*.

Rezumat

Considerând faptul că activitatea terapeutică a *Centaurea cyanus* este puţin cunoscută, studiul de faţă şi-a propus să compare activitatea acesteia cu a *Calendulei officinalis*. Extractele alcoolice ale celor două plante au fost evaluate din punct de vedere al compoziţiei, concentraţiile fiind exprimate în polifenoli totali (acid galic) şi flavonoide totale (quercetină). Studiile efectuate au demonstrat efectul protector al extractelor împotriva radicalilor liberi (determinarea activităţii SOD-mimetice *in vivo*), capacitatea lor antioxidantă (metodele DPPH, FRAP, CUPRAC) şi citotoxicitatea. *Centaurea cyanus* posedă activitate biologică superioară faţă de *Calendula officinalis*.

Keywords: antioxidant, SOD-mimetic activity, cytotoxicity

Introduction

The human body has a complex system of natural enzymatic and non-enzymatic antioxidant defence system which counteracts the harmful effects of free radicals and other oxidants [1]. Free radicals are responsible for causing a large number of diseases including cancer [15, 26], cardiovascular disease [27], neural disorders [25], mild cognitive impairment, Alzheimer's [10] and Parkinson's diseases, atherosclerosis and aging [14]. The impact of ROS (reactive oxygen species) on tissues is devastating regarding oxidative stress-induced cell death [19]. Protection against free radicals can be enhanced by the intake of dietary antioxidants. There is a growing consensus among scientists that a combination of antioxidants, rather than singles entities, may be more effective over the long period of time [13].

Gallic acid has been associated with a wide variety of biological actions including antioxidant, antibacterial, antifungal, antimalarial and antiherpetic action. The main interest in gallic acid is related to its anti-tumoural activity [20].

Previous studies have demonstrated that gallic acid exerts its activity against several types of tumour cells, including leukaemia [21], cervical [32], lung [22], colon and breast cancer cell lines [6]. Particularly, the studies have shown that the antitumor activity seems to be related to the induction of apoptosis involving different signalling pathways. Apoptosis induced by gallic acid may be associated with oxidative stress derived from ROS [29], mitochondrial dysfunction and an increase in intracellular Ca^{2+} levels. It also has been reported that the cell death promoted by gallic acid in different cell lines may

be related with glutathione (GSH) depletion [31]. Interestingly, gallic acid has both pro-oxidant and antioxidant properties [7].

Quercetin has also an antioxidant and anticancer effect, being therefore reported as an efficient free radical scavenger. Therefore, it is capable of preventing cancer induced by oxidative stress [3]. Previous studies have already demonstrated that quercetine exerts effects against several types of tumour cells, including hepatic cancer [5], leukaemia [23] and cervical cancer [24].

*Centaurea cyanus (*cornflower) is part of the *Compositae* family. It is an annual plant, silky hairy, with branched or simple stem, with linear alternate leaves and tubular, blue flowers.

Calendula officinalis (marigold) is part of the *Compositae* family. It is an annual, branched, pubescent herbaceous plant with characteristic smell. The leaves are lanceolate, alternate, the flowers are grouped in terminal trays.

In the present study we determined first the total content of polyphenols and flavonoids for the lyophilized extracts of *Centaurea cyanus* and *Calendula officinalis*. The second part of the study consists in the determination of the antioxidant activity of the extracts using three methods (DPPH, FRAP and CUPRAC) [22, 28]. The last part consists in the determination of the protective effect of plant extracts against free radicals (SOD-like activity in vivo) and cytotoxicity assays.

Materials and Methods

Plant material

Centaurea cyanus flowers were harvested from June to August 2016 at the beginning of the flowering period, from Bihor County, Romania. *Calendula officinalis* flowers were harvested from June to September 2016, from Bihor County, Romania.

Preparation of extracts

For the extraction of bioactive compounds from plant products, a 70% hydroalcoholic mixture (1:10 g/v) was used as an extraction solvent. The extraction mixture was subjected to a shaking operation using a magnetic homogenizer for 20 minutes followed by sonication for 5 minutes. At the end of the extraction, the hydroalcoholic extracts were subjected to centrifugation, and the supernatants evaporated to dryness in a rotary evaporator. The dry extract was dispersed in 10 mL distilled water and then frozen at -25°C. Finally, they were brought to the lyophilizer and the lyophilized extracts were weighed and transferred to a dry sealed container.

Physico-chemical characterization of extracts

Determination of total phenolic content: Folin Ciocâlteu reagent was used for analysis of total phenolics content (TPC) [3]. In a 10 mL volumetric flask, a 0.2 mL aliquot of the extracts and fractions in methanol (1.0 mg/mL) was mixed with 0.4 mL of Folin-Ciocâlteu reagent. The solution was allowed to stand at 25°C for 5 - 8 min before adding 0.2 mL of 4.0 mL of 7.0% sodium carbonate solution and made to 10.0 mL with bidistiled water. The mixture was allowed to stand for 2 h before its absorbance was measured at 725 nm. Gallic acid was used as standard for the calibration curve. TPC was expressed as mg gallic acid equivalents (GAE) *per* 100 grams dry weight (mg GAE/100 d.w.).

Determination of total flavonoid content: The total flavonoids content was assessed by a colorimetric assay [33, 34]. A 100.0 μL aliquot of extracts or fractions in methanol was added to a 10 mL volumetric flask containing 4 mL of distilled water. At zero time, 0.3 mL 5% sodium nitrite was added to the flask. After 5 min, 0.3 mL of 10% aluminium chloride was added [16]. At 6 min, 2 mL of 1 M sodium hydroxide was added to the mixture. Immediately, the mixture was diluted to volume with the addition of 2.4 mL distilled water and thoroughly mixed. The absorbance of the mixture was determined at 510 nm *versus* a blank containing all reagents except samples of extracts or fractions. Quercetin was used as standard for the calibration curve. The total flavonoid content of the extracts and fractions was expressed as mg quercetin equivalents (QE) per 100 grams dry weight (mg QEE/100 d.w.).

Measurement of DPPH-radical scavenging activity

DPPH (2,2-diphenyl-2-picryl-hydrazyl-hydrate) radical scavenging activity was assessed according to Kikuzaki *et al.* [18]. In this assay, 50 μL of extract or fraction solutions with different concentrations were added with 1.0 mL of 0.4 mM methanolic - DPPH and brought up with methanol to 5.0 mL. The mixture was shaken vigorously using a vortex and left to stand for 15 min, at room temperature, in dark. The scavenging effect on the DPPH radical was read using a spectrophotometer (Genesys-10) at 517 nm. The radical scavenging activity was expressed as the radical scavenging percentage using the following equation: percentage (%) of DPPH radical scavenging:

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \times 100,$$

where, A_B = absorption of blank sample (t = 0 min.), A_A = absorption of test extract solution (t = 15 min.). The DPPH solution was used as control.

FRAP method (ferric reducing antioxidant power)

FRAP method is a simple spectrophotometric method that assesses the antioxidant power of the studied samples, being based on the reduction of ferric tri-pyridyltriazine complex [Fe(III)-TPTZ] by a reductant, at an acid pH. The stock solutions included: 300 mM acetate buffer; 270 mg $FeCl_3$ x 6 H_2O dissolved in

50 mL distilled water; 150 mg TPTZ and 150 µL HCl, dissolved in 50 mL distilled water. The working FRAP solution was freshly prepared by mixing 50 mL acetate buffer, 5 mL $FeCl_3$ x 6 H_2O solution and 5 mL TPTZ solution. Trolox was used as a standard solution, the calibration curve was made for concentrations between 0 and 300 µM, having a correlation coefficient $R^2 = 0.9956$ and the regression equation ($y = 0.0017x + 0.0848$), where *y* represents the absorbance registered at 595 nm. The results are expressed as µmol Trolox equivalents (TE)/100 mL extract.

Cupric ions (Cu^{2+}) reducing - CUPRAC assay

In order to determine the cupric ions (Cu^{2+}) reducing antioxidant capacity the method proposed by Apak *et al.* [2] and Karaman *et al.* [17] was used with slight modifications. To this end, 0.25 mL $CuCl_2$ solution (0.01 M), 0.25 mL ethanolic neo-cuproine solution (7.5 x 10^{-3} M) and 0.25 mL CH_3COONH_4 buffer solution (1 M) were added to a test tube, followed by mixing with the plants extracts. Then, the total volume was adjusted to 2 mL with distilled water, and thoroughly mixed. The tubes were stoppered and kept at room temperature. Absorbance was measured at 450 nm against a reagent blank after 30 min. An increased absorbance of the reaction mixture indicates the enhancement of the reduction capability. Values were obtained using the calibration curve. As standard, Trolox was used, the standard curve was between 0 and 2500 µM, with a correlation coefficient $R^2 = 0.9935$. The antioxidant capacity of the extracts was calculated from the regression equation $y = 0.0006x$, where y represents the absorbance read at 450 nm, and x represents µmol of equivalent Trolox in 100 µL of extract [30].

In vivo SOD-like activity

The protective effect of the plant extracts against free radicals produced by oxidative agents has been determined.

The SOD-like activities of the plant extracts where evaluated using a strain of *S. cerevisiae* Δsod1 (ATCC 96687), which has the ability to delete/insert the SOD_1 gene encoding the synthesis of Cu_2Zn_2SOD. The characteristics of *S. cerevisiae* Δsod1 (ATCC 96687) are: MAT aura 3-52 trp1-289 his3-Δ1 leu 2-3 leu 2-112 sod1: URA3. The Cu_2Zn_2SOD is the main SOD in the cell and it is localised in the cytoplasm.

Yeast cells were grown in YPD reach medium (1% yeast extract, 2% peptone and 2% glycerol). The used culture medium does not contain glucose; it contains glycerol instead, because in its presence the levures can breathe. This is determinant, as free radicals are going to be generated during the breath processes taking place in the mitochondria [25].

Solid media contained 1.5% agar. Cell density from cultures grown overnight was determined by cell counting in a "Nebauer" hematimetre. 10^6 cells were resuspended in 15 mL of melted solid YPD media kept at 45°C. Solutions of the liofilizated extracts in a mixture of DMSO:EtOH (1:4) at increasing concentrations (30, 50, 70 µm, see Table III) were added to the growth medium. Cell suspensions were poured into Petri dishes and allowed to solidify at room temperature. Paper disks measuring 6 mm in diameter (Antibiotica test Blättchen) containing 5 µL of a 5 mM menadione solution in ethanol or 5 µL of 17.5% H_2O_2 have been used. The diameters of clear zones around the disks, measured after 3 days of incubation at 28°C, where taken as a quantitative estimate of the protective action.

Cell toxicity assay

Cell culture. The B16-F10 metastatic murine melanoma cell line was obtained from American Type Culture Collection (Rockville, MD, USA). B16-F10 cells were grown in DMEM, supplemented with 10% foetal bovine serum (Hyclone), 2 mM glutamine (Sigma-Aldrich), 1% penicillin and streptomycin, 0.1% amphotericin. The cells were cultured at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity.

Cytotoxicity assays. B16-F10 cells (8 x 10^3 cells/well) were seeded on 96 well plate and cultured in DMEM containing 10% FBS for 24 h. The medium was then replaced with complete medium containing or not the plant extracts at various concentrations (0, 15, 25, 35, 45, 60, 80, 100 µg/mL). A stock solution of plant extract was prepared containing 0.3% DMSO. The treatment was applied for 24 h at 37°C and 5% CO_2. The number of viable cells was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation reagent. Three PBS washing steps were followed by 1 h of incubation with MTT solution (0.5 mg/mL) in DMEM without phenol red. The formazan particles were solubilized with DMSO. The absorbance was read at 550 nm, respectively at 630 nm (for back-ground) with the microplate plate reader HT BioTek Synergy (BioTek Instruments, USA). The results were expressed as survival percent with respect to an untreated control [4, 11, 12].

Statistics

All the experiments were conducted in triplicate and data were displayed as mean ± SEM. Two-way analysis of variance (two-way ANOVA) followed by the Bonferroni post-test was performed for the multiple comparisons for normally distributed samples with homogenous variance. Statistical significant differences were set at $p < 0.05$. The IC_{50} values representing the concentration required to inhibit 50% of cell proliferation were calculated from the dose response curve using non-linear regression. Statistical values and figures were generated using GraphPad Prism version 5.0 for Windows, Graph-Pad Software, San Diego, California, USA.

Results and Discussion

Physico-chemical characterization of extracts

Determination of polyphenol and flavonoid content, phytocomplexes responsible for the therapeutic properties of the two plants, were performed using the Folin-Ciocâlteu method and the colorimetric method. The results obtained by the two methods are shown in Table I.

Table I

Total polyphenol and total flavonoid content for *Centaurea cyanus* and *Calendula officinalis*

Sample	Total polyphenolic content (mg GAE/100 d.w.)	Total flavonoid content (mg QE/100 d.w.)
Centaurea cyanus	718.81 ± 1.12	1.31 ± 2.72
Calendula officinalis	116.92 ± 0.04	4.21 ± 1.05

* – GAE = gallic acid equivalent, QE – quercetin equivalent

According to the data in Table I for the two analysed plants, the total content of polyphenols in *Centaurea cyanus* is higher, but the total flavonoid content is higher in *Calendula officinalis*.

Antioxidant activity

To evaluate the antioxidant activity of the two plant extracts three methods were used: DPPH, FRAP and CUPRAC. After the results of these determinations were examined, it can be concluded that the extract of *Centaurea flos* has a very good antioxidant activity, comparable to the extract of *Calendulae flos*, plant known and used for its antioxidant effect (Table II).

Table II

Antioxidant activity of plant extracts for *Centaurea cyanus* and *Calendula officinalis* by three methods: DPPH, FRAP and CUPRAC

Sample	FRAP (mg TE/mL)	DPPH%	Cuprac (mmol Trolox/100g d.w.)
Centaurea cyanus	78.01 ± 0.24	83.42	1.86 ± 0.21
Calendula officinalis	129.5 ± 0.11	41.70	1.56 ± 0.02

* – TE = trolox equivalent, d.w. – dry weight

By interpreting the obtained results, we have shown that the superior antioxidant activity for marigold (129.5 mg TE/mL for the marigold and 78.01 mg TE/mL for the cornflower) has been achieved by the FRAP method, but for the other two methods, cornflower has shown an antioxidant activity superior to those of marigold (by DPPH method 83.42% for cornflower and 41.70% for marigold respectively) and by the CUPRAC method 1.86 mmol Trolox/100 g dry weight *versus* 1.56 mmol Trolox/100 g dry weight).

In vivo SOD-like activity

The *in vivo* SOD-like activity of the liofilizated extracts was quantified by a method based on the protection against free radicals provided by the extracts to the yeast *S. cerevisiae* [8, 9]. The SOD-mimetic activity of the two plant extracts on cell growth with a Δsod1 mutant treated with menadione or H_2O_2 had been evaluated. Oxidative stress is produced by two oxidative agents: menadione due to the superoxide radical and H_2O_2 due to OH• radicals.

It will be considered that the extract has a SOD-like activity if a decrease of the diameter of the inhibition zone is registered *versus* the control zone. The efficacy will then be evaluated by comparison of the diameter of the inhibition area for the extract and control (Figure 1).

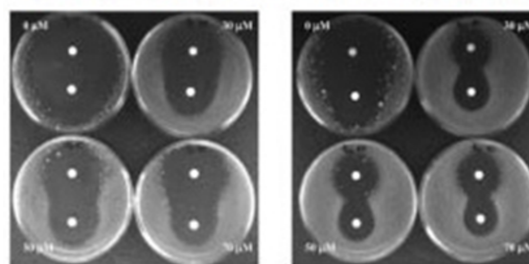

Figure 1.

Effect of plant extracts of *Centaurea cyanus* and *Calendula officinalis* on the growth of the Δsod1 mutant against free radicals produces by H_2O_2 (disk at the top of each Petri disk) and menadione (disk at the bottom of each Petri disk)

In the presence of the plant extracts at 30, 50 and 70 µM a significant reduction of the inhibition area was observed when the oxidative stress was produced by both menadione and H_2O_2.

The diameter of the inhibition area for both, *Centaurea cyanus* and *Calendula officinalis* plant extracts in different concentrations, using menadione and H_2O_2 are given in Table III.

The reduction of the inhibition area was between 38 - 45% for the *Centaurea cyanus* plant extract and 23 - 28% for the *Calendula officinalis* plant extract against oxidative stress generated by menadione. The protective activities of extracts do not seem to be dependent on the extract concentration.

Table III
The diameter of inhibition area for *Centaurea cyanus* and *Calendula officinalis* lyophilized extracts, in different concentrations, using menadione and H_2O_2

Lyophilized extract (μM)	*Centaurea cyanus* Diameter of the inhibition area (cm)		*Calendula officinalis* Diameter of the inhibition area (cm)	
	Menadione	H_2O_2	Menadione	H_2O_2
Control (Menadione 5 mM or H_2O_2 17.5%)	8	7.5	8	7.5
30	5	5.2	6.2	6.4
50	4.5	5	5.8	6.2
70	4.4	5	5.8	6

The protection of the extracts against free radicals generated by H_2O_2 is lower than in the case of free radicals generated by menadione. *Centaurea cyanus* extracts produced a reduction of the inhibition diameter of about 30 - 33% while *Calendula offi.* extracts only between 14 - 20%. Nor in this case the protective action produced by the extracts did not depend on the extract concentration.

As a conclusion, *Centaurea cyanus* extracts registered a higher SOD-like activity compared with *Calendula officinalis*.

The current study clearly suggests that both plant extracts are able to protect efficiently against superoxide anions and they could be considered as promising effective agents against the toxicity of the superoxide anion, improving significantly the growth of *Δsod1* strain. They supply the Cu_2Zn_2SOD deficiency of the mutant. For this reason they are potential therapeutic agents in the prevention and treatment of diseases mediated by free radicals.

Cell toxicity assay

The *in vitro* cytotoxicity of the plant extracts was tested on B16F10 melanoma cell line. The response was quantified using 3-(4,5-dimethyl-2-thioazolyl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay.

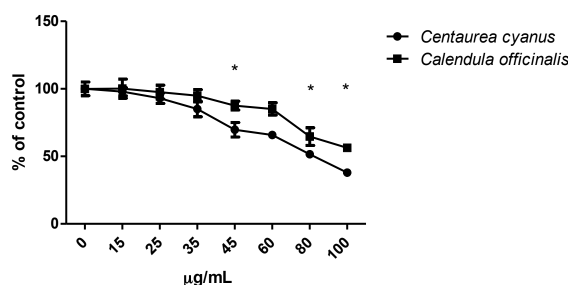

Figure 2.
Comparative cytotoxicities of *Centaurea cyanus* and *Calendula officinalis* on B16F10 melanoma cells after 24 hours exposure from 15 to 100 μg/mL (*versus* untreated cells) (* = $p < 0.05$) (mean ± SEM) (n = 3)

Both extracts exhibited cytotoxicity on B16F10 melanoma cells lines. At small concentrations of the plant extracts there were no significant differences between the two plant extracts but at higher concentrations (45; 60; 100 μg/mL) *Centaurea cyanus* showed a superior inhibitory effect (Figure 2). This pattern is clearly supported by the IC_{50} values (Table IV).

Table IV
IC_{50} values of *Centaurea Cyanus* and *Calendula officinalis* against B16F10 cells (mean ± SEM) (n = 3)

Plant extract	IC_{50} (μg/mL)/24 h
Centaurea cyanus	79.85 ± 0.77
Calendula officinalis	105.83 ± 3.36

As a conclusion, *Centaurea cyanus* plant extracts exhibited a higher cytotoxicity compared with *Calendula officinalis*.

Conclusions

Following the physicochemical characterization of the extracts, *Centaurea cyanus* has been shown to be higher in total polyphenols, but in *Calendula officinalis* the content of total flavonoids is higher.

The *Centaurea flos* extract has a very good antioxidant activity, comparable to the *Calendulae flos* extract, a plant widely used for its antioxidant effect.

Both plant extracts may be considered as potential therapeutic agents, higher SOD-like activity and cytotoxicity being registered for the *Centaurea cyanus* extract compared to the *Calendula officinalis*. This may be considered a premise in considering the *Centaurea cyanus* extracts as a potential therapeutic agent.

Considering the beneficial effects of both vegetal extracts, in the future, we would like to associate them with the purpose of formulating a mouthwash.

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