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IN VIVO AND *IN VITRO* ANTIOXIDANT ACTIVITY OF *Cnicus benedictus*

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Abstract

The aim of this study was to comparatively evaluate the in vivo and in vitro Cnicus benedictus L. antioxidant activity based on secondary metabolites (total polyphenolic content and flavonoids). The species is used as an antidepressant, anti-inflammatory, antiseptic, cardiotonic, antimicrobial and anti-proliferative. The samples used for biochemical analysis were represented by two type of in vitro regenerated callus and different parts of in vivo mother plant. The callus cultures were initiated from the leaf explants from potted plant, cultured on Murashige and Skoog (MS) medium supplemented with 2.4-Dichlorophenoxyacetic acid (2.4D) alone or in combination with 6-Benzylaminopurine (BAP). In our conditions, the antioxidant activity was correlated with total phenolic content. The level of flavonoids was higher in callus than in the mother plant. Through HPLC, the rutin presence was validated in callus, and a higher number of constituents were observed.

Key words*: callus, Cnicus, flavonoids, HPLC.*

INTRODUCTION

One of the most significant parameters which regulate the therapeutic effects of a plant is the antioxidant activity based on bioactive compounds represented by phenolics and flavonoids (Chandur et al., 2011).

The spontaneous medicinal plant species *Cnicus benedictus* L. (blessed thistle, Asteraceae family) has a great potential as an alternative oil crop, being liable to be cultivated on medium-quality soil with low agricultural costs or efforts (Ghiasy-Oskoee et al., 2018). In the past decade, the plant gained more interest, starting to be cultivated as a food source and additive in Turkey (Can et al., 2017) and Romania (Ministry of Agriculture and Rural Development - M.A.D.R., 2011).

The chemical constituents of this plant are represented by sesquiterpene lactone glycosides, cnicin, triterpenoids, lignans, flavonoids, tannins, essential and volatile oils, many nutritional components, minerals and trace elements (Al-Snafi, 2016). The oil of *C. benedictus* is attractive for cosmetics and human nutrition (Horn et al., 2014), being characterised by a high concentration of α-tocopherol (600-750 mg/kg), higher than in sunflower oil (432-730 mg/kg) (Bele et al., 2013; Grilo et al., 2014).

Different aspects concerning scientific literature, expert opinion, folkloric precedent, history, pharmacology, kinetics/dynamics, interactions, adverse effects, toxicology and dosing of this species were presented by Ulbricht et al. (2008). Recent studies about the pharmacological effects of *C. benedictus* are available (Păun et al., 2015; Chabane et al., 2013).

The aim of this study was to evaluate the *in vivo* and *in vitro* antioxidant activity of *Cnicus benedictus*, a medicinal plant species.

MATERIALS AND METHODS

Plant material was represented by *C. benedictus* seedlings obtined from seeds germinated at room temperature. In order to induce *in vitro* callus, leaves fragments were sterilized with $HgCl_2$ 0.1% and cultivated on two MS (Murashige et Skoog, 1962) media variants supplemented with 3% sucrose (w/v), 0.8% agar. In the first variant, MS was added with 1 mg/l 2.4D, and the second variant was supplemented with 1 mg/l 2.4D + 0.2 mg/l BAP. The culture dishes were placed for 30 days in a Weiss Gallenkamp Fitotron, at 25 ± 2°C, with a photoperiod of 16/8 and a 2*36W fluorescent lamp with maximum intensity ~ 90 µmol m^{-2} s^{-1}.

Biochemical analyses. The samples used for biochemical analyses were represented by parts of plant (achenes, roots, leaves, stem) and 4 weeks old callus induced *in vitro* from the same plant.

Extraction of *C. benedictus* antioxidant compounds. The samples were dried on filter paper, grinded with quartz sand and extracted in a ratio of 1: 1 (v/v) with 100% methanol for 24 h. The homogenates were centrifuged at 10,000 g, for 20 minutes and the supernatants were used for subsequent analysis.

Determination of antioxidant capacity by DPPH (2.2 diphenyl-1-picrylhydrazyl) method. According to the method proposed by Marxen et al. (2007) 100 µl of diluted (1:2) extract were mixed with 2.25 ml of methanol and 150 µl of 1.27 mM DPPH. In control variant the extract was replaced with the extraction solvent. After 30 minutes of incubation at room temperature, the absorbance at 515 nm was read. Antioxidant capacity was represented by the differences between samples and control against a standard curve that used Trolox (synthetic antioxidant α-tocopherol analogue) as standard antioxidant. Antioxidant capacity was expressed as Trolox equivalents/ fresh weight (mM Trolox/g sample).

Determination of the Total Phenolic Content (TPC). TPC was determined according to the method described by Mihailović et al. (2013) with some changes. The reaction mixture consisted in 0.5 ml of suitably diluted extract (1:2 for calus samples and 1:5 for *in vivo* samples), 2.5 ml of Folin-Ciocalteu reagent 11-fold diluted and 2 ml of 7.5% Na_2CO_3. This was kept for 30 minutes at room temperature, and then absorbance was measured at 765 nm. Three repetitions of the same variant were made and the average represents total phenol content expressed as gallic acid equivalents/ fresh weight (EAG µg/g sample).

Determination of the flavonoids content. Flavonoid content estimation of the methanol extracts was performed using a protocol described by Zhishen et al. (1999), with minor adjustments. Thus, suitably diluted 0.5 ml methanol extract of each sample was mixed with 2 ml of distilled water and 5% $NaNO_2$ solution and the mixture was equilibrated for 5 min. Then, 150 µl of 10% $AlCl_3$ solution was added, and after 6 minutes of reaction, 1 ml of 4% NaOH was added and brought to a total volume of 5 ml with distilled water. Optical density at 510 nm was recorded against a blank and flavonoid concentration estimation was done according to a calibration curve using as standard rutin. Three repetitions of the same variable were made and the average of flavonoid content was expressed as rutin equivalents/fresh weight (ERU mg/g sample).

High-performance liquid chromatography (HPLC). The callus induced on MS variant with 2.4D characterized by the highest flavonoids concentration represented the sample for HPLC.

A Jasco HPLC System (Jasco Europe, Cremella, Itally), equipped with a Nucleosil 100 C18 (Teknokroma) column and coupled with a Fluorescence Detector FP-2020 (Jasco) detector, was used. The elution was performed using a protocol described by Chuanphongpanich et al. (2006). The mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B) and methanol (solvent C). The system was run with the following elution gradient program: 0 min, 5% A/95% B; 10 min, 10% A/80% B/10% C; 20 min, 20% A/60% B/20% C and 30 min, 100% A. There was a 10 min post run at initial conditions for equilibration of the column. The flow rate was kept constant at 1 ml/min. The absorbance was monitored in the fluorescence canal, at 280 nm and 320 nm. Prior to HPLC analysis, all solutions were filtered through 0.45 µm membrane filters. Rutin was used as standard solution. A stock standard solution (50 µg/ml) of rutin was prepared in methanol and stored in the dark at 5°C up to three months.

Statistical analysis

The experimental measurements were done in triplicates for each sample. The results are presented as mean values ± standard deviations (Table 1).

For DPPH activity in relation to the total phenolic, flavonoid content and plant segments samples, analysis of covariance (ANCOVA) and Spearman's correlation were conducted using Xlstat addinsoft (Xlstat, 2013 version).

ANCOVA is a technique based on regression and analysis of variance (ANOVA) (Stevens, 1986) and highlights how the dependent

variable (DPPH in our case) was influenced by independent variables (total phenolic, flavonoid content as quantitative variables and plant segments as qualitative variables). For both techniques, the results with $p<0.05$ were considered statistically significant.

Spearman's correlation is a nonparametric statistic used as a measure of the power of association between two variables (Hauke and Kossowski, 2011).

RESULTS AND DISCUTIONS

There are many studies concerning the biological roles of secondary metabolites produced by plants through tissue cultures (Çetin et al., 2015).

Since flavonoids were recognized for their health beneficial effects, and the studied species may represent a cheap source (Ghiasy-Oskoee et al., 2018) of metabolites with antimicrobial, cytotoxic, anti-inflammatory and other activities (Al-Snafi, 2016), we have tested the antioxidant activity of various parts of the plant.

We also tried to obtain callus characterized by higher flavonoid content by using *in vitro* cultures. One of the advantages of *in vitro* techniques is represented by reducing the time required to obtain the plant biomass.

Table 1. The antioxidant activity, total phenolic content and flavonoids concentration of different parts of *in vivo* plant and *in vitro* callus of *C. benedictus*

Samples	DPPH (mM Trolox/g sample)	Total phenolic content (EAG µg/g sample)	Flavonoids (ERU µg/g sample)
Achenes	0.34	356.37 ± 1.4	233.27 ± 2.06
Roots	0.2 ± 0.02	274.525 ± 3 5.49	179.35 ± 3.42
Leaves	3.66 ± 0.6	1,483.75 ± 29.87	234.75 ± 0.56
Stem	1.58 ± 0.08	1,069.3 ± 22.45	409.5 ± 2.08
Callus induced on MS + 2.4D	0.99	802.56 ± 15.033	519.38 ± 0.91
Callus induced on MS + 2.4D+BAP	1.42 ± 0.3	563.42 ± 9.52	367.5 ± 1.09

In DPPH assay, the highest free-radical scavenging capacity was observed in leaves (3.665 ± 0.60 mM Trolox/g fresh sample), followed by stem (1.588 ± 0.08 mM Trolox/g fresh sample) and callus induced on MS supplemented with 2.4D and BAP (1.427 ± 0.3 mM Trolox/g fresh sample) (Table 1).

Analysis of secondary metabolites in different parts of *C. benedictus* plant showed that the highest total phenolic content was registered in leaves followed by stem, achenes and roots, while flavonoids concentration was highest in stem followed by leaves, achenes and roots (Table 1).

The total phenolic and flavonoids content from leaves are 5 times and respectively 1.3 times higher than roots extract**.** Our results are in accordance with Can (2017), who showed that contents of these compounds were 2 times higher in leaf methanolic extract than in root extract of *C. benedictus* cultivated in Aegean region from Turkey. The prevalence of polyphenols in leaves and stems can be explained by the higher involvement in gas exchange of plant aerial parts than roots, polyphenols being detected in the vacuoles of guard cells of different species (Karabourniotis et al., 2001), in epidermal and subepidermal cells of leaves and shoots (Lattanzio et al., 2008). Moreover, polyphenols have protective role against UV radiation due to their screening properties (Pereira et al., 2009) and also due to their involvement in antioxidant system adjustments (Lattanzio et al., 2008), thus in some species they usually accumulate in aerial parts, which are the most exposed to UV light.

In our study, a comparative analysis between *in vitro* callus and parts of the *in vivo* plant, showed that the total phenolic content was higher in leaves (1483.75 ± 29.87 EAG µg/g sample) than *in vitro* callus (802.56 ± 15.03 EAG µg/g sample). In the case of flavonoids, *in vitro* callus induced on the medium with 2.4D was characterized by a higher concentration (519.38 ± 0.91 ERU µg/g sample) than *in vivo* plant part (409.5 ± 2.08 ERU µg/g stem sample). *In vitro* callus had 2 times higher flavonoid concentration than originating inoculum (leaves from mother plant) (Table 1).

The ANCOVA has emphasized that DPPH potential was determined by quantitative variables ($R^2 = 98\%$; $F = 27.40$; $p = 0.01$). The results provided by Type I Sum of Squares of ANCOVA revealed that polyphenols are parameters that have significantly influenced ($F = 162.866$; $p = 0.01$) the antioxidant activity.

Instead, the flavonoid content had a marginal significance ($F = 9.140$, $p = 0.057$) on antioxidant activity. Spearman's correlation ($R = 0.888$; $R^2 = 0.789$; $p = 0.0003$) showed the strong association between DPPH and polyphenols.

The biotechnological approaches were used for numerous species from Asteraceae family for *in vitro* flavonoid production (Bharati and Bansal, 2014). Agarwal and Kamal (2007) observed a higher value of total flavonoid content in *Momordica charantia* callus compared to *in vivo* samples, with the maximum amount accumulated in 6-weeks-old callus. Plant growth factors which promote cell growth by stimulating cell division and elongation like auxins and cytokinins are usually used to induce callus (Coenen and Lomax, 1997). 2.4D has been widely used alone or combined with cytokinin (especially BAP) to stimulate callus induction, to obtain cellular mass and to produce bioactive compounds (Castro et al., 2016).

The efficiency of exogenous 2.4-D has also been reported with other Asteraceae medicinal plants like *Carthamus tinctorius* Linn. (Kumari et al., 2015), *Chrysanthemum morifolium* Ramat. (Swarna et al., 2016). The synergism of 2.4-D and BAP has good results in callus induction at *Helianthus annuus* (Inoka and Dahanayake, 2015). A B5 medium supplemented with BA (0.05 mg/l) and 2.4-D (0.5 mg/l) was the best culture medium for callus production for biomass of milk thistle as a potential source of milk clotting peptidases (Cimino et al., 2006).

In our case, the initiation of callus occurred from the tenth day. In terms of morphology, the calli did not differ, being friable and having a pale green colour. The induction rate was low in the first subculture, more exactly 18.18% on MS added with 2.4-D and 36.36% on MS added with 2.4D and BAP.

The obtained calli were clearly different from the metabolic point of view, callus induced on medium with 2.4-D being highly productive for both flavonoids and polyphenols. This may be explained by the herbicidal effect of 2.4D which induces abiotic stress and plant triggers its defense mechanism by producing more secondary metabolites (Ong et al., 2011). The callus induced on medium with 2.4-D in combination with BAP is distinguished by increased antioxidant activity although it does not possess a rich polyphenolic and flavonoid content (Table 1). This may be possible due to other metabolites which contribute to the total antioxidant activity, being known that this species have a high α-tocoferol content, a potent antioxidant.

In our case, medium supplementation with BAP resulted in a decreased level of polyphenols and flavonoids (Table 1). Our finding is in accordance with results where cytokinins inhibited rutin in callus and in adventitious root culture of *Morus alba* (Lee et al., 2011).

A HPLC was performed to check the presence of rutin in *in vitro* callus induced on MS+2.4D. The rutin peak (retention time = 24.0 minutes, λ =320 µm channel) was validated in *in vitro* callus; and also, a lot of different elution peaks can be observed in the chromatograms of *in vitro* callus extract (Figure 1).

Our results showed that *in vitro* techniques represent an efficient way to obtain secondary metabolites as flavonoids and/or polyphenols which may be produced in large quantities by increasing cellular biomass and subsequently exploited in the food, cosmetics, pharmaceutical industry.

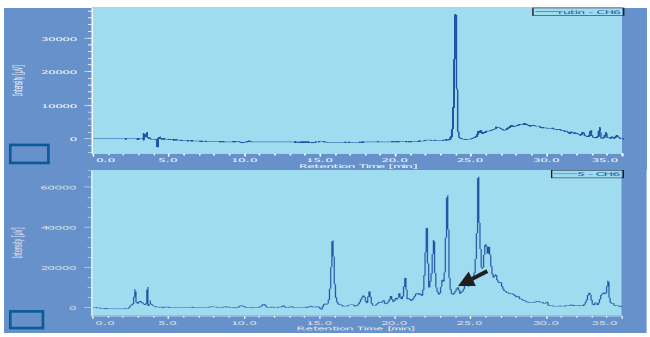

Figure 1. HPLC chromatograms of active principles present in callus sample in 320 µm channel. Arrow indicate the peak correspnding to rutin. A - standard solution, B - callus sample

CONCLUSIONS

In our case, antioxidant activity was correlated with polyphenols content, but there are also other metabolites whose synthesis can be induced *in vitro* and contribute to the total antioxidant activity.

Starting from the same type of explant, two types of callus different from biochemical point of view were obtained in four weeks: one that possesses high antioxidant activity and the second with a rich content of flavonoids and polyphenols.

It was evidenced that *in vitro* induced callus is effective in enhancing the production of flavonoids and polyphenols in a medium supplemented with 2.4-D.

Using HPLC technique, rutin was identified in callus induced through *in vitro* methods; numerous other metabolites were observed.

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REFERENCES

Agarwal, M., Kamal, R. (2007). Studies of flavonoid production using *in vitro* cultures of *Momordica charantia* L. *Indian J. Biotechnol*., 6, 277-279.

Al-Snafi, A.E. (2016). The constituents and pharmacology of *Cnicus benedictus*-A review. *Pharmaceutical and Chemical J.*, 3(2), 129-135.

Bele, C., Matea, C.T., Raducu, C., Miresan, V., Negrea, O. (2014). Tocopherol content in vegetable oils using a rapid HPLC fluorescence detection method. *Not Bot Horti Agrobo*., 41(1), 93-96.

Bharati, A.J., Bansal, Y.K. (2014). *In vitro* production of flavonoids: a review. *World Journal of Pharmacy and Pharmaceutical Science*s, 3(6), 508-536.

Can, Z., Baltaş, N., Keskin, Ş., Oktay, Y., Kolaylı, S. (2017). Properties of antioxidant and anti-inflammatory activity and phenolic profiles of şevketi bostan (*Cnicus benedictus* L.) cultivated in Aegean region from Turkey. *Turkish Journal of Agriculture - Food Science and Technology*, 5(4), 308-314.

Castro, A.H.F., de Queiroz Braga, K., de Sousa, F.M., Coimbra, M.C., Chagas, R.C.R. (2016). Callus induction and bioactive phenolic compounds production from *Byrsonima verbascifolia* (L.) DC. (Malpighiaceae). *Rev. Ciênc Agron*., 47(1), 143-151.

Çetin, B., Kurtuluş, B., Akanil, B.N. (2015). Effects of plant growth regulators on callus formation in different explant of *Calendula officinalis* L. *Journal of Applied Biological Sciences,* 9(3), 34-39.

Chabane, D., Assani, A., Mouhoub, F., Bourakba, C., Nazeli, N. (2013). Anatomical, phytochemical and pharmacological studies of roots of *Cnicus benedictus* L. *International Journal of Medicinal Plants Research,* 2(2), 204-208.

Chandur, U., Shashidhar, S., Chandrasekar, S.B., Bhanumathy, M., Midhun, T. (2011). Phytochemical evaluation and anti-arthritic activity of root of *Saussure alappa*. *Pharmacologia*, 2, 265-267.

Chuanphongpanich, S., Phanichphant, S., Bhuddasukh, D., Suttajit, M., Sirithunyalug, B. (2006). Bioactive glucosinolates and antioxidant properties of broccoli seeds cultivated in Thailand. *Nutraceutical and Functional Food*, 28(1), 55-61.

Cimino, C., Cavalli, S.V., Natalucci, C., Priolo, N. (2006). Callus culture for biomass production of milk thistle as a potential source of milk clotting peptidases. *Electron J. of Biotechnol*., 9(3), 237-240.

Coenen, C., Lomax, T.L. (1997). Auxin-cytokinin interactions in higher plants: old problems and new tools. *Trends Plant Sci*., 2(9), 351-356.

Ghiasy-Oskoee, M., Agha Alikhani, M., Mokhtassi-Bidgoli, A., Sefidkon, F., Ayyari, M. (2019). Seed and biomass yield responses of blessed thistle to nitrogen and density. *Agronomy Journal*, 3(2), 1-11.

Grilo, E.C., Costa, P.N., Gurgel, C.S.S., Beserra, A.F.L., Almeida, F.N.S., Dimenstein, R. (2014). Alpha-tocopherol and Gamma-tocopherol concentration in vegetable oils. *Food Sci. Technol*, Campinas., 34(2), 379-385.

Hauke, J., Kossowski, T. (2011). Comparison of values of Pearson's and Spearman's correlation coefficients on the same sets of data. *Quaestiones geographicae*, 30(2), 87-93.

Horn, G., Kupfer, A., Rademacher, A., Kluge, H., Kalbitz, J., Eißner, H., Dräger, B. (2014). *Cnicus benedictus* as a potential low input oil crop: *Cnicus benedictus* oil. *European Journal of Lipid Science and Technology*, 117, 561-566.

Inoka, K.P.I., Dahanayake, N. (2015). Effect of plant growth regulators on micro-propagation of Sunflower (*Helianthus annuus* L.). *International Journal of Scientific and Research Publications*, 5(1), 1-5.

Karabourniotis, G., Tzobanoglou, D., Nikolopoulos, D., Liakopoulos, G. (2001). Epicuticular Phenolics Over Guard Cells: Exploitation for in situ Stomatal Counting by Fluorescence Microscopy and Combined Image Analysis. *Annals of Botany*, 87, 631-639.

Kumari, S., Pandey, R.K., Kumar, U. (2015). *In-vitro* callus induction from two different explants stem and leaf in *Carthamus tinctorius* Linn. *Euro J. Exp. Bio*., 5(2), 1-4.

Lattanzio, V., Kroon, P.A., Quideau, S., Treutter, D. (2008). Plant Phenolics - Secondary Metabolites with Diverse Functions. In Daayf, F., Lattanzio V. (Eds.) *Recent Advances in Polyphenol Research*, (1-24),Wiley-Blackwell Publishing.

Lee, Y., Lee, D.E., Kim, K.S., Lee, W.S., Kim, S.H., Kim, M.W. (2011). Influence of auxins, cytokinins and nitrogen on production of rutin from callus and adventitous roots of the mulberry tree (*Morus alba* L.). *Plant Cell Tiss Organ Cult*.,105, 9-19.

Marxen, K., Vanselow, K.H., Lippemeier, S., Hintze, R., Ruser, A., Hansen, U.P. (2007). Determination of DPPH radical oxidation caused by methanolic extracts of some microalgal species by linear regression analysis of spectrophotometric measurements. *Sensors*, 7, 2080-2095.

Mihailović, V., Matić, S., Mišić, D., Solujić, S., Stanić, S., Katanić, J., Stanković, N. (2013). Chemical composition, antioxidant and antigenotoxic activities of different fractions of *Gentiana asclepiadea* L. roots extract. *EXCLI Journal*, 12, 807-823.

Murashige, T., Skoog, F. (1962). A revised medium for rapid growth and biossays with tabacco tissue cultures. *Physiol Plant*.,15(3), 473-497.

Ong, S.L., Ling, A.P.K., Poospooragi, R., Moosa, S. (2011). Production of flavonoid compounds in cell cultures of *Ficus deltoidea* as influence by medium composition. *Int. J. Med. Arom. Plants*, 1(20), 62-74.

Păun, G., Neagu, E., Albu, C., Radu, G.L. (2015). Inhibitory potential of some Romanian medicinal plants against enzymes linked to neurodegenerative diseases and their antioxidant activity. *Phcog Mag*.,11(S1), 110-116.

Pereira, D.M., Valentão, P., Pereira, J.A., Andrade, P.B. (2009). Phenolics: From Chemistry to Biology. *Molecules*, 14, 2202-2211.

Stevens, J. (1986). *Applied Multivariate Statistics for the Social Sciences*. Hillsdale, NJ: Lawrence Erlbaum Associates.

Swarna, R.J., Dilruba, Y., Mostafizur, R., Firoz, A. (2016). Callus induction and indirect organogenesis in *Chrysanthemum morifolium* Ramat. *International Journal of Biosciences*, 9(3), 139-149.

Ulbricht, C., Basch, E., Dacey, C., Dith, S. et al. (2008). An evidence-based systematic review of blessed thistle (*Cnicus benedictus*) by the natural standard research collaboration. *Journal of Dietary Supplements*, 5(4), 422-437.

Zhishen, J., Mengcheng, T., Jianming, W. (1999). The determination of flavonoid content in mulberry and their scavenging effects on superoxide radicals. *Food Chem*., 64, 555-559.

***Ministry of Agriculture and Rural Development - Ministerul Agriculturii şi Dezvoltării Rurale (2011). Ghid de bună practică pentru culivarea plantelor medicinale şi aromatice, Monitorul oficial, 527.

***XLSTAT pro, Data Analysis and Statistical Solution for Microsoft Excel (2013). Addinsoft, Paris, France.