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Antioxidant Potential of Marigold (*Calendula officinalis* Linn.) Flowers Grown in Slovakia and Bulgaria

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ABSTRACT

Marigold plants (*Calendula officinalis* Linn) var. 'Plamen Plus' were grown under field conditions during the 2009 season in Trebišov, Slovakia and in Sofia, Bulgaria. The antioxidant potential in Slovak marigold flowers was due to the content of glutathione and ascorbate, two peroxidases (glutathione peroxidase and guaiacol peroxidase) and monodehydroascorbate reductase. The antioxidant capacity in Bulgarian marigold flowers was determined by higher levels of flavonoids, catalase, ascorbate peroxidase, glutathione-*S*-tansferase and dehydroascorbate reductase. Marigold grown in Slovakia had a higher dry biomass of flower heads with a higher carotenoid content. Different soil-climatic conditions mobilize different compounds of the antioxidant defense system, and accumulate biomass and carotenoids in marigold plants grown in both countries.

Keywords: antioxidants, carotenoids, enzymes, marigold (*Calendula officinalis* Linn.), secondary metabolites
Abbreviations: **APX**, ascorbate peroxidase; **ASC**, ascorbate; **CAT**, catalase; **DHAR**, dehydroascorbate reductase; **DHASC**, dehydroascorbate; **GPO**, guaiacol peroxidase; **GPX**, glutathione peroxidase; **GR**, glutathione reductase; **GSH**, reduced gluthatione; **GST**, glutathione-*S*-transferase; **MDHAR**, monodehydroascorbate reductase; **MDA**, malondialdehyde; **ROS**, reactive oxygen species

INTRODUCTION

The species *Calendula officinalis* or pot marigold has been cultivated for many years as an attractive garden plant (Duke *et al*. 2002). This plant has a long history of medicinal use; it is mentioned in many ancient herbals for use in the treatment of headaches, toothache, swellings and for strengthening the heart. Marigold petals are used to make eyewash. This medicinal plant species is also used in both homeopathic and conventional medicine as a healing ointment for cuts and grazes. Marigold has anti-septic, stimulant and anti-fungal properties (Muley *et al*. 2009). Plants produced bioactive compounds with several uses. These include the essential oils and pigments from its flowers, especially carotenoids. Carotenoids are known as biologically active compounds with multiple applications in therapy. The very important attribute of these natural components is their antioxidative activity, which protects organisms against reactive oxygenic radicals. Compounds that might contribute to the total lipid antioxidant capacity include carotenoids, flavonoids and cinnamic aid derivates (Wu *et al*. 1984). Vitamin E (tocopherol) is a major contributor to overall lipid-soluble extracts (Schwants *et al*. 1996). Antioxidant potential is a mobilization of the antioxidant reserves in the plant that reacts both enzymatically and non-enzymatically with these toxic free radicals making them less harmful. Non-enzymatic antioxidants include ascorbate (ASC) and glutathione (GSH) and enzymatic antioxidants are superoxide dismutase (SOD), different specific peroxidases, catalase (CAT) and enzymes of ascorbate-glutathione cycle, as reviewed by Ahmad *et al*. (2008). It is well known that ascorbate-glutathione cycle plays a vital role in the detoxification of reactive oxygen species (ROS). The development of oxidative stress symptoms is judged by the accumulation of H_2O_2 and the oxidative damage to lipids. There is an increasing interest in the use and measurement of antioxidants in the food, pharmaceutical and cosmetic industries.

The aim of the present study was to evaluate carotenoid contents, non-enzymatic antioxidants and antioxidant enzymes in flowers of marigold grown in Bulgaria and Slovakia.

MATERIALS AND METHODS

Plant material and soil-climatic conditions

Field experiments were carried out in Slovakia (Trebišov) and Bulgaria (Sofia). Trebišov is an area of the Easter Slovakian Lowland. The altitude is about 107 m above sea level, a warm and dry climatic region during summer and colder winter. The average annual temperature ranges from 9 to 10°C and annual precipitation is 550–600 mm. The experimental site is situated on private plots with neutral soil (pH = 7.1–7.2), which has a higher content of phosphorus (320 mg kg^{-1}), magnesium (730 mg kg^{-1}) and potassium (285 mg kg^{-1}) (Kobza *et al*. 1999). The relief is formed by an undulating plain on fluvio-eolian substrate.

The study in Bulgaria was conducted at an experimental field, on a leached cinnamonic meadow soil (Chromic Luvisols, according to FAO - Unesco-Isric legend). The experimental field of the Institute of Plant Physiology is situated in a mild and rainy climatic region of sub-mountain areas in West-Central Bulgaria. The altitude is about 564 m above sea level. The average annual temperature ranges from 8 to 10°C and annual precipitation is 224–304 mm. The slightly acid soil is characterized by the following agrochemical characteristics: pH (H_2O) = 6.2, 8 mg kg^{-1} soil total mobile nitrogen ($N\text{-}NO_3^-$ + $N\text{-}NH_4^+$), 30 mg kg^{-1} soil P_2O_5, 120 mg kg^{-1}soil K_2O and 1.88% of organic matter (Geneva *et al*. 2006).

Marigold seeds were sown in lines with an inter-row spacing of 0.5 m. Flower heads of var. 'Plamen Plus' were picked during the 2009 season in Trebišov (Slovakia) and in Sofia (Bulgaria).

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Original Research Paper

Var. 'Plamen Plus' was selected because of the high fresh and dry biomass and β-carotene content of flower heads (Salamon 2006).

Flower clusters were cleaned of field debris and subsequently dried for 6-12 days in a dark room at 21-23°C at 40–60% relative humidity. Dry matter was calculated from 3 g of filtered mixture, which was dried in an electrical dryer at 80°C. For accuracy, all assays were performed sequentially, and the data were evaluated separately.

Reagents

All solvents were of analytical grade. Potassium iodide KJ, trichloracetic, nitric and perchloric acids were obtained from Merck (Darmstadt, Germany). Ascorbate oxidase and glutathione reductase were purchased from Sigma (Munich, Germany). 1-chloro-2,4-dinitrobenzene (CDNB) and cumene hydroxyperoxide were obtained from Fluka Chemie AG (Buchs, Switzerland). All other chemicals were of analytical grade.

Carotenoid evaluation

For chemical analysis of carotenoids in flower inflorescences, 1 g of dry matter from each harvest was used (Kishimoto *et al*. 2005). The crushed material was mixed with 50 ml of methanol. The extract was boiled under reflux for 30 min with recooling. Subsequently, the solution was filtered; 2 ml of the solution was diluted with methanol to 20 ml. The absorbance was measured at 455 nm against methanol. The proportion of carotenoids was calculated with reference to the absorbance of a standard 1% carotenoid solution in methanol. The analytical accuracy was verified by reference to concentrated carotenoids. The weight of methanol and dry matter were taken into account when calculating the mass of extractable substances.

Activities of antioxidant enzymes

In order to prepare crude extracts for determination of enzymes of the ascorbate-glutathione cycle – glutathione peroxidase (GPX), glutathione *S*-transferase (GST) and glutathione reductase (GR), as well as guaiacol peroxidase (GPO) and catalase (CAT) dry flower petals were ground with 4 cm^3 of extraction buffer (100 mM K_2HPO_4, pH 7.8; 5 mM EDTA; 2% PVP (MW = 44,000) that was added to 0.3 g of tissue powder. The extraction buffer for the determination of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) contained: 50 mM K_2HPO_4, pH 7.0; 1 mM ascorbate; 1 mM EDTA; 0.2% PVP and was added to 0.15 g of tissue powder. The suspensions were centrifuged (16,000 × *g*, 15 min, 4°C). All enzymes were assayed spectrophotometrically by tracing the changes in absorbance at 27°C using UV-VIS SPECORD or SPECOL 11. GPO (EC 1.11.1.7) was assayed according to Polle *et al*. (1994), CAT (EC 1.11.1.6) according to Aebi (1984), GPX (EC 1.11.1.9) according to Edwards (1996), GST (EC 2.5.1.18) according to Li *et al*. (1995), GR (EC 1.6.4.2) according to Sherwin and Farrant (1998), APX (EC 1.11.1.11) according to Nakano and Asada (1981), MDHAR (EC 1.6.5.4.) according to Miyake and Asada (1992), DHAR (EC 1.8.5.1.) according to Doulis *et al*. (1997). The protein content was determined after Lowry *et al*. (1951).

Other antioxidants

Low molecular antioxidants were determined in flower extracts as described by Foyer *et al*. (1983). Reduced forms of glutathione were measured in extracts according to Doulis *et al*. (1997). Ascorbate was assayed according to Foyer *et al*. (1983). The level of lipid peroxidation as 2-thiobarbituric acid reactive metabolites, chiefly malondialdehyde (MDA) and H_2O_2 content were determined as described previously (Heath and Packer 1968). For determination of phenols and flavonoids fresh flower samples (1 g) were ground and exhaustively extracted with 96% (v/v) methanol. The content of phenolic compounds was determined spectrophotometrically using Folin-Ciocalteu reagent and calculated as caffeic acid equivalents (Pfeffer *et al*. 1998). Flavonoids in plant tissues were measured by Jia *et al*. (1999) spectrophotometrically using a standard curve of catechin.

Quantitative determination of antioxidant capacity, including vitamin C (ascorbic acid), reduced glutathione and vitamin E (tocopherols), was performed according to Prieto *et al*. (1999). Total antioxidant capacity (free radicals scavenging activity) was measured from the bleaching of the purple-colored methanol solution of free stable radical (diphenylpycril- hydrazyl-DPPH$^{\bullet}$) inhibition after Tepe *et al*. (2006). DPPH$^{\bullet}$ radical is a stable radical with a maximum absorption at 517 nm that can readily undergo reduction by an antioxidant. The inhibition of free radical DPPH$^{\bullet}$ in percent (I%) was calculated in the following way:

$$I\% = (A_{blank}\text{-}A_{sampe}/A_{blank}) \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), A_{sample} is the absorbance of the test compound, i.e. marigold extracts.

Data are expressed as means ±SE, where n = 3. Comparison of means was performed by Fisher's LSD test ($P \leq 0.05$) after performing ANOVA analysis (Statgraphics Plus, v. 2.1).

RESULTS AND DISCUSSION

The level of antioxidant metabolites (**Fig. 1**) and enzymes of the ascorbate-gluthatione cycle (**Fig. 2**) indicates the antioxidant potential of marigold flower heads. The cycle involves the antioxidant metabolites: ascorbate, glutathione and NADPH and the enzymes linking these metabolites (Noctor and Foyer 1988). The levels of the GSH and ASC were higher in the Slovak samples (**Fig. 1**).

Free radicals scavenging activity (DPPH$^{\bullet}$ inhibition) was also higher in the Slovak marigold. The contents of H_2O_2, total phenols and vitamin E were equal in both samples, MDA was higher in the Slovak petals, and only the flavonoids accumulation was higher in the Bulgarian marigold petals. Reactive oxygen species are generated in the plant cells under normal metabolism and predominantly under stress conditions, but ROS such as O_2^- and H_2O_2 are synthesized at high rates even under optimal conditions (Noctor and Foyer 1988). H_2O_2 can also be produced by a number of non-enzymatic and enzymatic processes in cells while mitichondria and chloroplasts are the major sources of H_2O_2 in the cells, peroxisomes and glyoxysomes also contain SOD and APX, which are responsible for its production and scavenging (Jiménez *et al*. 1997). CAT and APX, two potential scavengers of H_2O_2, maintain its level and prevented uncontrolled export of this toxic species from organelles to cytosol.

The higher ASC content and APX activity would result in faster removal of H_2O_2 through the CAT and ascorbate-gluthatione cycle, helping to alleviate oxidative damage (Wu *et al.* 2006). Toxic O_2 species can initiate lipid peroxidation and increased levels of MDA as a result. Preservation of lipidic fractions of natural foods from oxidative deterioration is very important for organoleptic quality and safety. Among the low-molecular-weight antioxidants, ASC and GSH are of great importance. They fulfill multiple roles in defense reactions. Glutathione in plants has a great physiological significance in defense. Glutathione is a precursor of the phytochelatins, which are crucial in controlling cellular heavy metal concentration (Grill *et al.* 1985), therefore increased GSH levels are connected with enhanced plant tolerance to stress. Glutathione is also consumed and degraded in order to protect cellular membranes from lipid peroxidation.

Flowers of marigold grown in Bulgaria accumulated more flavonoids, while total phenols did not differ significantly in the two samples. It is known that total phenols and flavonoids are also involved in plant cell antioxidant defense. It has already been reported that the antioxidant mechanism of flavonoids may also come from the interaction between transition-metal ions and flavonoids to produce complexes that keep the metal ions from their participation in free-radical generation (Jia *et al.* 1999). At the same time, as natural metal chelators, flavonoids show a

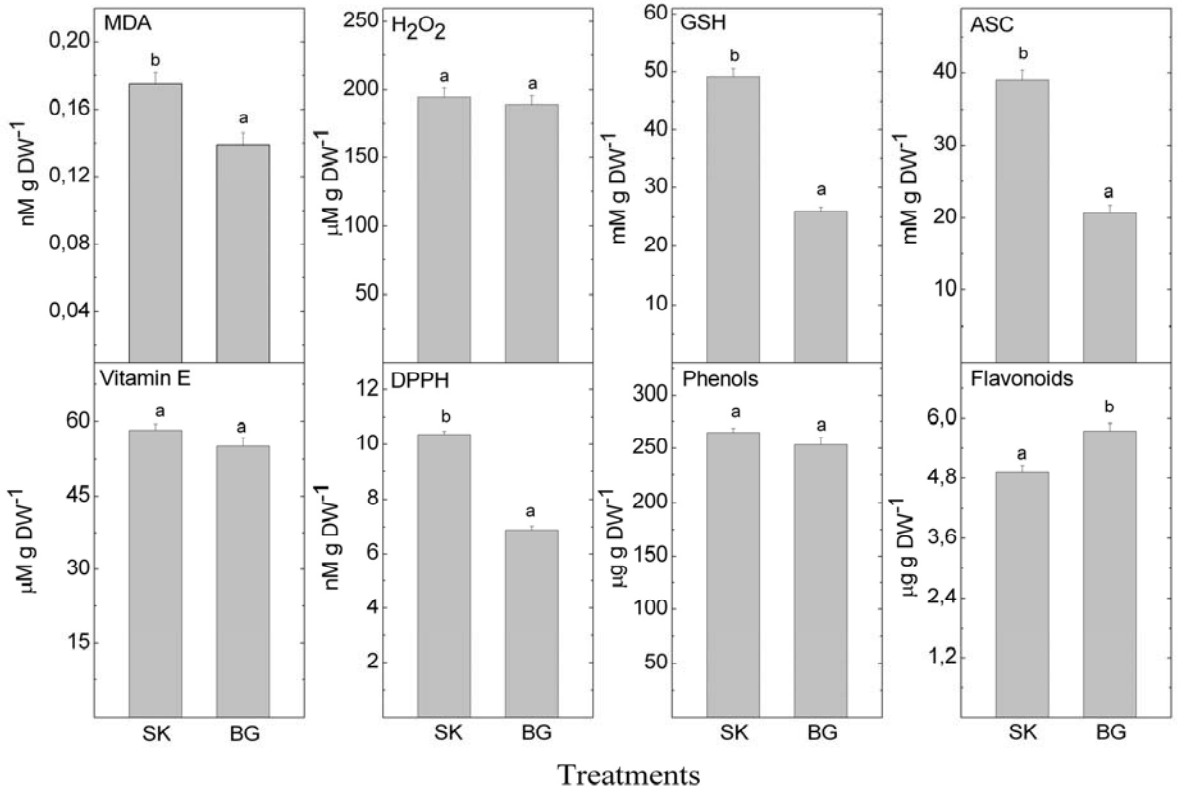

Fig. 1 Content of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), reduced glutathione (GSH), ascorbate (ASC) and dehydroascorbate (DHASC), phenols and flavonoids in the petals of *Calendula officinalis* grown in Slovakia and Bulgaria. Values are means ± SE, $n = 3$; different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

significant function on the bio-utilization of metal and anti-metal-toxicosis (Jia *et al.* 1999). According to some authors (Milić *et al.* 1998; Ćetković *et al.* 2003) phenolic compounds such as flavonoids and phenolic acids are principal contributors to the free radicals scavenging activity in pant cells.

Stable DPPH radical has been used to investigate scavenging activity of marigold extracts. Free radical-scavenging capacities from both extracts measured by DPPH$^{\bullet}$ assay are shown in **Fig. 1** and expressed as the percentage reduction of the initial DPPH$^{\bullet}$ absorption by the antioxidants. As confirmed by the test system results, higher antioxidant activity was exhibited by the flower extracts from Slovakia.

The antioxidant systems consist also of antioxidant enzymes shown in **Fig. 2**. In the Bulgarian samples, higher activities of CAT, GST, DHAR and APH were measured. Increased activities in the Slovak samples were observed regarding GPX, GPO and MDHAR. GR which is catalyzed NADPH-dependent conversion of oxidized to reduced glutathione, is equal in both samples.

These antioxidant systems can be divided into two categories: one that reacts with ROS and keeps them at low levels, (peroxidases, superoxide dismutase and CAT), and one that regenerates the oxidized antioxidants (APX and GR) (Smirnoff 1993). Increases of the antioxidant enzyme activities could indicate increased oxidative stress, but they give no indication of changes in overall flux through ascorbate-gluthathione cycle. Despite of equal levels of H_2O_2 in both samples, CAT activity is about 2 times higher in the Bulgarian marigold flowers. It is well known that CAT has extremely high maximum catalytic rates but low substrate affinities and is induced by the high H_2O_2 levels only. Ascorbate peroxidase that uses two molecules of ASC to reduce H_2O_2 is higher in the Bulgarian marigold (**Fig. 2**) which corresponds with lower ASC content (**Fig. 1**). Increased levels of GPX and GPO in the Slovak marigold petals allow an assumption that antioxidant enzymatic defense is due to these enzymes activities.

Peroxidases belong to a large family of enzymes that are ubiquitous in fungi, plants, and vertebrates. These proteins usually contain a ferriprotoporphyrin prosthetic group and oxidize several substrates in the presence of H_2O_2 (Vianello *et al.* 1997). DHAR, using GSH as the reducing substrate, is also higher in the Bulgarian samples where GSH is reduced. Higher activity of MDHAR in the Slovak marigold coincides with its higher ASC level (**Figs. 1, 2**). Despite the possibility enzymatic and nonenzymatic regeneration of ASC directly from MDHAR, its rapid transformation means that some quantities of DHAR are always produced when ASC is oxidizes under stress conditions. GST activity is higher in the Bulgarian marigold extracts (**Fig. 2**). GSTs are considered, among several others, to contribute to the biotransformation of xenobiotics. GSTs catalyse the conjugation of reduced glutathione – via a sulfhydryl group – to electrophilic centers on a wide variety of substrates (Douglas 1987). This activity detoxifies endogenous compounds such as peroxidised lipids (Leaver and George 1998), as well as breakdown of xenobiotics.

Therefore, antioxidant potential in the Slovak marigold flowers is based on the content of glutathione and ascorbate, two peroxidases (GPX and GPO) and MDHAR. The antioxidant capacity of the Bulgarian marigold flowers is determinated by the higher flavonoid levels, CAT, APX, GST, DHAR catalase, ascorbate peroxidase, glutathione-*S*-tansferase and dehydroascorbate reductase.

The results of total carotenoid content are presented in **Table 1**. This implies changes in extracts of flower heads of var. 'Plamen Plus', picked during the 2009 season in Trebišov (Slovakia) and in Sofia (Bulgaria). The results show the average content and composition of carotenoids in extracts of dry flower heads of this variety, picked within the same growth period during July and August 2009 in Slovakian and Bulgarian fields. The total carotenoid content of the marigold flower heads varied from 0.06 to 0.10 % during the experiment. Muley *et al*. (2009) reported about carotenoid compositions in methanol extracts of leaves, petals and pollen of *C. officinalis* flowers. The authors

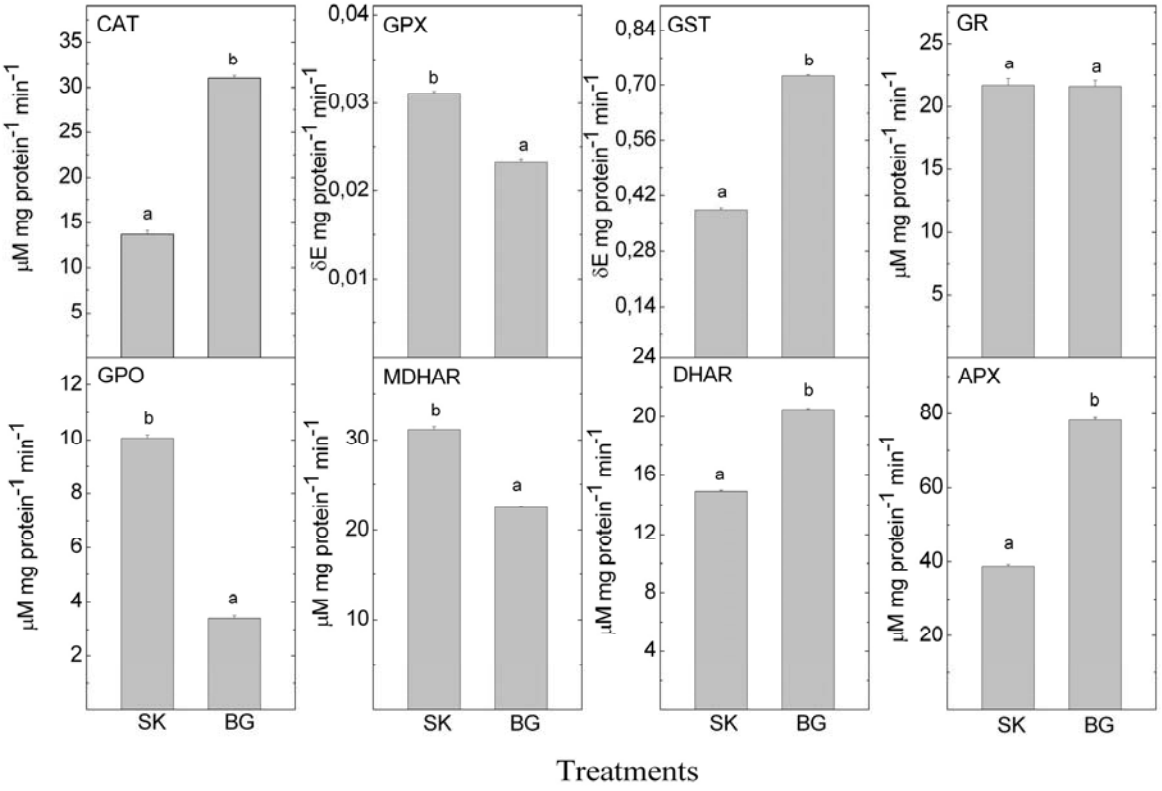

Fig. 2 Activity of catalase (CAT), glutatione peroxidase (GPX), glutathione *S*-transferase (GST) and glutathione reductase (GR), guaiacol peroxidase (GPO), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and ascorbate peroxidase (APX) in the petals of *Calendula officinalis* grown in Slovakia and Bulgaria. Values are means ± SE, $n = 3$; different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

Table 1 Dry biomass and total carotenoids content in petals of marigold grown in Slovakia and Bulgaria.

Treatments	DW (%)	Absorbance 455 nm	Total carotenoids content in drug (%)	% of extractable components in extract (%)	% Carotenoids in extractable components
flowers - Slovak	0.7 b	0.448 b	0.09 b	23 b	0.42 a
flowers- Bulgarian	0.4 a	0.316 a	0.06 a	14 a	0.4 a
LSD	0.06	0.044	0.009	1.97	0.05

Data are presented as means from four replications. Different letters indicate significant differences assessed by Fisher's LSD test ($P \leq 0.05$) after performing ANOVA analysis.

showed that total carotenoids in petals were 5 times more than in pollens and 9 times more than in leaves.

Dry biomass percentage and total carotenoids are higher in the Slovak marigold. The findings show a relationship between higher dry mass and higher % of carotenoids. In general, the results are determined a high carotenoid content of the marigold variety 'Plamen Plus', which was breeding to this special attribute.

CONCLUSIONS

It was concluded that marigold plants (*Calendula officinalis* Linn) of var. 'Plamen Plus' grown under field conditions in Slovakia and in Bulgaria showed different carotenoid contents and antioxidant response to the specific soil-climatic conditions. The antioxidant potential of the Slovak marigold flowers is due to content of GSH and ASC, two peroxidases (GPX and GPO) and MDHAR. The antioxidant capacity of the Bulgarian marigold flowers is determined by the higher levels of flavonoids, CAT, APX, GST and DHAR. Higher dry biomass level corresponded with higher carotenoid contents.

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