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THE EFFECTS OF OXIDATIVE STRESS ON PHENOLIC COMPOSITION AND ANTIOXIDANT METABOLISM IN CALLUS CULTURE OF COMMON DAISY

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Abstract

Background: Exogenous pretreatment of oxidative stress on callus cultures of *Bellis perennis* L. (common daisy) induced catalase (CAT), superoxide dismutase (SOD), total phenolic, total flavonoid, total protein and selected commercial phenolic compounds production and accumulation.
Materials and Methods: The callus culture obtained from *B. perennis* pedicel explants was incubated on Murashige and Skoog medium (MS) containing 10 mM H_2O_2 or 0 mM H_2O_2 (for control group) for 10 hours. Twenty phenolic compounds (apigenin, caffeic acid, *p*-coumaric acid, gallic acid, genistein, kaempferol, luteolin, myricetin, procyanidin-*C1*, quercetin, rutin hydrate, vanillic acid, ferulic acid, salicylic acid, sinapic acid, chlorogenic acid, hesperedin, naringenin, rosmarinic acid and isorhamnetin) were detected by LC-ESI-MS/MS analysis in methanolic extracts of 10 mM H_2O_2 and control treatments.
Results: A predominant phenolic compound was chlorogenic acid followed by rutin hydrate, caffeic acid, luteoline, isorhamnetin, quercetin, myricetin, apigenin, *p*-coumaric acid and kaempferol. No gallic acid, genistein, procyanidin-*C1*, vanillic acid, sinapic acid, hesperidin and naringenin were detected in H_2O_2 treatment and control groups of *B. perennis*. The total phenolic contents estimated were in the order of H_2O_2 treatment (285.36 µg/g dw) and control (220.79 µg/g dw) groups. The biosynthesis and accumulation of kaempferol, myricetin, quercetin and isorhamnetin were only determined in H_2O_2 treatment callus materials. The H_2O_2 pretreatment clearly showed in a raise in enzymatic and non-enzymatic antioxidant activities. Finally, a significant positive correlation between phenolic accumulation and comprehensive activities of CAT, SOD, total phenolic, total flavonoid and proline was accessible.
Conclusion: The present results suggest that using H_2O_2 as an elicitor or a stimulant plays a significant enhancement role in special phenolic molecules biosynthesis and activation of antioxidant metabolism on callus cultures of *B. perennis*.

Key words: *Bellis perennis*, pedicel explant, callus culture, SOD, CAT, antioxidant activity, hydrogen peroxide

Introduction

Reactive oxygen species (ROS), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), are frequently generates during metabolic pathways in all living organisms. Expose to normal physiological situation, cellular ROS generation is reverse equilibrium by the action of antioxidant enzymes and other redox molecules. On the other hand, high value of ROS production will cause to cellular damage, such as disruption to DNA, protein, and lipid membrane. Dangerous effects of high ROS level must be quickly inhibited from the cells by different antioxidant defense systems. The superoxide dismutase (SOD) enzyme is one of them, which catalyzes the detoxification of superoxide anion into H_2O_2 and molecular oxygen, is one of the most valuable antioxidative enzyme. The H_2O_2 is a potentially dangerous side effect of the metabolism of oxygen, a process that occurs in most living organisms after many metabolic pathways (Cingoz et al., 2014).

By traditional agriculture, producing of biologically active secondary metabolites for commercial purposes is not a sufficient process. Generally, methods related with *in vitro* culture protocols have been investigated to develop the generation of these important medicinal compounds. Whensoever numerous abiotic and biotic factors were applied on plant tissue cultures, amount of secondary compounds was enhanced in a number of scientific reports (Cingoz et al., 2014). The H_2O_2 is one of the most important molecules which are caused to increase in plants by biotic and abiotic stress conditions. Higher levels of H_2O_2 generally terminate in toxicity to cell membrane system and damages to plant cells (He et al., 2009). However, the raised data evidence the biological activity of H_2O_2 as a stress signal molecule in plants (Hung et al., 2005) and H_2O_2 signaling functions significantly in plant development and defense system against environmental stresses (He et al., 2009; Cingoz et al., 2014). The H_2O_2 affects plant defense system and induced biosynthesis of glutathione-S transferase, CAT, SOD, and phenylalanine ammonia lyase (PAL) as antioxidant enzymes, transcription factors and defense proteins (Kovtun et al., 2000; Hung et al., 2005). Nonetheless, with specific elicitor, there are no data on *B. perennis* callus cultures in which improvements in phenolic production have been accomplished yet. Cingoz et al., (2014) showed that exogenous H_2O_2 treatment increased cardiotonic glycoside production in *Digitalis* species. Until now, there is no too much information on the use of H_2O_2 as an elicitor producing medicinally important secondary metabolites.

Bellis perennis L. (common daisy) is a medicinal perennial herb in the family Compositae (Panda, 2004). Aerial parts with flowers of plant material have been used in the remedy of many types of wounds (Karakaş et al., 2012), headache (Uzun et al., 2004), common cold (Cakilcioglu et al., 2010), rheumatism, muscular pain (Morikawa et al., 2011), expectorant and anti-inflammatory (Siatka and Kasparova, 2010) in folk medicine for ancient times. Aerial part of the plant material include phenolic compounds (Karakas and Turker, 2013), triterpenoid saponins (Yoshikawa et al., 2008; Karakas et al., 2014), and essential oils (Kavalcioglu et al., 2010). The plant extracts obtained from *B. perennis* have many biological activities such as wound healing (Karakaş et al., 2012), spatial memory opener (Karakas et al., 2011), antitumor (Karakas et al., 2014), antimicrobial (Kavalcioglu et al., 2010), antihyperlipidemic (Morikawa et al., 2010), antioxidant (Siatka and Kasparova, 2010) and cytotoxic activities against some human cancer cell lines (Li et al., 2005; Karakas et al., 2015).

Despite these scientific endeavors in the literature, the phenolic compounds accumulation and some activities such as the CAT, SOD, total phenolic, total flavonoid, proline activity in *in vitro*-propagated callus cultures of *B. perenn*is are still open to investigation because of a lack of the experimental research in the literature. Thus the aim of the present study was to identify how the phenolic compounds accumulation and the CAT, SOD, total phenolic, total flavonoid, proline activity in *B. perenn*is callus cultures affected abiotic stress factor such as oxidative stress by using H_2O_2 as an elicitation.

Material and Methods

Plant Material and Culture Conditions

Fresh and wild-grown pedicel parts with flowers of *B. perennis* were collected from Abant Izzet Baysal University Campus, Bolu, Turkey in May. Surface sterilization of the plant materials was made according to Karakas and Turker (2013).

The sterile pedicel explants for callus production were transferred to petri plates containing MS medium with 0.5 mgL^{-1} thidiazuron (TDZ) and 0.5 mgL^{-1} indole-3-acetic acid (IAA) for 30 days (Karakas and Turker, 2013). The calli (5 × 15 petri) of both treatments that have same sizes were put into the MS medium supplement with zero (control) and 10 mM H_2O_2 for forming oxidative stress for 10 hours. Petri dishes containing untreated (control) and oxidative stress treated calli were maintained at 23 ± 2 °C under a 16 h photoperiod from cool white fluorescent lamps.

Preparation of Extracts

Untreated (control) and oxidative stress treated calli were collected from *in vitro*-cultured common daisy. The freeze-dried callus materials were powdered with grinder and weighted. 100 mg of callus material of *B. perennis* was put into the plastic centrifuge tube including 2 mL of 80% methanol (MeOH) for 16 h at dark and room temperature. After 30 min application in an ultrasonic bath at 40 °C, it was centrifuged at 10,000 rpm for 12 min. The supernatant was filtrated and transferred to new centrifuge tube. The extract solutions of stress-treated and untreated (control) calli were kept at -80 °C deep-freeze until using in all analyses.

Determination of Total Phenolic Content

The amount of total phenolic content was determined in 80% methanol extracts of calli obtained from *B. perennis* using the Folin-Ciocalteu assay (Slinkard and Singleton, 1977), with some modifications. Therefore, 20 µL of each calibration solution or sample or blank, 100 µL Folin-Ciocalteu reagent (Sigma®) and 1.58 mL of deionized water were transferred in a 10 mL test tube and mixed thoroughly. After 2 minutes, 300 µL of 20% Na_2CO_3 solution was added in the test tube. The solutions were incubated at 20 ± 2 ºC for 2 hours and measured the absorbance of each solutions at 765 nm against the blank (the "0 mL" solution) using the spectrophotometer (Hitachi U-1900, UV-VIS Spectrophotometer 200V, JAPAN). Gallic acid was used as a standard (0-500 mg/L). The total phenol content of 80% methanolic extracts from callus was expressed as mg gallic acid equivalents (GAE)/ g dried weight (dw). Three measurements of one sample were performed at same time.

Determination of Total Flavonoid Content

Total flavonoid content of methanolic extracts of *B. perennis* calli was measured by aluminum chloride ($AlCl_3$) colorimetric assay (Chang et al., 2002). Catechol was used as a reference flavonoid. The 0.0125 g catechol was dissolved in 25 mL of 80% methanol and this stock solution was adjusted to concentration as 500 mg/mL. In order to obtain calibration curve of catechol, 20, 40, 60, 80 and 100 mg/mL concentrations were prepared. 500 µL of extract solution or standard solution of catechol was added to a 10 mL test tube containing 2 mL distilled water. At zero time, 150 µL of 5 % sodium nitrate ($NaNO_2$) was added to the test tubes. After 5 min, 150 µL of 10% $AlCl_3$ was added. At 6 min, 1000 µL of 1M sodium hydroxide (NaOH) was added to the mixture. Immediately, the reaction tube was diluted to volume 5 mL with the addition of 1200 µL distilled water and thoroughly mixed. Absorbance of the mixture, pink in color, was determined at 510 nm versus a blank (Chang et al., 2002). Samples were analyzed in three replications. The total flavonoid content of methanolic extracts of *B. perennis* was expressed as mg catechol equivalents (CE)/ g dw.

Enzyme Extraction and Protein Determination

Freeze-dried untreated (control) and oxidative stress treated calli (0.1 g) of *B. perennis* were grinded and mixed in 2 mL of ice cold 50 mM phosphate buffer (pH 7.0) which included 2 mM sodium ethylenediaminetetraacetic acid (Na–EDTA) and 1% (w/v) polyvinyl-polypirrolidone (PVP). Well mixed materials were centrifuged at 12,000 rpm and 4 ºC for 10 min. Then, the callus extracts of *B. perennis* were kept at -80 ºC for antioxidant enzyme analyses of the SOD and the CAT activity. Lowry method (Lowry et al., 1951) was used for the detection of the soluble protein content in oxidative stress treated and untreated callus extracts. The amount of the soluble protein in extracts was calculated with standard curve of bovine serum albümin ($R^2 = 0.997$).

Catalase (CAT; EC 1.11.1.6) activity

CAT activity of the freeze-dried callus materials was described by using the method of Lartillot et al. (1988). Shortly, amount of the CAT activity was measured at 240 nm (using a specific absorption coefficient at 0.0392 cm^3 $mmol^{-1}$ H_2O_2) spectrophotometrically and the CAT activity was showed as mmol H_2O_2 decomposed/mg protein/min.

Superoxide dismutase (SOD; EC 1.11.1.1) activity

SOD activity of callus enzyme extracts of *B. perennis* was determined by photochemical nitroblue tetrazolium (NBT) test method (Sun et al. 1988). The SOD activity required inhibition of NBT reduction was detected using xanthine-xanthine oxidase that

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as a generator of superoxide anions (O_2^-) (Sun et al., 1988). A unit of the SOD was determined as the quantification of protein that prevents the ratio of NBT decreasing by 50%.

Proline analysis

The proline analysis was detected with respect to the method of Bates et al. (1973). The proline content of callus materials was measured spectrophotometrically at 520 nm as µmol/g dw against standart proline curve ($R^2 = 0.99$).

Quantification of the selected phenolic compounds

The quantification of the chosen 20 phenolics (apigenin, caffeic acid, *p*-coumaric acid, gallic acid, genistein, kaempferol, luteolin, myricetin, procyanidin-C1, quercetin, rutin hydrate, vanillic acid, ferulic acid, salicylic acid, sinapic acid, chlorogenic acid, hesperedin, naringenin, rosmarinic acid and isorhamnetin) in 80% MeOH extracts of oxidative stress treated and untreated (control) groups was determined using Liquid Chromatography- Electro Spray Ionization-Multi Stage/Mass Spectrometry (LC-ESI-MS/MS) method. Analysis was made by "METU Central Laboratory, Molecular Biology-Biotechnology Research and Development Center, Mass Spectroscopy Laboratory, Ankara, Turkey", with Agilent 6460 Triple Quadrupole System (ESI+Agilent Jet Stream) coupled with Agilent 1200 Series HPLC. All standard compound solutions and samples were kept at -20 °C through the lab and bench work. The amount of standard phenolic molecules in callus extracts were detected from the peak areas by using the equilibrium for linear regression taken from the calibration curves (R^2; 0.99) (Karakas and Turker, 2013).

Statistical analysis

All experiments were performed in three different sets, with each set in triplicate. The results were expressed as mean values ± standard deviation (SD). Statistically significant differences between untreated (control) and oxidative stress treated groups were identified by Paired-Samples t-test using statistical software SPSS Version 22.0 program (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant at $p < 0.05$.

Results

In this study, calli were efficiently produced from pedicel explant of field-grown *B. perennis* within two weeks when cultured on MS medium supplemented with 0.5 mgL^{-1} IAA and 0.5 mgL^{-1} TDZ. After four weeks, callus cultures were maintained on MS medium with or without 10 mM H_2O_2 for 10 hours to investigate the effect of H_2O_2 on phenolic accumulation, and enzymatic and nonenzymatic antioxidant activities.

The Quantification of the Selected Phenolic Compounds

The quantification of the chosen twenty phenolic molecules in H_2O_2 treated and untreated (control) callus extracts of *B. perennis* was determined using LC-ESI-MS/MS analysis. Retention times of the standard phenolic compounds and the amount of the standard compounds in callus culture of *B. perennis* were shown in Table 1.

Table 1: Amount of the chosen phenolic molecules in oxidative stress treated (H_2O_2 treatment) and untreated (control) callus cultures of *B. perennis*. Values are means ± SD of three measurements. Nd; not detected.

No	Phenolic molecules	Retention time (min)	Amount of phenolics (µg/g of dry weight)	
			H_2O_2 Treatment	Control
1	Apigenin	6.998	0.0335 ± 0.0004	0.1092 ± 0.0004
2	Caffeic acid	3.150	1.8628 ± 0.0279	2.6401 ± 0.0054
3	*p*-Coumaric acid	3.959	0.0299 ± 0.0039	0.0783 ± 0.0007
4	Gallic acid	0.943	Nd	Nd
5	Genistein	6.493	Nd	Nd
6	Kaempferol	6.864	0.022 ± 0.000	Nd
7	Luteolin	6.489	0.3524 ± 0.0079	0.2267 ± 0.0004
8	Myricetin	5.347	0.0506 ± 0.0003	Nd
9	Procyanidin-C1	3.181	Nd	Nd
10	Quercetin	6.135	0.0691 ± 0.0060	Nd
11	Rutin hydrate	4.985	6.3895 ± 0.5941	5.4110 ± 0.2338
12	Vanillic acid	3.120	Nd	Nd
13	Ferulic acid	4.271	0.0820 ± 0.0011	0.1866 ± 0.0019
14	Salicylic acid	3.933	Nd	Nd
15	Sinapic acid	4.377	Nd	Nd
16	Chlorogenic acid	2.807	276.25 ± 2.9046	212.08 ± 0.0667
17	Hesperidin	5.169	Nd	Nd
18	Naringenin	6.235	Nd	Nd
19	Rosmarinic acid	4.949	0.1398 ± 0.0205	0.0556 ± 0.0004
20	Isorhamnetin	7.003	0.0761 ± 0.0026	Nd
	Total Phenolics		285.358	220.788

Whenever the 80% MeOH extracts of oxidative stress treated and untreated control groups were matched as per the results of LC-ESI-MS/MS analysis, high total phenolic content was detected in H_2O_2 treatment group (285.36 µg/g of dw) (Table 1). The considerable amounts of the chlorogenic acid (276.25 µg/g of dw), rutin hydrate (6.39 µg/g of dw), luteolin (0.35 µg/g dw), rosmarinic acid (0.14 µg/g dw), isorhamnetin (0.08 µg/g of dw), quercetin (0.07 µg/g of dw), myricetin (0.05 µg/g of dw), and kaempferol (0.02 µg/g of dw) were detected in methanol extract of H_2O_2 treated group (Table 1). However, the caffeic acid (2.64 µg/g of dw), ferulic acid (0.19 µg/g of dw), apigenin (0.11 µg/g of dw) and *p*- coumaric acid (0.08µg/g of dw) had high value in untreated (control) group. Both callus extracts obtained from oxidative stress treated and untreated groups included chlorogenic acid, rutin hydrate, caffeic acid, ferulic acid, rosmarinic acid, luteolin, apigenin and *p*- coumaric acid as dominant compounds in our study. Gallic acid, genistein, procyanidin-*C1*, vanillic acid, hesperidin, salicylic acid, sinapic acid and naringenin were lower than the limit of detection in both groups (data not shown). Total phenolic content ranged from 220.79 µg/g of dw for MS-control (untreated callus) to 285.36 µg/g dw for H_2O_2 treated group. Remarkably, we detected the effect of oxidative stress on chlorogenic acid ranged from 212.08 µg/g of dw for control group to 276.25µg/g of dw for H_2O_2 treatment group (Table 1). While 8 phenolic compounds were detected in untreated callus (control) group, 12 of 20 phenolic compounds were detected in H_2O_2 treated callus group (Table 1). Isorhamnetin, quercetin, myricetin and kaempferol were detected only in H_2O_2 treated group.

Antioxidant Enzymes

Catalase (CAT) and Superoxide Dismutase (SOD) Activity

The exogenous oxidative stress treatment caused a certain rise in CAT activity in callus cultures of *B. perennis* (Figure 1). The CAT activity was ranged from 32.52 mmol H_2O_2/min/mg protein for control group to 42.57 mmol H_2O_2/min/mg protein for H_2O_2 treated group. The oxidative stress treatment in callus cultures of *B. perennis* increased SOD activity from 0.56 (untreated group) to 0.72 U/mg protein (H_2O_2 treated group) (Figure 2). Soluble protein concentration was affected by H_2O_2 treatment in callus cultures (Figure 1 and 2).

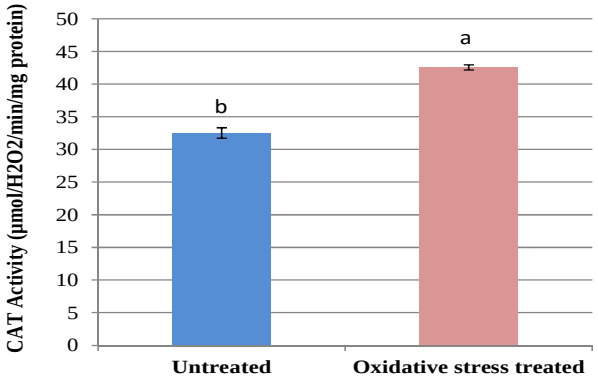

Figure 1: CAT activity of oxidative stress treated (H_2O_2 treatment) and untreated (control) groups of *B. perennis*. Data represented are means (n = 3) ± SD of three measurements. Columns marked with different letters indicate statistically different values ($p < 0.05$).

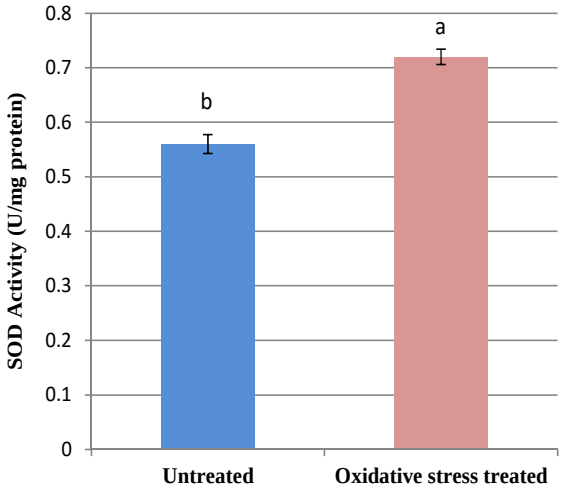

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Figure 2: SOD activity of oxidative stress treated (H_2O_2 treatment) and untreated (control) groups of *B. perennis*. Data represented are means (n = 3) ± SD of three measurements. Columns marked with different letters indicate statistically different values ($p < 0.05$).

Proline, Total Phenolic and Flavonoid Contents

Antioxidant activity of methanol extracts of oxidative stress treated and untreated (control) calli was assessed. Treatment of callus samples with 10 mM H_2O_2 for 6 hours drastically increased proline, total phenolic and flavonoid contents. The exposure of H_2O_2 treatment noticeably increased the proline content from 0.086 mmol/g dw (control) to 0.139 mmol/g dw (H_2O_2 treatment) (Figure 3).

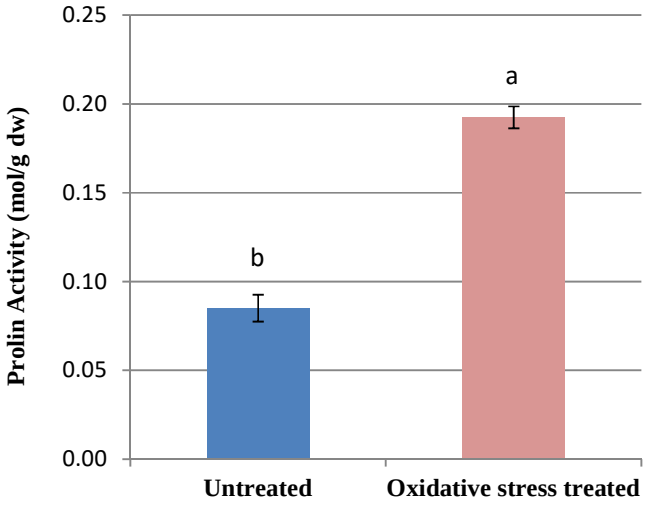

Figure 3: Proline content of oxidative stress treated (H_2O_2 treatment) and untreated (control) groups of *B. perennis*. Data represented are means (n = 3) ± SD of three measurements. Columns marked with different letters indicate statistically different values ($p < 0.05$).

Total phenolic content of callus extracts was assessed using the Folin-Ciocalteu assay (Slinkard and Singleton, 1977). The results were expressed as mg gallic acid equivalents (GAE)/g dry weight of callus (Figure 4). The methanolic extracts of calli obtained from oxidative stress treated and control groups contained 17.65 and 12.56 mg GAE/ g dried weight, respectively. The results presented in Figure 4 indicated that methanolic extract of H_2O_2 treated callus contained higher concentrations of phenolic content than untreated callus. There was a significant positive correlation among total phenolics, total flavonoids and LC-ESI-MS/MS results. Total flavonoid content in methanolic extracts of oxidative stress treated and untreated calli were determined spectrophotometrically by aluminium chloride method. The content of flavonoids was expressed as mg catechol equivalents (CE)/g dried weight. The results presented in Figure 5 indicated that methanolic extract of H_2O_2 treated group (14.51 mg CE/g dw) contained higher concentration of flavonoid content than the control group (9.07 mg CE/g dw) (Figure 5).

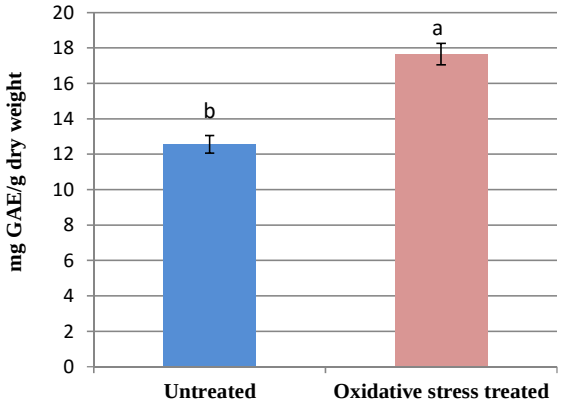

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Figure 4: Total phenolic content of oxidative stress treated (H_2O_2 treatment) and untreated (control) callus cultures of *B. perennis*. Data represented are means (n = 3) ± SD of three measurements. Columns marked with different letters indicate statistically different values ($p < 0.05$).

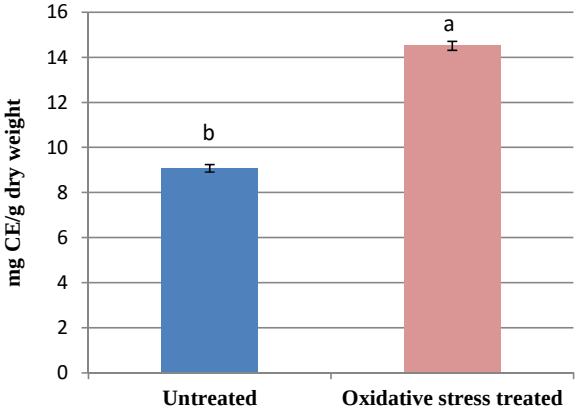

Figure 5: Total flavonoid content of oxidative stress treated (H_2O_2 treatment) and untreated (control) callus cultures of *B. perennis*. Data represented are means (n = 3) ± SD of three measurements. Columns marked with different letters indicate statistically different values ($p < 0.05$).

Discussion

Abiotic stimulants or signal molecules increase the production or induce de novo synthesis of medicinally important secondary metabolites in *in vitro* plant tissue cultures. The various types of elicitors have been widely used for the enhancement of secondary metabolite production in cultures of plant cell, tissue and organ (Ramakrishna and Ravishankar, 2011).

The analysis of phenolic profile points out that *B. perennis* mainly includes phenolics containing the kaempferol, isorhamnetin, quercetin and apigenin (Nazaruk and Gudej, 2001; Karakas and Turker, 2013). An increased rate of the phenolic molecules can be observed under many environmental stresses such as temperature, high light, UV-irradiation, pathogen attack, nutrient deficiencies and herbicide treatments (Ramakrishna and Ravishankar, 2011). Some studies showed that exogenous application of H_2O_2 was capable of increasing secondary metabolites, such as capsidiol production in pepper fruits (*Capsicum annuum* L.) (Areceli et al., 2007), monoterpenoid oxindole alkoloids production in *Uncaria tomentosa* (Huerta-Heredia et al., 2009) and cardenolides production in some *Digitalis* species (Cingoz et al., 2014). Furthermore, Cingoz et. al (2014) reported that H_2O_2 increased phenolic content in different *Digitalis* species. The existence of essential phenolic molecules in callus of *B. perennis*, such as chlorogenic acid, rutin hydrate, caffeic acid, luteolin, rosmarinic acid, ferulic acid, isorhamnetin, quercetin, myricetin, apigenin, *p*-coumaric acid and kaempferol in present study can be beneficial for further investigation of *B. perennis*.

The SOD and CAT are basic antioxidant enzymes required for the direct inhibition of ROS. The SOD enzyme catalyzes the detoxification of superoxide anions and CAT enzyme catalyzes the decrease of H_2O_2 and prevents tissues of organism from largely reactive hydroxyl radicals. The CAT and SOD are essential defense enzymes against oxygen radicals in biotic and abiotic stress conditions (Cingoz et al., 2014). Some investigations demonstrated the increased CAT and SOD antioxidant enzyme activities in callus of *Digitalis* species (Cingoz et al., 2014), wheat (Li et al., 2011) and *Triticum aestivum* (He et al., 2009) seedlings with exogenous application of H_2O_2. Similar result was also found in our study that CAT (42.57 mmol H_2O_2/min/mg protein) and SOD activity (0.72 U/mg protein) significantly increased in H_2O_2 treated callus cultures of *B. perennis*. This study recommended that H_2O_2 pretreatment could induce the activation of antioxidant enzymes and concerned genes as an abiotic stress trigger molecule.

The accumulation of proline under different stress conditions, such as salt, drought and heavy metals has been demonstrated in many plant species (Kishor et al., 2005). Yang et al. (2009) found that exogenous H_2O_2 treatment led to a significant accumulation of proline in coleoptiles and radicles of maize seedlings. Similar result was also observed by He et al. (2009) that exogenous H_2O_2 pretreatment significantly increased free proline content in *T. aestivum* seedlings. Ozden et al. (2009) also showed that H_2O_2 treatment enhanced in endogenous proline production in grapevine leaves. Similarly, our study apparently demonstrated that exogenous application of H_2O_2 triggered a rapid production of proline in *B. perennis* callus cultures. Cingoz et al. (2014) showed that treatment of H_2O_2 enhanced the total phenolic contents and antioxidant enzyme production. Similar result was also detected in our present experiment that treatment of oxidative stress induced phenolic production and antioxidant activity in callus cultures of *B. perennis*.

Our data recommended that the exogenous pretreatment of oxidative stress for 10 hours on callus culture of *B. perennis* might be a powerful method to enhance the phenolic compounds, enzymatic and non-enzymatic antioxidant activities. Moreover, it was found that CAT, SOD, total phenolic, total flavonoid and proline activity had a significant positive correlation with the phenolic accumulation under H_2O_2 pretreatment. Generation of medicinally bioactive secondary metabolites by classic agricultural methods is an insufficient strategy. The *in vitro* culture methods under controlled laboratory conditions have been broadly investigated to develop the production of specific plant originated bioactive molecules. In this study, we can conclude that application of oxidative

stress as an effective elicitor is a very useful method for the enhancement of phenolics in *B. perennis*. In this study, enhancement of phenolic compounds with the application of oxidative stress was demonstrated with callus culture. Generally, callus culture can be used for the starting material of cell suspension cultures for the large scale production of phenolic compounds. The antioxidant potential of this plant can provide an advantage in food, medicine and cosmetic industries.

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