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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 H2O2 or 0 mM H2O2 (for control group) for 10 hours. Twenty phenolic compounds (apigenin, cafffeic 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, rosamarinic acid and isorhamnetin) were detected by LC-ESI-MS/MS analysis in methanolic extracts of 10 mM H2O2 and control treatments.

Results: A predominant phenolic compound was chlorogenic acid followed by rutin hydrate, cafffeic acid, luteoline, isorhamnetin, quercetin, myricetin, apigenin, p-coumaric acid and kaempferol. No gallic acid, genistein, procyanidin-C1, vanillic acid, sinapic acid, hesperedin and naringenin were detected in H2O2 treatment and control groups of B. perennis. The total phenolic contents estimated were in the order of H2O2 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 H2O2 treatment callus materials. The H2O2 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 H2O2 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 (O2·-) and hydrogen peroxide (H2O2), 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 H2O2 and molecular oxygen, is one of the most valuable antioxidative enzyme. The H2O2 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. Whessoever 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 H2O2 is one of the most important molecules which are caused to increase in plants by biotic and abiotic stress conditions. Higher levels of H2O2 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 H2O2: as a stress signal molecule in plants (Hung et al., 2005) and H2O2 signaling functions significantly in plant development and defense system against environmental stresses (He et al., 2009; Cingoz et al., 2014). The H2O2 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 H2O2 treatment increased cardiotonic glycoside production in Digitalis species. Until now, there is no too much information on the use of H2O2 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 (Cakicioglu 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 (Karakaş and Turker, 2013), triterpenoid saponins (Yoshikawa et al., 2008; Karakaş et al., 2014), and essential oils (Kavalcıoglu 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 (Karakaş et al., 2011), antitumor (Karakaş et al., 2014), antimicrobial (Kavalcıoglu et al., 2010), antihiperlipidemic (Morikawa et al., 2010), antioxidant (Siatka and Kasparova, 2010) and cytotoxic activities against some human cancer cell lines (Li et al., 2005; Karakaş et al., 2015).
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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. perennis 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. perennis callus cultures affected abiotic stress factor such as oxidative stress by using H₂O₂ 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 x 15 petri) of both treatments that have same sizes were put into the MS medium supplement with zero (control) and 10 mM H₂O₂ 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-Ciocalteau assay (Slinkard and Singleton, 1977), with some modifications. Therefore, 20 μL of each calibration solution or sample or blank, 100 μL Folin-Ciocalteau 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% Na₂CO₃ solution was added in the test tube. The solutions were incubated at 20 ± 2 °C 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 (AlCl₃) 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 (NaNO₃) was added to the test tubes. After 5 min, 150 μL of 10% AlCl₃ 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 °C for 10 min. Then, the callus extracts of B. perennis were kept at -80 °C 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 albumin (R² = 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 cm³ mmol⁻¹ H₂O₂) spectrophotometrically and the CAT activity was showed as mmol H₂O₂ 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
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$_2$O$_2$ for 10 hours to investigate the effect of H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ treatment) and untreated (control) callus cultures of $B. perennis$. Values are means ± SD of three measurements. Nd; not detected.
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$_2$O$_2$ 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$_2$O$_2$ 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 (0.08 µg/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 of dw for H$_2$O$_2$ 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$_2$O$_2$ treatment group (Table 1). While 8 phenolic compounds were detected in untreated callus (control) group, 12 of 20 phenolic compounds were detected in H$_2$O$_2$ treated callus group (Table 1). Isorhamnetin, quercetin, myricetin and kaempferol were detected only in H$_2$O$_2$ 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$_2$O$_2$/min/mg protein for control group to 42.57 mmol H$_2$O$_2$/min/mg protein for H$_2$O$_2$ 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$_2$O$_2$ treated group) (Figure 2). Soluble protein concentration was affected by H$_2$O$_2$ treatment in callus cultures (Figure 1 and 2).

**Figure 1:** CAT activity of oxidative stress treated (H$_2$O$_2$ 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).
Antioxidant activity of methanol extracts of oxidative stress treated and untreated (control) calli was assessed. Treatment of callus samples with 10 mM H$_2$O$_2$ for 6 hours drastically increased proline, total phenolic and flavonoid contents. The exposure of H$_2$O$_2$ treatment noticeably increased the proline content from 0.086 mmol/g dw (control) to 0.139 mmol/g dw (H$_2$O$_2$ treatment) (Figure 3).

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 dw, respectively. The results presented in Figure 4 indicated that methanolic extract of H$_2$O$_2$ 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$_2$O$_2$ 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).
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 Turkler, 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 H2O2 was capable of increasing secondary metabolites, such as capsidiol production in pepper fruits (Capsicum annuum L.) (Arececi 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 H2O2 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 H2O2 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 H2O2. Similar result was also found in our study that CAT (42.57 mmol H2O2/min/mg protein) and SOD activity (0.72 U/mg protein) significantly increased in H2O2 treated callus cultures of B. perennis. This study recommended that H2O2 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 H2O2 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 H2O2 pretreatment significantly increased free proline content in T. aestivum seedlings. Ozden et al. (2009) also showed that H2O2 treatment enhanced endogenous proline production in grapevine leaves. Similarly, our study apparently demonstrated that exogenous application of H2O2 triggered a rapid production of proline in B. perennis callus cultures. Cingoz et al. (2014) showed that treatment of H2O2 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 1 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 H2O2 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...
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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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References

1. Araceli, A.C., Eldaia, C.M., Edmundob, L.G. and Ernesto, G.P. (2007). Capsidiol production in pepper fruits (Capsicum annuum L.) induced by arachidonic acid independent of an oxidative burst. Physiol. Mol. Plant Path. 70: 69-76.
2. Bates, L.S. (1973). Rapid determination of free proline for water stress studies. PlantSoil 39: 205-207.
3. Cakılcıoglu, U., Sengun, M.T. and Turkoglu, I. (2010). An ethnobotanical survey of medicinal plants of Yazykonak and Yurbat districts of Elazığ province, Turkey. J. Med. Plants. Res. 4: 567-572.
4. Chang, S.T. and Cheng, S.S. (2002). Antitermiate activity of leaf essential oils and their constituents from Cinnamomum osmophloeum. J. Agric. Food Chem. 50: 1389-1392.
5. Cingoz, G.S., Verma, S.K. and Gurel, E. (2014). Hydrogen peroxide-induced antioxidant activities and cardiotonicglycoside accumulation in callus cultures of endemic Digitalis species. Plant Physiol. Biochem. 82: 89-94.
6. Gudej, J. and Nazaruk, J. (2001). Flavonol glycosides from the flowers of Bellis perennis. Fitoterapia 72: 839-840.
7. He, L., Gao, Z. and Li, R. (2009). Pretreatment of seed with H2O2 enhances drought tolerance of wheat (Triticum aestivum L.) seedlings. Afr. J. Biotecnol. 8: 6151-6157.
8. Huerta-Heredia, A.A., Marron-Lopez, R., Ponce-Noyola, T., Cerda-Garcia-Rojas, C.M., Trejo-Tapia, G. and Ramos-Valdivia, A.C. (2009). Oxidative stress induces alkaloid production in Uncaria tomentosa root and cell cultures in bioreactors. Eng. Life Sci. 3: 211-218.
9. Hung, S.H., Yu, C.W. and Lin, C.H. (2005). Hydrogen peroxide functions as a stress signal in plants. Bot. Bull. Acad. Sin. 46: 1-10.
10. Karakas, F.P., Şöhretoğlu, D., Liptaj, T., Stujber, M., Turker, A.U., Marak, J., Çalış, I. and Yalçın, F.N. (2014). Isolation of an efficient 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.
11. Karakas, F.P., Karakas, A., Coşkun, H. and Turker, A.U. (2011). Effects of common daisy (Bellis perennis L.) aqueous extracts on anxiety-like behaviour and spatial memory performance in wistar albino rats. Afr. J. Pharm. Pharmac. 5: 1378-1388.
12. Koval, T., Cingoz, G., Verma, S.K. and Gurel, E. (2014). Isolation of an oleanane-type saponin active from Bellis perennis through antitumor bioassay-guided procedures. Pharm. Biol. 52: 951-955.
13. Koval, T. and Turker, A.U. (2013). An efficient in vitro regeneration system for Bellis perennis L. and comparison of phenolic contents of field-grown and in vitro-grown leaves by LC-MS/MS. Ind. Crop. Prod. 48: 162-170.
14. Koval, T., Karakas, A., Boran, Ç., Turker, A.U., Yalcın, F.N. and Bilensoy, E. (2012). The evaluation of terminal administrations of Bellis perennis fractions on circular excision wound healing in Wistar albino rats. Pharm. Biol. 50 (8): 1031-1037.
15. Koval, T., Karakas, A., Coşkun, H. and Turker, A.U. (2011). Effects of common daisy (Bellis perennis L.) aqueous extracts on anxiety-like behaviour and spatial memory performance in wistar albino rats. Afr. J. Pharm. Pharmac. 5: 1378-1388.
16. Koval, T., Yildirim, A.B., Bayram, R., Yavuz, M.Z., Gepdiremen, A. and Turker, A.U. (2015). Antiproliferative activity of some medicinal plants on human breast and hepatocellular carcinoma cell lines and their phenolic contents. Trop. J. Pharm. Res. 14: 1787-1795.
17. Koval, T., Cingoz, G., Verma, S.K. and Gurel, E. (2014). Isolation of an oleanane-type saponin active from Bellis perennis through antitumor bioassay-guided procedures. Pharm. Biol. 52: 951-955.
18. Koval, T. and Turker, A.U. (2013). An efficient in vitro regeneration system for Bellis perennis L. and comparison of phenolic contents of field-grown and in vitro-grown leaves by LC-MS/MS. Ind. Crop. Prod. 48: 162-170.
19. Koval, T., Karakas, A., Boran, Ç., Turker, A.U., Yalcın, F.N. and Bilensoy, E. (2012). The evaluation of terminal administrations of Bellis perennis fractions on circular excision wound healing in Wistar albino rats. Pharm. Biol. 50 (8): 1031-1037.
20. Koval, T., Karakas, A., Coşkun, H. and Turker, A.U. (2011). Effects of common daisy (Bellis perennis L.) aqueous extracts on anxiety-like behaviour and spatial memory performance in wistar albino rats. Afr. J. Pharm. Pharmac. 5: 1378-1388.
21. Koval, T., Yildirim, A.B., Bayram, R., Yavuz, M.Z., Gepdiremen, A. and Turker, A.U. (2015). Antiproliferative activity of some medicinal plants on human breast and hepatocellular carcinoma cell lines and their phenolic contents. Trop. J. Pharm. Res. 14: 1787-1795.
22. Li, J.T., Qiu, Z.B., Zhang, X.W. and Wang, L.S. (2011). Exogenous hydrogen peroxide can enhance tolerance of wheat seedlings to salt stress. Acta. Physiol. Plant. 33: 835-842.
23. Li, W., Asada, Y., Koike, K., Nikaido, T., Furuya, T. and Yoshikawa, T. (2005). Bellisosides A–F, six novel acylated triterpenoid saponins from Bellis perennis (compositae). Tetrahedron, 61: 2921-2929.
24. Lowny, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with Folin-phenol reagent. J. Biol. Chem. 193: 265-295.
25. Morikawa, T., Miura, N., Nakamura, S., Ninomiya, K., Matsuda, H., Hamao, H., Muraoka, O., Hayakawa, T. and Yoshikawa, M. (2011). Medicinal Flowers. XXXII. Structures of Oleane-type Triterpene Saponins, Perennisosides VIII, IX, X, XI, and XII, from the Flowers of Bellis perennis. Chem. Pharm. Bull. 59: 889-895.
26. Morikawa, T., Muraoka, O. and Yoshikawa, M. (2010). Pharmaceutical food science: Search for anti-obese constituents from medicinal foods-anti-hyperlipidemic saponin constituents from the flowers of Bellis perennis. Yakugaku Zasshi 673-678.
27. Nazaruk, J. and Gudej, J. (2001). Qualitative and quantitative chromatographic investigation of flavonoids in Bellis perennis L. Acta Pol. Pharm. 58. 401-404.
28. Ozden, M., Demirel, U. and Kahraman, A. (2009). Effects of proline on antioxidant system in leaves of grapevine (Vitis vinifera L.) exposed to oxidative stress by H2O2. Sci Hortic. 119: 163-168.
29. Panda, H. (2004). Handbook on Medicinal Herbs with Uses. Asia Pacific Business Pres. India, pp. 188-189.
27. Ramakrishna, A. and Ravishankar, G.A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. Plant Signal. Behav. 6: 1720-1731.
28. Siatka, T. and Kasparova, M. (2010). Seasonal variation in total phenolic and flavonoid contents and DPPH Scavenging activity of Bellis perennis L. flowers. Molecules 15: 9450-9461.
29. Slinkard, K. and Singleton, V.L. (1977). Total phenol analysis: automation and comparison with manual methods. Am. J. Enol. Vitic. 28(1): 49.
30. Sun, Y., Oberley, L.W. and Li, Y. (1988). A simple method for clinical assay of superoxide dismutase. Clin. Chem. 34: 497-500.
31. Uzun, E., Sariyar, G., Adsersen, A., Karakoc, B., Otükg, G., Oktayoglu, E. and Pirildar, S. (2004). Traditional medicine in Sakarya province (Turkey) and antimicrobial activities of selected species. J. Ethnopharmacol. 95: 287-96.
32. Yang, S.L., Lan, S.S. and Gong, M. (2009). Hydrogen peroxide-induced proline and metabolic pathway of its accumulation in maize seedlings. J. Plant Physiol. 166: 1694-1699.
33. Yoshikawa, M., Li, X., Nishida, E., Nakamura, S., Matsuda, H., Muraoka, O. and Morikawa, T. (2008). Medicinal flowers.XXI. Structures of perennisaponins A, B, C, D, E, and F, acylated oleanane-type triterpene oligoglycosides, from the flowers of Bellis perennis. Chem. Pharm. Bull. 56: 559-568.