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ASSESSMENT OF BIOMARKERS FOR ANTIOXIDANT DEFENSE IN THE EQUINE ERYTHROCYTES AFTER INCUBATION WITH *BEGONIA PSILOPHYLLA* IRMSCH. LEAF EXTRACT

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In this study, the investigation of the effect of *Begonia psilophylla* Irmsch. leaf extract on the antioxidant defenses [catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) activity, ceruloplasmin level, and total antioxidant capacity (TAC)] in the equine erythrocyte suspension and plasma was undertaken. In relation to blood cells, circulating erythrocytes are regularly exposed to stress conditions and are especially vulnerable as they have no membrane repair mechanism or regenerative capacity. Freshly sampled *B. psilophylla* leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in ratio 1 : 19, w/w) at room temperature. The extract was then filtered and used for analysis. A volume of 0.1 ml of the plant extract was added to 1.9 ml of clean equine erythrocytes or 1.9 ml of plasma. For positive control, the 0.1 M phosphate buffer was used. After incubation, the mixture at 37 °C for 60 min it was centrifuged at 3,000 rpm for 5 min with continuous stirring. Erythrocytes and plasma aliquots were used in the study. The presence of the extract during incubation of erythrocyte suspension and plasma caused a non-considerable increase of catalase and glutathione peroxidase activity, while the activity of glutathione reductase was not changed compared to control samples. At the same time, *B. psilophylla* extract caused a statistically significant decrease in ceruloplasmin level by 47.6% (p <0.05). The total antioxidant capacity in the equine erythrocytes' suspension and plasma after *in vitro* incubation with *B. psilophylla* leaf extract was non-significantly changed. Based on the collected data, positive trends were observed in the regressions of GPx activity against catalase activity (r = 0.809, p = 0.0005), and ceruloplasmin level (r = 0.553, p = 0.017) for in the equine erythrocytes' suspension after *in vitro* incubation with *B. psilophylla* leaf extract. Therefore, our study suggests that crude extract obtained from *B. psilophylla* leaves has an effective antioxidant and anti-inflammatory effect after the treatment of equine erythrocytes. It could be interpreted that *B. psilophylla* extract showed the anti-inflammation effect expressed as decreasing the ceruloplasmin level in the plasma. The pronounced effect of *B. psilophylla* leaf extract, probably, could be attributed to its secondary metabolites content. However, the components responsible for the antioxidative activity of *B. psilophylla* extract is currently unclear. Therefore, further investigations need

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to be carried out to isolate and identify the phytochemical constituents and antioxidant compounds present in the plant extract.

Keywords: *Begonia psilophylla* extract, equine erythrocytes, total antioxidant capacity, superoxide dismutase, catalase, glutathione peroxidase, ceruloplasmin

Introduction

The health benefits of plants and plant food-based diets could be related to both integrated antioxidant and anti-inflammatory mechanisms exerted by a wide array of phytochemicals present in fruit, vegetables, herbs, and spices. Therefore, there is increasing interest in identifying foods, food extracts and phytochemical formulations from plant sources that are able to efficiently modulate oxidative and inflammatory stress to prevent diet-related diseases (Serafini and Peluso, 2016). Antioxidants from plants are a large group of bioactive compounds (i.e., flavonoids, phenolic compounds, sulfur-containing compounds, tannins, alkaloids, phenolic diterpenes, and vitamins) demonstrating different antioxidant activities. For example, flavonoids have the ability to scavenge free radicals and can form complexes with catalytic metal ions rendering them inactive. Studies have shown that spices and herbs such as rosemary, sage, and oregano are excellent sources of antioxidants with their high content of phenolic compounds (Yashin et al., 2017).

Antioxidants help prevent cellular damage caused by reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and the superoxide anion radical ($O_{2.}–$) (Halliwell and Gutteridge, 1989). Antioxidants can be enzymes or molecules such as vitamins E and C, urea, glutathione, etc. Antioxidant enzymes include superoxide dismutase (SOD), which catalyzes the dismutation of $O_{2.}–$ to water and oxygen, catalase (CAT), which reduces H_2O_2 to water and oxygen, and glutathione reductase (GR), which regenerates reduced glutathione (GSSG) used as a direct scavenger of ROS or as a substrate for the antioxidant enzyme glutathione peroxidase (GPx) (Halliwell and Gutteridge, 1989).

A diverse group of antioxidants such as polyphenols, ascorbic acid, vitamin A, α-lipoic acid, thioredoxin, glutathione, melatonin, coenzyme Q, beta carotenoids, alpha-tocopherols as well as antioxidant enzymes has been widely investigated for the prevention and treatment of diseases resulting from oxidative damage (Ighodaro and Akinloye, 2017).

Begonia is a mega-diverse genus containing more than 1800 species, with a very high proportion of microendemics and hotspots of diversity in the Andes and Southeast Asia (Hughes et al., 2018). The first living plant in *Begonia* was introduced to Europe during the eighteenth century, and thereafter over 400 natural species have been introduced for horticulture and many cultivars have been developed (Tebbitt, 2005). Begonias are among the most popular ornamental plants in the world thanks to their large, showy, and long-lasting multicolor flowers (Sakhanokho et al., 2013; Twyford et al., 2014). They are used as garden plants and potted plants, in hanging baskets, and as greenhouse flowers, as well as potherbs or leafy vegetables in many parts of the world. The roots and tubers of some species have been reported to possess antimicrobial activities and are used to treat various ailments (Sakhanokho et al., 2013).

In our previous study, we have assessed the percentage of equine erythrocyte hemolysis induced by treatment with extracts of various species of *Begonia* genus to exemplify their further potential development and use as a drug against metabolic diseases in medicine and veterinary (Tkachenko et al., 2017). Our study demonstrated that among 30 species of *Begonia* genus, the most species screened possessed anti-hemolytic activity. The results of these biological assays demonstrated that compounds present in *B. glabra*, *B. aconitifolia*, *B. sanguinea*, *B. thiemei*, *B. masoniana*, B. × *credneri*, *B. oxyphylla*, *B. subvillosa*, *B. ulmifolia*, *B. conconvulaceae* can prevent the formation of methemoglobin and reduce hemolysis, while *B. erythrophylla*, *B. psilophylla*, and *B. arborescens* var. oxyphylla extracts can facilitate the formation of methemoglobin and hemolysis in healthy equine blood. Extracts from leaves of *B. foliosa*, *B. rex*, *B. solimutata*, *B. mexicana*, *B. goegoensis*, *B. imperialis* var. *smaragdina*, *B. pustulata*, *B. peltata*, *B. cucullata*, *B. angularis*, *B. boisiana*, *B. venosa* exhibited the decrease of percentage hemolysis of equine erythrocytes, but these alterations were non-significant (Tkachenko et al., 2017).

Moreover, we also assessed the antioxidant effect of leaf extract obtained from *Begonia rex* Putz. on oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), carbonyl derivatives content of protein oxidative modification] and antioxidant defenses [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activity, ceruloplasmin level, and total antioxidant capacity (TAC)] using the equine erythrocytes model. The extract during incubation of erythrocyte suspension caused a non-considerable TBARS formation (by 18%, $p > 0.05$), while the content of aldehydic and ketonic derivatives of oxidatively modified proteins was decreased (by 7 and 8%, $p > 0.05$, respectively) compared to control. The aqueous leaf extract of *B. rex* has proven to be the most effective to increase the catalase and GPx activity. The increase of the catalase and GPx activity was induced by TAC enhancement by 34% ($p > 0.05$). The SOD activity was non-significantly decreased by 17% ($p > 0.05$). *B. rex* extract caused a statistically significant decrease in ceruloplasmin level by 64% ($p < 0.05$). These *in vitro* assays indicate that *B. rex* leaf extract screened is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. However, the components responsible for the antioxidative activity of *B. rex* extract is currently unclear (Buyun et al., 2018). *In vitro* microbiological investigation of ethanolic extracts obtained from leaves of various *Begonia* species against bacteria and fungi strain was revealed the antibacterial properties of these plants (Tkachenko et al., 2016, 2017a, b).

In this study, we have focused on the antioxidant effect of leaf extract obtained from *Begonia psilophylla* on antioxidant defenses biomarkers [catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) activity, ceruloplasmin level, and total antioxidant capacity] using the equine plasma and erythrocytes model. Thus, equine erythrocytes were proved to be a good tool for analyzing the oxidative stress biomarkers as a mechanism of antioxidant action of *B. psilophylla* leaf extract.

Material and methodology

Collection of Plant Materials

The leaves of *Begonia psilophylla*, cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine. The biochemical screening of *Begonia* leaf extracts has been carried out in the laboratory of the Institute of Biology and Earth Sciences, Pomeranian University in Slupsk (Poland). Our current scientific project has been undertaken in the frame of the cooperation program between the Institute of Biology and Earth Sciences (Pomeranian University in Slupsk, Poland) and M.M. Gryshko National Botanic Gardens of National Academy of Sciences of Ukraine, aimed at assessment of medicinal properties of tropical plants. It has encompassed some tropical mega-diverse genera, including genus *Begonia* with a near pantropical distribution.

Preparation of Plant Extracts

Freshly collected leaves *B. psilophylla* were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in ratio 1 : 19, w/w) at room temperature. The extracts were then filtered and used for analysis. All extracts were stored at -20 °C until use.

Horses

Eighteen healthy adult horses from the central Pomeranian region in Poland (village Strzelinko, N 54º 30’ 48.0” E 16º 57’ 44.9”), aged 8.9 ±1.3 years old, including 6 Hucul pony, 5 Thoroughbred horses, 2 Anglo-Arabian horses and 5 horses of unknown breed, were used in this study. All horses participated in recreational horseback riding. Horses were housed in individual boxes, with feeding (hay and oat) provided twice a day, at 08.00 and 18.00 h, and water available *ad libitum*. All horses were thoroughly examined clinically and screened for hematological, biochemical and vital parameters that were in the reference ranges. The females were non-pregnant.

Collection of blood samples

Blood was drawn from the jugular vein of the animals in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM). Blood was stored in tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3,000 rpm for 5 min to remove plasma. The pellet of blood was resuspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 ml of the plant extract was added to 1.9 ml of clean equine erythrocytes or 1.9 ml of plasma. For positive control (phosphate buffer) was used. After incubation of the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3,000 rpm for 5 min. Erythrocytes and plasma aliquots were used in the study.

Assay of Superoxide dismutase activity

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was assessed by its ability to dismutate superoxide produced during quercetin auto-oxidation in an alkaline medium (pH 10.0) by Kostiuk and co-workers (1990) method. Activity is expressed in units of SOD per mL of blood.

Assay of Catalase activity

Catalase (CAT, E.C. 1.11.1.6) activity was determined by measuring the decrease of H_2O_2 in the reaction mixture using a spectrophotometer at the wavelength of 410 nm by the method of Koroliuk et al. (1988). One unit of catalase activity is defined as the amount of enzyme required for decomposition of 1 mmol H_2O_2 per min per L of blood.

Assay of Glutathione peroxidase activity

Glutathione peroxidase (GPx, EC 1.11.1.9) activity was determined by detecting the non-enzymatic utilization of GSH (the reacting substrate) at an absorbance of 412 nm after incubation with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) according to by the method of Moin (1986). The assay mixture contained 0.8 mL of 0.1 M Tris-HCl buffer with 6 mM EDTA and 12 mM sodium azide (pH 8.9), 0.1 mL of 4.8 mM GSH, 0.2 mL of hemolyzed erythrocytes (1 : 20), 1 mL of 20 mM t-butyl hydroperoxide, and 0.1 mL of 0.01 M 5.5-dithiobis-2-nitrobenzoic acid. The rate of GSH reduction was followed spectrophotometrically at 412 nm. GPx activity is expressed as μmol GSH per min per mL of blood.

Assay of the Ceruloplasmin level

The ceruloplasmin (CP, EC 1.16.3.1) level in the plasma was measured spectrophotometrically at 540 nm, as described by Ravin (1961). The assay mixture contained 0.1 mL of plasma, 0.4 M sodium acetate buffer (pH 5.5), and 0.5% *p*-phenylenediamine. The mixture was incubated at 37 °C for 60 min. Before cooling at 4 °C for 30 min, the mixture was added to 3% sodium fluoride for inhibition. Ceruloplasmin was expressed in mg per L of plasma.

Measurement of Total antioxidant capacity (TAC)

The TAC level in the plasma and erythrocytes' suspension was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al., 1998). Sample inhibits the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. The level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

Statistical analysis

The mean ± S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p >0.05). The significance of differences between the parameters (significance level, p <0.05) was examined using the Mann-Whitney *U*-test (Zar, 1999). In addition, the relationships between antioxidant defense biomarkers were evaluated using Spearman's correlation analysis. All statistical calculation was performed on separate data from each individual with Statistica 8.0 software (StatSoft, Krakow, Poland).

Results and discussion

Figure 1A summarizes the results obtained by incubating equine erythrocyte suspension in the presence of the aqueous extract of *B. psilophylla*. As seen, the presence of the extract during incubation of erythrocyte suspension and plasma caused a non-considerable increase of catalase and glutathione peroxidase activity, while the activity of glutathione reductase was non-changed compared to control samples.

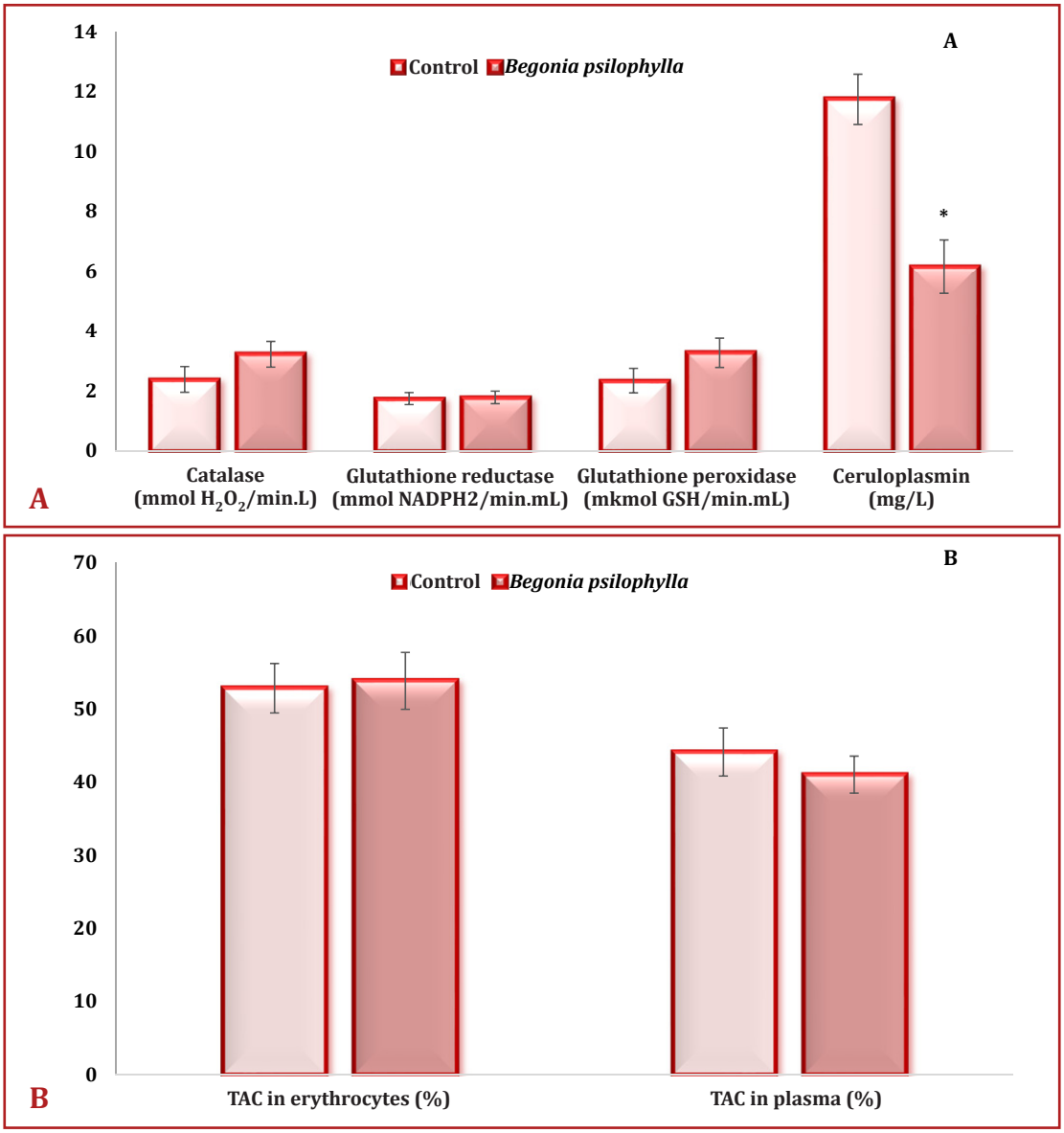

Figure 1 The catalase, glutathione reductase, glutathione peroxidase, and ceruloplasmin activity (A), as well as total antioxidant capacity (TAC) in the equine erythrocytes' suspension and plasma (B) after *in vitro* incubation with *Begonia psilophylla* leaf extract (M ±m, *n* = 18)

In our study, the aqueous leaf extract of *B. psilophylla* has proven to be the most effective to increase the catalase and GPx activity (by 35.3%, p >0.05 and 39.7%, p >0.05) (Figure 1A). Else, *B. psilophylla* extract caused a statistically significant decrease in ceruloplasmin level by 47.6% (p <0.05) (Figure 1A).

Ceruloplasmin is a serum ferroxidase that contains greater than 95% of the copper found in plasma. This protein is a member of the multicopper oxidase family, an evolutionarily conserved group of proteins that utilize copper to couple substrate oxidation with the four-electron reduction of oxygen to water (Hellman and Gitlin, 2002). It has been proposed to function in copper transport, oxidation of organic amines, Fe^{2+}–oxidation and the regulation of cellular iron levels, and regulation of catechols metabolism, radical scavenging and other antioxidant processes (Healy and Tipton, 2007). Therefore, we suggested that *B. psilophylla* extract could exhibit the anti-inflammatory effect decreasing ceruloplasmin level after *in vitro* incubation with leaf extract.

The total antioxidant capacity (TAC) in the equine erythrocytes' suspension and plasma after *in vitro* incubation with *B. psilophylla* leaf extract was non-significantly changed (increased by 1.9% in the erythrocytes and decreased by 7% in the plasma) (Figure 1B).

Based on the collected data, positive trends were observed in the regressions of GPx activity against catalase activity (r = 0.809, p = 0.0005), and ceruloplasmin level (r = 0.553, p = 0.017) for in the equine erythrocyte suspension after *in vitro* incubation with *B. psilophylla* leaf extract (Figure 2).

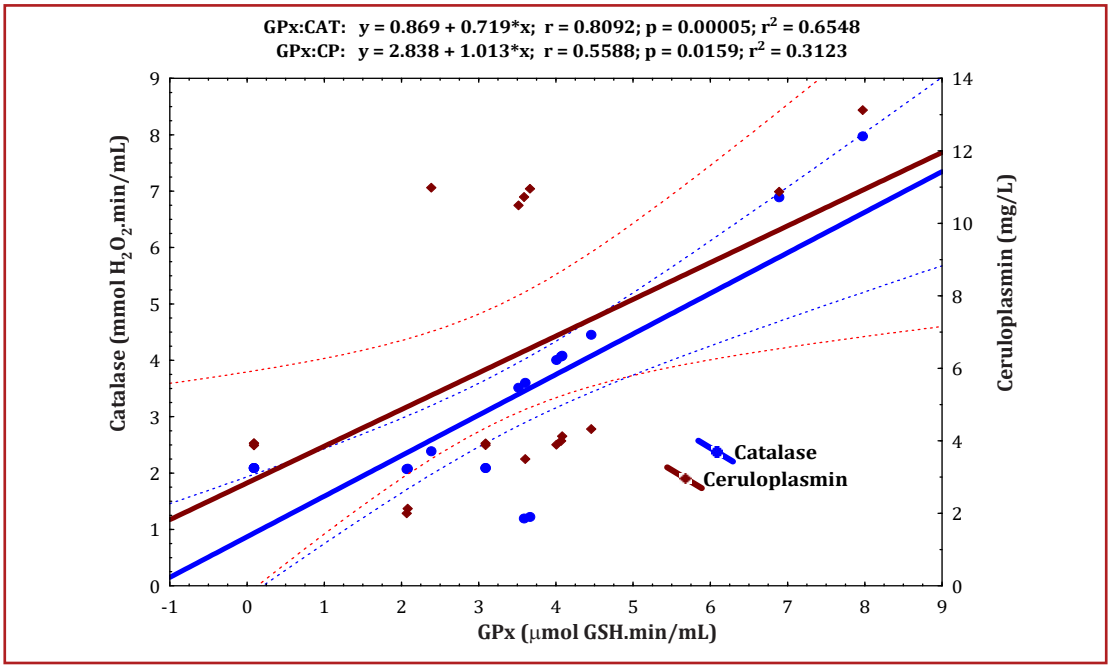

Figure 2 Correlations between oxidative stress biomarkers GPx activity, catalase activity and ceruloplasmin level in the equine erythrocyte suspension after *in vitro* incubation with *Begonia psilophylla* leaf extract

Our study demonstrates changes in the antioxidant defenses biomarkers of equine erythrocytes incubated with *B. psilophylla* leaf extract (Figures 1 and 2). Accordingly, our study suggests that crude extract obtained from *B. psilophylla* leaves has an effective antioxidant and anti-inflammatory effect after the treatment of equine erythrocytes. *B. psilophylla* extract showed the anti-inflammation effect expressed as decreasing on the ceruloplasmin level in the plasma. The pronounced effect of leaf *B. psilophylla* extract could be attributed to its secondary metabolites, e.g. polyphenols, flavonoids, anthocyanin contents.

It has become increasingly clear that all secondary metabolite components in *Begonia* species displayed antioxidant and antimicrobial properties through different biological mechanisms (Hossain and Nagooru, 2011). It is reasonable to suggest, that variation in the chemical profile of plants could influence their biological activities. Therefore, it was important to know the chemical composition of extracts to correlate with their antioxidant activities.

Aswathy et al. (2016) have demonstrated that *Begonia rex-cultorum* (Baby rainbow) and *Begonia malabarica* exhibited the highest antioxidant activities. The anthocyanin concentration positively correlates with the antioxidant activities among the cultivars. The diversity in radical scavenging in these assays may be due to factors like stereoselectivity of the radicals or due to the differential solubility of anthocyanin molecules in the crude extract (Aswathy et al., 2016).

A study conducted by Kalpanadevi and Mohan (2012) has shown that the extracts of *B. malabarica* and *B. floccifera* contain higher quantities of phenolic compounds, which exhibit antioxidant and free radical scavenging activity. The methanol extracts of whole plants of *B. malabarica* and *B. floccifera* showed potent *in vitro* antioxidant activities using various models, i.e. DPPH, hydroxyl, superoxide and ABTS radical scavenging activity. *B. malabarica* and *B. floccifera* whole plant extracts (methanol) exhibited potent *in vitro* antioxidant activity in DPPH radical scavenging, hydroxyl radical scavenging, superoxide radical scavenging, ABTS radical cation scavenging and reducing power in comparison to the known antioxidants, such as ascorbic acid and Trolox. It was observed that methanol extracts of the whole plant of *B. malabarica* had higher activity than that of the whole plant extract of *B. floccifera*. At a concentration of 1 mg/mL, the scavenging activity of methanol extract of the whole plant of *B. malabarica* reached 96.14% while at the same concentration, that of the *B. floccifera* was 63.51%. At a concentration of 1 mg/mL, the scavenging activity of methanol extract of the whole plant of *B. malabarica* exhibited higher activity than ascorbic acid. Superoxide radical scavenging activity of *B. malabarica* and *B. floccifera* whole plant extracts was studied and compared with ascorbic acid. It was observed, that the superoxide radical scavenging activity of *B. malabarica* and *B. floccifera* extracts increased with increasing concentration. At a concentration of 1 mg/mL, the superoxide radical scavenging activity of methanol extracts of *B. malabarica* and *B. floccifera* were found to be 81.55% and 62.56%, respectively. Among plant extracts screened, *B. malabarica* exhibited higher activity (79.11%) at a concentration of 1 mg/mL than Trolox. The reducing power of extracts increased with an increase in concentration. Nevertheless, the reducing power values of the methanol extracts of *B. malabarica* whole plant was slightly higher than that of ascorbic acid (Kalpanadevi and Mohan, 2012).

Many studies have suggested that plant secondary metabolites obtained from Begoniaceae representatives are responsible for their antioxidant activity. Literature data confirmed that extracts from various parts of the *Begonia* plants exhibited strong antioxidant properties, effectively deactivating the stable, synthetic DPPH radical. For example, Indrakumar and co-workers (2014) have evaluated the antimicrobial and *in vitro* antioxidant potential of extracts of *B. dipetala*. Antimicrobial activity, DPPH free radical scavenging activity, Superoxide anion scavenging activity, Nitric oxide scavenging activity, and Ferric reducing antioxidant power assay were carried out on different concentration of the extracts. The reducing power assay of the ethanolic extract showed a reduction at various concentrations similar to that of standard ascorbic acid. The *in vitro* antioxidant studies clearly indicate that the ethanolic extract of *B. dipetala* has significant antioxidant activity (Indrakumar et al., 2014).

Methanol and ethyl acetate extract of *B. trichocarpa* has shown a marked dose-dependent antioxidant activity in both 2.2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method and Nitric acid scavenging method in the study of Sindhu et al. (2016). Additionally, total phenol content of different extracts of *B. trichocarpa* was estimated. It was established that methanol leaf extracts contain 49.96% of phenol content and 23.71% anthocyanin content.

Consequently, it was assumed that the antioxidant activity of *B. trichocarpa* may be due to the high phenol content and the presence of anthocyanin (Sindhu et al., 2016).

Previous studies have reported both protective and destabilizing effects of various plant extracts on erythrocyte membrane stability (Awe et al., 2009; Chikezie et al., 2011). It was suggested that the presence of phytochemicals such as tannins, saponin was responsible for destabilizing effects of certain plant extracts on erythrocyte membrane. *Carica papaya* L. leaf extracts, on the contrary, exhibited a significant inhibition of hemolysis *in vitro* and could have a potential therapeutic effect on disease processes causing destabilization of biological membranes (Ranasinghe et al., 2011).

The results of this research indicated that crude extract obtained from *B. psilophylla* leaves was highly effective for the control of oxidative stress. Protective effect of *B. psilophylla* extracts was evident by amelioration in plasma antioxidant enzymes' activities as compared to control. The antioxidant defense system was improved through suppression on the ceruloplasmin level by treatment of equine plasma with *B. psilophylla* leaf extract. Finally, we can report that *B. psilophylla* crude extract is a good antioxidant agent for oxidative stress prevention.

Conclusions

The results of current study revealed that crude extract obtained from *B. psilophylla* leaves exhibited a marked antioxidant effect after the treatment of a suspension of equine erythrocytes and plasma. The findings suggest that protective effect of *B. psilophylla* extract is evident by amelioration in antioxidant enzymes' activities. The antioxidant defense system was determined by the increase of glutathione peroxidase and catalase activity, as first line defense antioxidant enzymes, after the treatment with *B. psilophylla* extract. Moreover, *B. psilophylla*

extract showed the anti-inflammation effect exhibited as a decreasing ceruloplasmin level in the plasma. The pronounced effect of *B. psilophylla* leaf extract, probably, could be attributed to its secondary metabolites content. However, the phytochemicals responsible for the antioxidative activity of *B. psilophylla* extract is currently unclear. Therefore, further investigations need to be carried out to isolate and identify the phytochemical content and antioxidant compounds present in the *B. psilophylla* leaf extract, which can effect the erythrocyte function.

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