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IN VITRO ASSESSMENT OF ANTIOXIDANT EFFECT OF *BEGONIA REX* PUTZ. LEAF EXTRACT ON OXIDATIVE STRESS BIOMARKERS IN THE EQUINE ERYTHROCYTES MODEL

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The main goal of our study was to assess 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 defences [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activity, ceruloplasmin level, and total antioxidant capacity] using the equine erythrocytes model. Freshly collected *B. rex* leaves were washed, weighted, crushed, and homogenized in 0.1M phosphate buffer (pH 7.4) (in ratio 1: 19, w/w) at room temperature. The extracts were 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 (phosphate buffer) was used. After incubation, the mixture at 37 °C for 60 min with continuous stirring, erythrocytes, and plasma aliquots was used in the study. 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 the most effective to increase the catalase and GPx activity (by 44%, p >0.05 and 62%, p <0.05). The increase of the catalase and GPx activity was induced by TAC enhancement by 34% (p >0.05). SOD activity was non-significantly decreased by 17% (p >0.05). *B. rex* extract caused the 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. Therefore, further investigations need to be carried out to isolate and identify the antioxidant compounds present in the plant extract.

Keywords: *Begonia rex*, equine erythrocytes, lipid peroxidation, oxidatively modified proteins, antioxidant defence

Introduction

Begonia L. 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

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(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). *Begonia*s are among the most popular ornamental plants in the world thanks to their large, showy, and long-lasting multicolor flowers, ranging from white to pink, red, and yellow (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 leaf 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 (Tkachenko et al., 2016; Buyun et al., 2017), we have assessed the anti-*Escherichia coli* activity of the ethanolic extracts from the leaves of *Begonia* species, i.e. *B. solimutata* L.B. Sm. & Wassh., *B. goegoensis* N.E.Br., *B. foliosa* Kunth, *Begonia* × *bunchii* L.H. Bailey (syn. *Begonia* × erythrophylla Hérincq), *B. thiemei* C.DC., *B. peltata* Otto & Dietr., *B. heracleifolia* Cham. & Schltdl., *B. dregei* Otto & Dietr., *B. mexicana* G. Karst. ex Fotsch. In our study, ethanolic extracts obtained from leaves of *Begonia* species had an average activity against *E. coli*. The inhibition zone diameter observed for *B. solimutata* was 14 mm, 11.5 mm for *B. goegoensis*, 13 mm for *B. foliosa*, 13.5 mm for *Begonia* × *bunchii*, 15 mm for *B. thiemei*, 19 mm for *B. peltata*, 12 mm for *B. heracleifolia*, 11.5 mm for *B. dregei*, and 16 mm for *B. mexicana*. The highest antimicrobial effect was recorded for *B. peltata*, *B. mexicana*, and *B. thiemei*. The most antimicrobial effective plant against *E. coli* was *B. peltata*, being highly active with the ethanolic extract (inhibition zone diameter 19 mm). The obtained results highlighted the interesting antimicrobial potency of various *Begonia* species and provided a scientific basis for the traditional use of these plants in the treatment of microbial infections (Tkachenko et al., 2016; Buyun et al., 2017). Moreover, the highly active antimicrobial effects of various *Begonia* species against *Candida albicans* and *Pseudomonas aeruginosa* isolates are worthy of highlighting (Buyun et al., 2016; Tkachenko et al., 2017). Furthermore, the antimicrobial activity showed by *Begonia* species screened is in agreement with previous findings on the antimicrobial effects produced by numerous *Begonia* species.

We also 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 of plants investigated 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. convolvulaceae* 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).

In erythrocytes, interactions between biomolecules and the components of plant extract take place. As a result, alterations in oxidative balance, as well as changes in cellular membrane properties, may appear. A disturbance in pro-oxidative-antioxidative balance (the increase in methemoglobin content and reactive oxygen species formation) leads to erythrocyte damage, including changes in cytoskeleton and cell's membrane such as formation and tearing off the bubbles. In the consequence of these processes, the erythrocytes are excessively eliminated from blood (Bors et al., 2012). Equine erythrocytes are more sensitive to oxidant-induced damage due to the use of inefficient mechanisms to correct and protect against oxidative damage, i.e. methemoglobin formation, alteration of aggregation, and reduction of cellular deformability (Baskurt and Meiselman, 1999). Therefore, the high susceptibility of equine erythrocytes to oxidant damage and the resulting hemorheologic alterations may have important consequences for tissue perfusion and cardiovascular adequacy in horses (Baskurt and Meiselman, 1999; Walter et al., 2014).

Oxidants typically damage erythrocytes by oxidizing the heme iron in hemoglobin, reactive sulfhydryls, or unsaturated lipids in the membranes. The oxidation of the heme iron in hemoglobin to the ferric (Fe^{3+}) state generates methemoglobin, which is incapable of transporting oxygen. Methemoglobin can be enzymatically reduced back to the functional ferrous (Fe^{2+}) state, primarily by nicotinamide adenine dinucleotide (NADH)-dependent methemoglobin reductase (Wright et al., 1999; Walter et al., 2014). Sulfhydryl groups in proteins and unsaturated lipids in erythrocyte membranes are especially susceptible to oxidation. Oxidative denaturation and the precipitation of the globin portion of hemoglobin into large aggregates result in the formation of Heinz bodies that can bind to and alter membranes. Membrane structure also is altered by the oxidation of sulfhydryl groups and by lipid peroxidation (Harvey, 1997). NADPH is produced by the initial enzyme reactions of the pentose phosphate pathway, and erythrocytes increase pentose phosphate–pathway metabolism in response to oxidants to provide the NADPH necessary for the regeneration of reduced glutathione (GSH). In healthy humans and animals, most glutathione in erythrocytes is maintained as GSH, with low concentrations of oxidizing glutathione (GSSG) being present (Harvey, 1997; Harvey et al., 2003). Erythrocytes from horses are slower than erythrocytes from other species studied in their ability to regenerate GSH after it has been oxidized *in vitro* (Agar et al., 1974; Harvey et al., 2003). Horse erythrocytes also appear less able to protect themselves against oxidative injury induced by incubation with high levels of ascorbate, which stimulates the GR reaction by the oxidation of GSH (Harvey and Kaneko, 1977; Harvey et al., 2003). Therefore, though many model systems are frequently used to study the biochemical alterations under the condition of oxidative stress including the tissues from various parts of the body, erythrocytes, as the most common type of blood cells, get superiority amongst them (Pandey and Rizvi, 2010). Red blood cell along with its membrane has always been an important medium for the study due to the important role it plays in varied physiological and metabolic processes (Karabulut et al., 2009).

In this study, we have focused on 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 defences [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activity,

ceruloplasmin level, and total antioxidant capacity (TAC)] using the equine 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. rex* leaf extract.

Material and methodology

Collection of plant material

The leaves of *Begonia rex* Putz., 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 Institute of Biology and Environmental Protection, Pomeranian University in Slupsk (Poland). Our current scientific project has been undertaken in the frame of cooperation programme between the Institute of Biology and Environmental Protection (Pomeranian University in Slupsk, Poland) and NBG, aimed at assessment of medicinal properties of tropical plants has encompassed some tropical mega-diverse genera, including genus *Begonia* with a near pantropical distribution.

Preparation of plant extracts

The *B. rex* has striking metallic patterns with variations of red and silver over a green background on a rippled surface (Zhang et al., 2009). Freshly collected leaves were washed, weighted, crushed, and homogenized in 0.1M 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 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 which 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 3000 rpm for 5 min to remove plasma. The pellet of blood was re-suspended 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 the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3000 rpm for 5 min. Erythrocytes and plasma aliquots were used in the study.

2-Thiobarbituric acid reactive substances (TBARS) assay

The level of lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reacting substances (TBARS) with the Kamyshnikov (2004) method for determining the malonic dialdehyde (MDA) concentration. This method is based on the reaction of the degradation of the lipid peroxidation product, MDA, with TBA under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. Briefly, 0.1 mL of sample (blood, plasma, and erythrocytes' suspension) was added to 2 mL of distilled water, 1 mL of 20% TCA and 1 mL of 0.8% TBA. The mixture was heated in a boiling water bath for 10 minutes. After cooling, the mixture was centrifuged at 3.000 g for 10 minutes. The μmol of MDA per l L was calculated using $1.56 \cdot 10^5$ mM^{-1} cm^{-1} as the extinction coefficient.

The carbonyl derivatives content of protein oxidative modification (OMP) assay

To evaluate the protective effects of the extract against free radical-induced protein damage in equine erythrocytes, a carbonyl derivatives content of protein oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the erythrocytes' suspension was performed. The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with 2,4-dinitrophenylhydrazine (DNFH) as described by Levine et al. (1990) and as modified by Dubinina et al. (1995). DNFH was used for determining carbonyl content in soluble and insoluble proteins. Briefly, 1 mL of 0.1M DNPH (dissolved in 2 M HCl) was added to 0.1 mL of the sample after denaturation of proteins by 20% trichloroacetic acid (TCA). After addition of the DNPH solution (or 2M HCl to the blanks), the tubes were incubated for a period of 1 h at 37 °C. The tubes were spun in a centrifuge for 20 min at 3.000 g. After centrifugation, the supernatant was decanted and 1 mL of ethanol-ethylacetate solution was added to each tube. Following the mechanical disruption of the pellet, the tubes were allowed to stand for 10 min and then spun again (20 min at 3.000 g). The supernatant was decanted and the pellet washed thrice with ethanol-ethylacetate. After the final wash, the protein was solubilized in 2.5 mL of 8M urea solution. To speed up the solubilization process, the samples were incubated in a 90 °C water bath for 10–15 min. The final solution was centrifuged to remove any insoluble material. The carbonyl content was calculated from the absorbance measurement at 370 nm and 430 nm, and an absorption coefficient 22.000 $M^{-1} \cdot cm^{-1}$. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehyde derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}).

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 et al. (1990) method. Briefly, 1.0 mL of C reagent was mixed with 0.1 mL of a blood sample (dilution in water 1 : 1000). C reagent was made *ex tempore* (a mixture of equal volumes of 0.1 M K, Na-phosphate buffer, pH 7.8 and 80 mM EDTA solution); pH of C reagent was adjusted to 10.0 by adding TEMED. Distilled water (0.1 mL) was added to blank vials instead of the blood sample. The total volume of all samples was brought up to 2.4 mL using distilled water. The reaction was initiated by adding 0.1 mL of quercetin (1.4 μM dissolved

in dimethyl sulfoxide). Absorbance at 406 nm was measured immediately and after 20 min addition of quercetin solution. 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). The reaction was initialized by adding 0.1 mL of plasma into the incubation medium (2 mL of 0.03% H_2O_2 solution) and to 1.0 mL of 4% ammonium molybdate dissolved in 12.5 mM H_2SO_4 solution (blank sample). The duration of the reaction was 10 min at room temperature. The reaction was terminated by rapid adding 1.0 mL of 4% ammonium molybdate dissolved in 12.5 mM H_2SO_4 solution to incubation medium and 1 mL of 125 mM H_2SO_4 to all samples. All samples were centrifuged at 3.000 g for 5 min. The absorbance of the obtained solution was measured at 410 nm and compared with that of the blank. 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 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 sample 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. Briefly, 0.1 mL of sample was added to 2 mL of 1% Tween 80 reagent, 0.2 mL of 1 mM $FeSO_4$, and 0.2 mL of 10 mM ascorbic acid. In the blank assay, 0.1 mL of distilled water was used instead of the sample. The mixture was heated in a water bath for 48 hrs at 37 °C. After cooling, 1 mL of 20% trichloroacetic acid was added. The mixture was centrifuged at 3.000 g for 10 min. After centrifugation, 2 mL of supernatant and 2 mL of 0.25% 2-thiobarbituric acid were mixed.

The mixture was heated in a water bath at 95 °C for 15 min. The absorbance of the obtained solution was measured at 532 nm. The absorbance of the blank was defined as 100%. 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 oxidative stress 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

In this study, the investigation of the effect of *B. rex* extract on the lipid peroxidation and oxidatively modified protein biomarkers, as well as antioxidant defences in the equine erythrocyte suspension 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 (Savignone and Palacios, 2017)

Figure 1A summarizes the results obtained by incubating equine erythrocyte suspension in the presence of the aqueous extract of *B. rex*. As seen, the presence of 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.

It is generally assumed, that membrane phospholipids of aerobic organisms are continually subjected to oxidant challenges from endogenous and exogenous sources, while peroxidized membranes and lipid peroxidation products represent constant threats to aerobic cells. The most widely used assay for lipid peroxidation is malondialdehyde (MDA) formation as a secondary lipid peroxidation product, with the thiobarbituric acid reactive substances test (Draper et al., 1993; Valavanidis et al., 2006). Protein oxidation reactions involve various propagating radicals and ROS and the results are oxidative modifications of amino acid side chains, reactive-oxygen-species-mediated peptide cleavage, reactions of peptides with lipids and carbohydrate oxidation products, and formation of carbonyl derivatives of proteins (Valavanidis et al., 2006). Of the various indices of protein oxidation, protein carbonyl formation is the best studied with increases in tissues and organs of organisms (Stadtman and Berlett, 1998).

Antioxidants help prevent cellular damage caused by reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and the superoxide anion radical ($O_2^{\cdot-}$) (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 $O_2^{\cdot-}$ 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). In our study, the aqueous leaf extract of *B. rex* has proven the most effective to increase the catalase and GPx activity (by 44%, p >0.05 and 62%, p <0.05) (Figure 1A). The increase of the catalase and GPx activity was induced by TAC enhancement by 34% (p >0.05) (Figure 1B). SOD activity was non-significantly decreased by 17% (p >0.05) (Figure 1B).

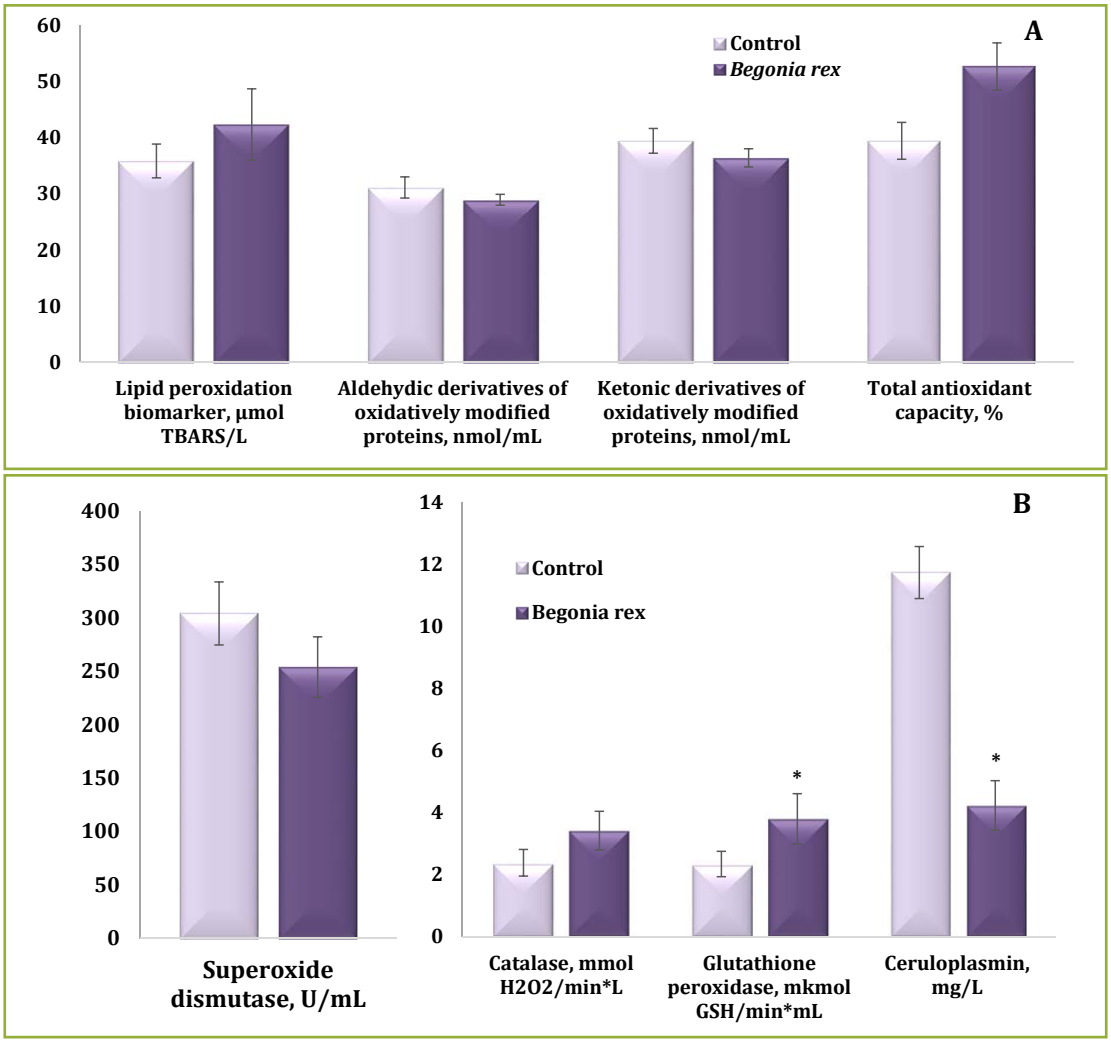


Figure 1 The TBARS content as biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity (A), superoxide dismutase, catalase, glutathione peroxidase, and ceruloplasmin activity (B) in the equine erythrocytes suspension after *in vitro* incubation with *Begonia rex* leaf extract (M ±m, n = 18)

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 ceruloplasmin 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). In our study, *B. rex* extract caused the statistically significant decrease in ceruloplasmin level by 64% (p <0.05) (Figure 1B).

Based on the collected data, positive trends were observed in the regressions of TBARS level against ketonic derivatives of OMP level (r = 0.477, p = 0.045), and SOD activity (r = 0.553, p = 0.017) for in the equine erythrocyte suspension after *in vitro* incubation with *B. rex* leaf extract (Figure 2A); in the case of SOD activity *vs.* ketonic derivatives of OMP level the regression was positive and significant (r = 0.540, p = 0.021) (Figure 2B). The SOD activity *vs.* TAC level regression was significant and reversible, which showed the relationship between increased TAC level and decreased SOD activity (r = -0.547, p = 0.019) (Figure 2B). The same results were obtained when regressions were performed on GPx activity (r = -0.489, p = 0.039) (Figure 2C), except in case the GPx activity *vs.* catalase activity regression was significant and positive (r = 0.802, p = 0.000) (Figure 2C).

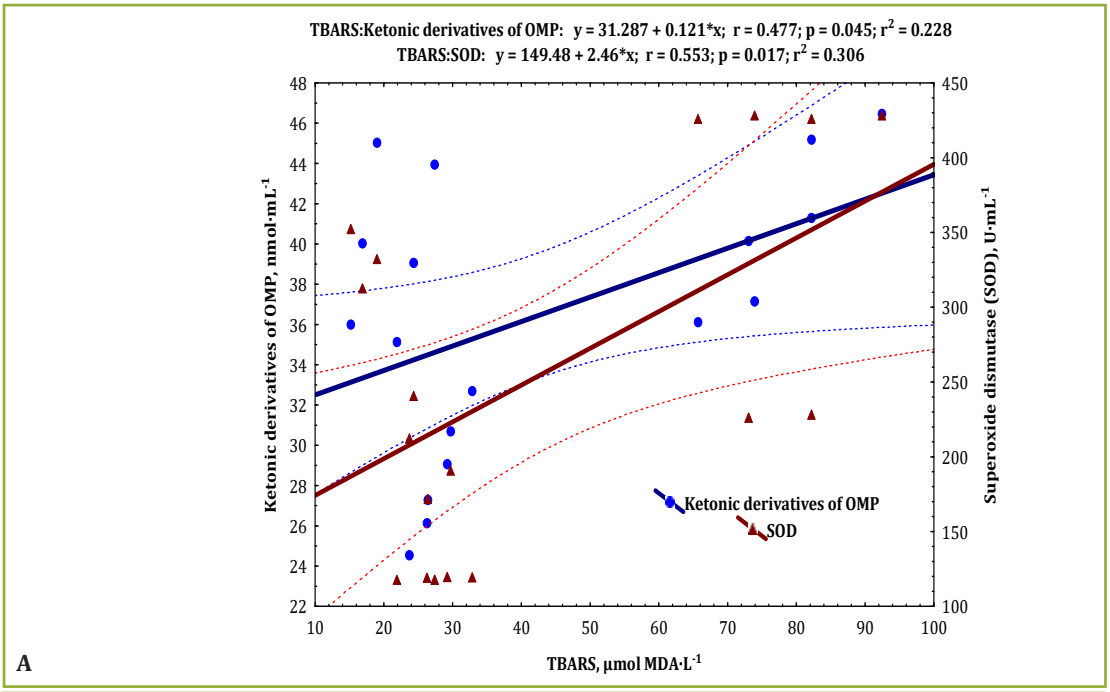
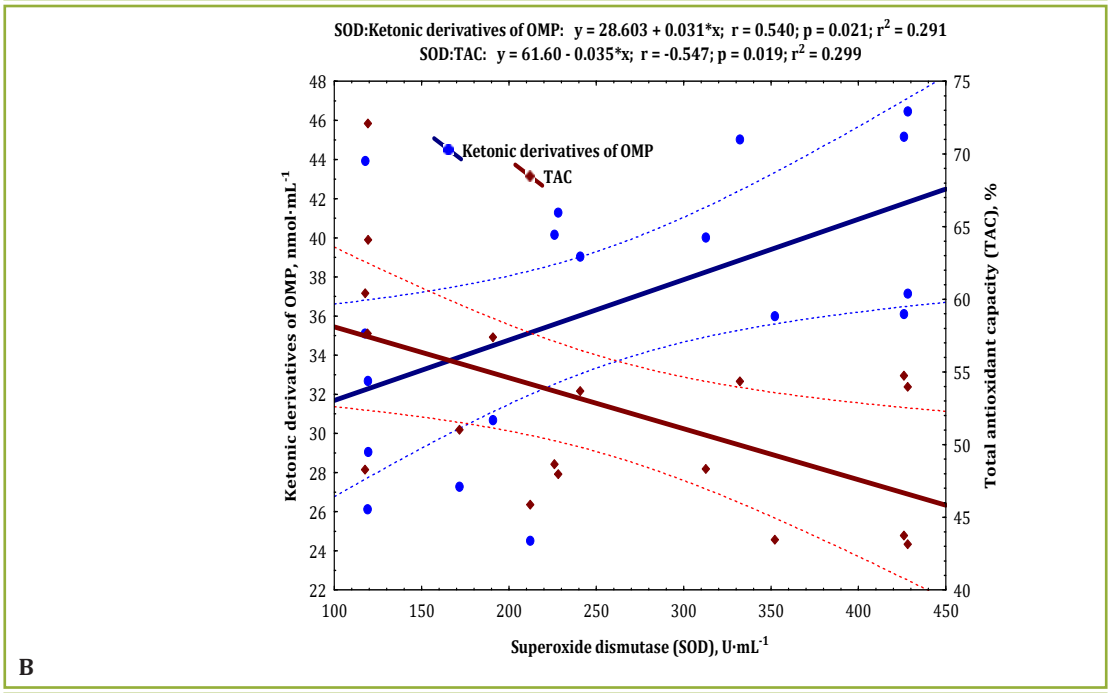


Figure 2A Correlations between oxidative stress biomarkers TBARS level, ketonic derivatives of OMP level, and SOD activity (A), SOD activity, ketonic derivatives of OMP level, and TAC activity (B), TAC level, catalase and GPx activity (C) in the equine erythrocyte suspension after *in vitro* incubation with *Begonia rex* leaf extract



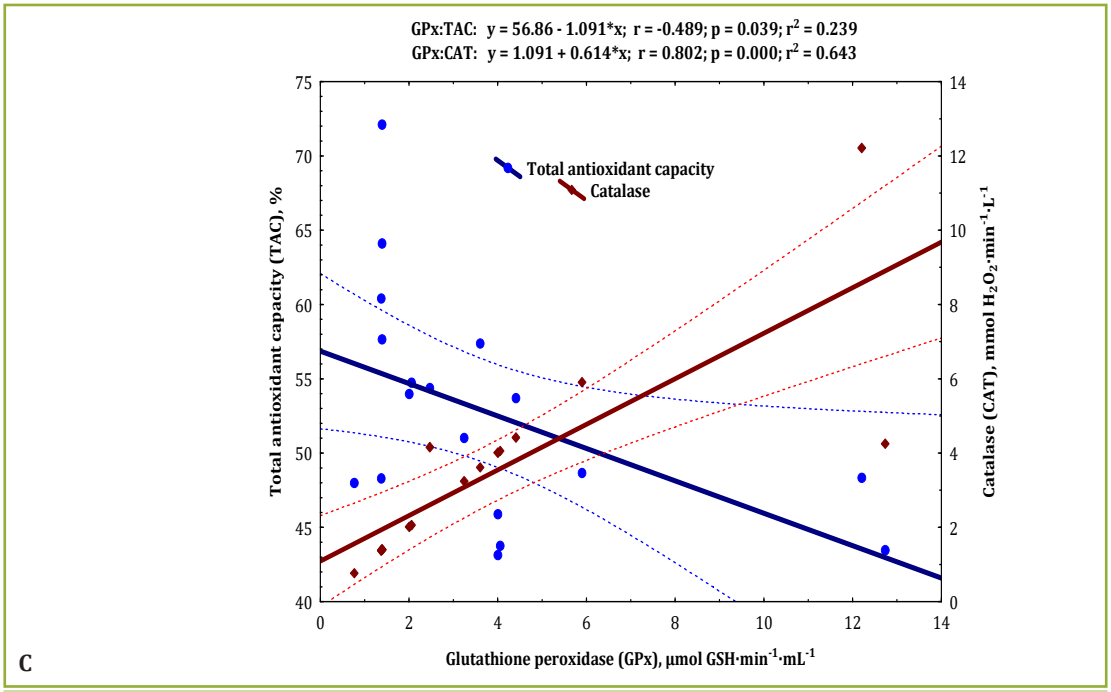


Figure 2B–C Correlations between oxidative stress biomarkers TBARS level, ketonic derivatives of OMP level, and SOD activity (A), SOD activity, ketonic derivatives of OMP level, and TAC activity (B), TAC level, catalase and GPx activity (C) in the equine erythrocyte suspension after *in vitro* incubation with *Begonia rex* leaf extract

This paper demonstrates changes in the oxidative stress biomarkers of equine erythrocytes incubated with *B. rex* leaf extract (Figures 1 and 2). Accordingly, our study suggests that crude extract obtained from *B. rex* leaves has the effective antioxidant effect after treatment of equine erythrocytes. Protective effects of *B. rex* leaf extracts became apparent by amelioration in antioxidant enzymes' activities and the increase of total antioxidant capacity. The antioxidant defence system was improved concurrence with suppression of aldehydic and ketonic derivatives of oxidatively modified proteins by treatment of *B. rex* extract. *B. rex* extract showed anti-inflammation effect represented as decreasing on ceruloplasmin level in the plasma. The pronounced effect of leaf *B. rex* extract could be attributed to its secondary metabolites, e.g. polyphenols and flavonoids contents.

Phytochemical constituents in *Begonia* species are known to be biologically active compounds and they are responsible for different activities such as antioxidant, antimicrobial, antifungal, and anticancer (Suresh and Nagarajan, 2009). It has become increasingly clear that all secondary metabolite components displayed antioxidant and antimicrobial properties through different biological mechanisms (Hossain and Nagooru, 2011). Variation in the chemical profile of extracts could influence their biological activities. Therefore, it was important to know the chemical composition of extracts to correlate with their antioxidant activities. A study conducted by Kalpanadevi and Mohan (2012) has shown that the extracts of *B. malabarica* Lam. and *B. floccifera* Bedd. contain higher quantities of phenolic compounds, which exhibit antioxidant and free radical scavenging activity. Kalpanadevi and Mohan (2012) have evaluated the total phenolic, flavonoid contents and *in vitro* antioxidant activity of methanol extracts of *B. malabarica* and *B. floccifera* whole plant. 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 $mg.mL^{-1}$, 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 $mg.mL^{-1}$, 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 $mg.mL^{-1}$, 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 the studied plant extracts, *B. malabarica* exhibited higher activity (79.11%) at a concentration of 1 $mg.mL^{-1}$ than Trolox. The reducing power of extracts increased with 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).

Preliminary phytochemical screening of *B. floccifera* and *B. malabarica* conducted by Ariharan et al. (2012) showed the presence of a number of bioactive constituents, i.e. vitamin C. The contents of flavonoids (including glycosides of quercetin and kaempferol), anthocyanins and ascorbic acid in overground part of plants of 7 species and cultivars of *Begonia* genus (*B. bahiensis*, *B. bowerae*, *B. carolineifolia*, *B. fischeri*, *B. heracleifolia*, *B.* '*Erythrophylla*', *B.* '*Helen Teupel*') were determined by Karpova et al. (2009). The contents of flavonoids were 24–650 mg% of dry weight, including glycosides of quercetin – 3–76 mg%. Kaempferol and glycosides were detected only in species of section Gireoudia (1.2–5.7 mg%). The contents of anthocyanins were between 60 and 157 mg%, ascorbic acid – 5–43 mg% of fresh weight. Studied plants of *Begonia* can be considered as the sources of biologically active compounds with antioxidant and antimicrobial activity (Karpova et al. 2009). The results of the phytochemical screening of the methanolic flower extracts of *B. floccifera* revealed that phenol, tannins, xanthoproteins, steroids, tannins, phytosterols, triterpenoids, sapogenins, coumarins and carbohydrates (Jeeva and Marimuthu Antonisamy, 2012).

Many studies have suggested that plant secondary metabolites obtained from *Begonia*ceae 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 (1,1-diphenyl-2-picrylhydrazyl). For example, Indrakumar et al. (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 ethanolic extract showed a reduction at various concentrations similar to that of standard ascorbic acid. DPPH scavenging activity of the ethanolic extract showed the IC_{50} value of 32.34 when compared to that of standard BHT which was 20.3. Scavenging activity showed IC_{50} Value of 165.45, nitric oxide scavenging activity showed the IC_{50} value of 134.20 when compared to that of standard ascorbic acid which was 32.14. The DPPH radical scavenging activity was higher (93.3%) when the concentration was increased. The reducing power of the extract increased with the increasing concentration. The *in vitro* antioxidant studies clearly indicate that the ethanolic extract of *B. dipetala* has significant antioxidant activity (Indrakumar et al., 2014).

The results of the Aswathy et al. (2016) study suggested the health-promoting properties of anthocyanin in *Begonia* cultivars (*B. heracleifolia* Cham. & Schltdl. and *B. malabarica* Lam. and three cultivars of *B. rex* (*B. rex* 'Baby Rainbow' L.H.Bailey, *B. rex* 'Black Beauty' & *B. rex* 'Sir Percy') in terms of their antioxidant activity, stable over time. Morphologically the cultivars showed variation among each other and showed variation in anthocyanin content, with the *B. rex* 'Baby Rainbow' and *B. rex* 'Black Beauty' possessing highest anthocyanin content which is morphologically distinguishable. Anthocyanin was found to be an effective antioxidant in different *in vitro* assays when compared to the standard antioxidants. The extracts of *Begonia* may have excellent potential as functional ingredients representing the potential source of natural antioxidant (Aswathy et al., 2016).

Methanol and ethyl acetate extract of *B. trichocarpa* has shown a marked dose-dependent antioxidant activity in both DPPH free radical scavenging method and Nitric acid scavenging method in the study of Sindhu et al. (2016). DPPH assay of methanol extract and ethyl acetate extract shows maximum% inhibition 53.0 and 50.93% at the concentration of 400 $\mu g.mL^{-1}$ respectively, whereas ascorbic acid exhibit 70.36% and IC_{50} values were 335.23, 370.74 and 16.84 $\mu g.mL^{-1}$ respectively. Nitric acid scavenging activity of methanol extract and ethyl acetate extract shows maximum% inhibition, i.e. 46.53% 27.36% respectively, while ascorbic acid exhibit 53.34%, IC_{25} values were found 150.87, 509.16 and ascorbic acid 0.633 $\mu g.mL^{-1}$. Total phenol content of different extracts of *B. trichocarpa* was estimated; out of this methanol extracts contain 49.96% of phenol content and 23.71% anthocyanin content present in the leaf. The antioxidant activity of *B. trichocarpa* may be due to the high phenol content and the presence of anthocyanin in the leaf give a supporting evidence for this (Sindhu et al., 2016).

The components derived from other species belonging to the Cucurbitaceae family also exert significant antioxidant activity both *in vivo* and *in vitro* study. For example, Arawwawala et al. (2011) have determined whether aerial parts of *Trichosanthes cucumerina* extracts can exert significant antioxidant activity in CCl_4-induced toxicity model in rats. The antioxidant activity of a hot water extract and a cold ethanolic extract of *T. cucumerina* aerial parts was evaluated by assessing its (a) radical scavenging ability and prevention effect of lipid peroxidation *in vitro*, and (b) effects on lipid peroxidation and antioxidant enzyme activities, *in vivo*. *In vitro* antioxidant assays (DPPH, TBARS, and carotene-linoleic acid assays) clearly demonstrated the antioxidant potential of *T. cucumerina* aerial parts extract. Moreover, hot water extract increased SOD activity (by 91.2%) and GPx activity (by 104.4%), while cold ethanolic extract increased SOD activity (by 115.5%) and GPx (by 96.4%) in CCl_4-induced toxicity model in rats. Treatments with hot water extract and cold ethanolic extract prevented the accumulation of lipid peroxidation products by 30.5 and 33.8%, respectively, in liver tissues compared to the rats exposed only to CCl_4 (Arawwawala et al., 2011).

Melon (*Cucumis melo* L.) concentrate exhibits an antioxidant capacity partly due to its high level of SOD activity, maybe in conjunction with other antioxidant compounds present in this melon extract associated with a relevant angiotensin 1-converting enzyme-inhibitory activity. Carillon et al. (2012) have assayed *in vitro* the antioxidant capacity and angiotensin 1-converting enzyme (ACE) inhibitory activity of a melon concentrate rich in superoxide dismutase. The total antioxidant capacity (TAC) was measured by the Trolox equivalent antioxidant capacity assay (TEAC), the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay, and the ferric reducing antioxidant power assay (FRAP). The ability of the extract to scavenge three specific reactive oxygen species [superoxide radical anion ($O_2^{\cdot-}$), hydroxyl radical (HO·) and hydrogen peroxide (H_2O_2)] was also investigated in order to better evaluate its antioxidant properties. Even if the measures of TAC were relatively low, results clearly established an antioxidant potential of SOD-Melon concentrate that exhibited the highest radical-scavenging activity towards, with an IC_{50} 12-fold lower than that of H_2O_2 or HO·. This lets the hypothesis that the antioxidant potential of SOD-Melon concentrates could be mainly due to its high level of SOD (Carillon et al., 2012).

Chen et al. (2014) have delineated the antioxidant activity of Radix Trichosanthis (RT), the dry root tuber of *Trichosanthis kirilowii* Maxim (Cucurbitaceae) both *in vitro* and *in vivo* models by using ethyl acetate (EtOAc), n-butanol, and the mixture of n-butanol and EtOAc fractions. The *in vitro* antioxidant activity was detected by using DPPH free radical, hydrogen peroxide scavenging, and reducing power assays. After pretreatment with different fractions saponins at 2 and 3 $mg.kg^{-1}$/d of crude drug, respectively, an established CCl_4 induced acute cytotoxicity model was used to evaluate the *in vivo* antioxidant potential by detection of superoxide dismutase (SOD), malonaldehyde (MDA), lactate dehydrogenase (LDH), and total antioxidant capacity (T-AOC) levels. The *in vitro* assay showed that the antioxidant activity of all the three fractions was promising. The reducing power of the EtOAc and the mixture of n-butanol and EtOAc extracts increased in a dose-dependent manner. As for hydrogen peroxide scavenging capability, the n-butanol fraction mainly demonstrated a time-dependent manner, whereas the EtOAc fraction showed a dose-dependent manner. However, in case of *in vivo* assay, an increase of SOD and T-AOC and the decrease of MDA and LDH levels were only observed in n-butanol (2 $mg.kg^{-1}$/d of crude drug) extracts pretreatment group. RT saponins in n-butanol fraction might be a potential antioxidant candidate, as CCl_4-induced oxidative stress has been found to be alleviated, which may be associated with the time-dependent manner of n-butanol saponins in a low dose (Chen et al., 2014).

Conclusions

The results of this research indicated that crude extract obtained from *B. rex* leaves has the effective antioxidant effect after treatment of a suspension of equine erythrocytes lysed. Protective effect of *B. rex* extract is evident by amelioration in antioxidant enzymes' activities and the increase of total antioxidant capacity. The antioxidant defencse system was improved concurrence with suppression of aldehydic and ketonic derivatives of oxidatively modified proteins by treatment of *B. rex* extract. *B. rex* extract showed anti-inflammation effect exhibited as decreasing of ceruloplasmin level in the plasma. The pronounced effect of *B. rex* leaf extract, probably, could be attributed to its secondary metabolites content, e.g. polyphenols and flavonoids contents. Finally, further investigation is necessary to reveal the exact cellular mechanisms of the effect of *B. rex* extract on the erythrocyte function. These *in vitro* assays indicate that plant extract screened is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. In addition, suspension of equine erythrocytes lysed is a sensitive assay applied to detect the antioxidant effect of leaf extract obtained from *Begonia rex* Putz. on oxidative stress biomarkers. However, the components responsible for the antioxidative activity of *B. rex* extract is currently unclear. Therefore, further investigations need to be carried out to isolate and identify the antioxidant compounds present in the plant extract.

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