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Oxidative stress biomarkers in equine erythrocytes and plasma after *in vitro* treatment with an aqueous leaf extract of *Coelogyne brachyptera* Rchb. f. (Orchidaceae)

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Many orchids have been used as medicinal plants for many years in China, Japan, India, and some other countries. They showed many health-beneficial functions, such as the protection of cells against free radicals and oxidative stress possessing hepatoprotection, cardioprotection, gastroprotection, neuroprotection, and other properties. This study aimed to investigate the oxidative stress biomarkers [2-thiobarbituric acid reactive substances (TBARS), aldehydic and ketonic derivatives of oxidatively modified proteins (OMP), and total antioxidant capacity (TAC)] in the equine erythrocytes and plasma after *in vitro* incubation with an extract derived from leaves of *Coelogyne brachyptera* Rchb. f. The leaves of *C. brachyptera* plants, cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine. Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1M phosphate buffer (pH 7.4) (in the proportion of 1:19, w/w). The equine plasma and erythrocyte aliquots were used in the study. The pellet of blood was re-suspended in phosphate buffer (pH 7.4). A volume of 0.1 ml of the *C. brachyptera* extract was added to 1.9 ml of clean equine erythrocytes or 1.9 mL of plasma. For positive control (blank), 0.1M phosphate buffer (pH 7.4) added to erythrocytes or plasma was used. Results of our study revealed that erythrocytes were more sensitive to the action of an extract derived from leaves of *C. brachyptera*. The levels of aldehydic and ketonic derivatives of oxidatively modified proteins in the treated erythrocytes were significantly decreased, while these parameters were no-changed in the equine plasma. The treatment of equine erythrocytes by extract derived from leaves of *C. brachyptera* increased lipid peroxidation. On the other hand, plasma TBARS level after treatment by extract derived from leaves of *C. brachyptera* was at the same level as in untreated controls. The level of total antioxidant capacity was not-significantly changed after treatment both in equine plasma and erythrocytes. Studies concerning the antioxidant properties of orchids are continued in our laboratory.

Keywords: leaf extract, equine erythrocytes and plasma, lipid peroxidation, oxidatively modified proteins, total antioxidant capacity

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Introduction

Orchidaceae is one of the largest and more diverse families of flowering plants with approximately 25,000 species in 736 genera currently recognized, as well as widely distributed as epiphytes, lithophytes, or terrestrials (Chase et al., 2015). Orchids have been used all over the world in traditional healing and treatment systems for various diseases, such as chest pain, arthritis, syphilis, jaundice, cholera, acidity, eczema, tumor, piles, tuberculosis, wounds, stomach disorders, boils, inflammation, menstrual disorders, spermatorrhea, leucoderma, slantendicular, muscular pain, earache, sexually transmitted diseases, blood dysentery, hepatitis, bone fractures, rheumatism, asthma, malaria, paralysis, and dyspepsia (Kong et al., 2003; Pant, 2013; Rahman et al., 2022). It is suggested that the pharmaceutical properties of orchids are due to the activities of many phytochemicals, including alkaloids, bibenzyl derivatives, flavonoids, carotenoids, phenanthrenes, phenanthropyrans, stilbenes, anthocyanins, glycosides, sterols, and terpenoids, which are present in various parts of orchid plants (Zhang et al., 2015; Axiotis et al., 2021). In recent years, the assessment of the anti-diabetic, anti-inflammatory, and antioxidant properties of orchids has received considerable attention (Li et al., 2018; Warinhomhoun et al., 2021; Zhang et al., 2021). Some orchid species are used as a potent antioxidant and cytotoxic activities and also proved to be potent antioxidant agents (Paudel et al., 2018, 2019; Robustelli Della Cuna et al., 2019; Li et al., 2021).

Previously, we have given considerable attention to the evaluation of the antibacterial activity of ethanolic extracts derived from leaves and pseudobulbs of plants belonging to various *Coelogyne* species, maintained under glasshouse conditions (Buyun et al., 2016-2019a,b, 2021). For example, the assessment of the antifungal potential of orchids species, i.e. *Coelogyne cristata* Lindl., *C. fimbriata* Lindl., *C. flaccida* Lindl., *C. huettneriana* Rchb.f., *C. ovalis* Lindl., *C. speciosa* (Blume) Lindl., *C. tomentosa* Lindl. and *C. viscosa* Lindl. against fungus strain, *Candida albicans* was conducted by Buyun et al. (2018). Marked antifungal efficacy was observed in the case of ethanolic extracts derived from leaves of *C. flaccida* (mean diameter of inhibition zones was 19.5 mm), *C. viscosa* (18.6 mm), *C. huettneriana* (18.2 mm), and *C. fimbriata* (17.5 mm). Extracts of *C. cristata*, *C. ovalis*, and *C. tomentosa* displayed less profound inhibitory activity against test fungus (mean diameter of inhibition zones ranging from 16 to 17.5 mm). Similarly, the ethanolic extracts from the pseudobulbs of eight *Coelogyne* species exhibited strong activity against *C. albicans* (inhibition zone diameter ranged from 16 to 23.5 mm). Moreover, it has been observed that ethanolic extract from pseudobulbs of *C. speciosa* revealed the highest antibacterial activity (21 mm as the diameter of the inhibition zone) among various *Coelogyne* species screened. The results also indicate that scientific studies carried out on medicinal plants having traditional claims of effectiveness might warrant fruitful results (Buyun et al., 2018).

Later, we also assessed the oxidative stress biomarkers (2-thiobarbituric acid reactive substances (TBARS), aldehydic and ketonic derivatives of oxidatively modified proteins (OMP), total antioxidant capacity (TAC)) in the equine erythrocytes and plasma after treatment *in vitro* by extract derived from leaves of *Dendrobium parishii* Rchb. F. (Buyun et al., 2019b). The levels of TBARS as a biomarker of lipid peroxidation, aldehydic and ketonic derivatives of OMP, as well as TAC, were non-significantly altered in the erythrocyte suspension after *in vitro* incubation with an extract derived from leaves of *D. parishii*. More significant changes were observed in the equine plasma. The *D. parishii* extract caused to increase in the formation of intracellular aldehydic and ketonic derivatives of OMP in the extract-treated plasma, but these results were non-significant. Total antioxidant capacity was non-significant decreased both in plasma and erythrocytes (Buyun et al., 2019b).

The current study is a continuation of our cooperation with M.M. Gryshko National Botanic Garden, National Academy of Science of Ukraine (Kyiv, Ukraine) concerning investigations of antibacterial and antioxidant properties of extracts derived from leaves and pseudobulbs of some species belonging to the Orchidaceae family using different cell models *in vitro*. We have chosen equine erythrocytes as a model for the evaluation of the antioxidant properties of plant extract because 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). Equine erythrocytes are slower than erythrocytes from other species studied in their ability to regenerate glutathione (GSH) after it has been oxidized *in vitro* (Harvey et al., 2003). Moreover, sulfhydryl groups in proteins and unsaturated lipids in 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).

Thus, this study aimed to investigate the oxidative stress biomarkers (2-thiobarbituric acid reactive substances, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity) in the equine erythrocytes and plasma after *in vitro* incubation with an extract derived from leaves of *Coelogyne brachyptera* Rchb. f.

Material and methodology

Collection of plant materials

The leaves of *C. brachyptera* plants cultivated under glasshouse conditions were sampled at M.M. Gryshko National Botanical Garden of the National Academy of Sciences of Ukraine (NBG, Kyiv, Ukraine) in September 2016 (Figure 1). Since 1999, the whole collection of tropical and subtropical plants (including orchids) has had the status of a National Heritage Collection of Ukraine and is supported through State Funding. Besides, the NBG collection of tropical orchids was registered at the Administrative Organ of CITES in Ukraine (Ministry of Environment Protection, registration No. 6939/19/1-10 of 23 June 2004).

Various databases are available for searching collections of living plants, confirming the taxonomic identity of having been reviewed, e.g. World Checklist of Orchidaceae (Govaerts et al., 2016), International Plant Names Index, The Plant List, the IUCN Red List (IUCN, 2013).

Coelogyne brachyptera is found in Burma, Thailand, Cambodia, Laos, and Vietnam. It grows epiphytically in the primary mountain forest, the most frequent at an altitude of 1,000 to 2,500 meters above sea level

Figure 1 Vegetative shoot with inflorescence of *Coelogyne brachyptera* Rchb. f. plant, cultivated at NBG's glasshouses (Kyiv, Ukraine)
Photo: Oleksandr Gyrenko

(Averyanov et al., 2003). It is a sympodial orchid with pseudobulbs of one internode, narrowly conical, 4-angled, slightly grooved, pale green, carrying 2 leaves. The leaves are elliptic to elliptic-lanceolate, subacute, plicate, 7-nerved, with an undulate margin. The flowering of *C. brachyptera* under glasshouse conditions at NBG was observed in March – April. The duration of anthesis of a single inflorescence did not exceed 2 weeks.

Preparation of plant extracts

Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in the proportion of 1:19, w/w) at room temperature. The extracts were then filtered and used for analysis. All extracts were stored at -25 °C until use. All biochemical assays were conducted at the Department of Biology, Institute of Biology and Earth Sciences, Pomeranian University in Słupsk (Poland).

Horses

Eighteen clinically healthy adult horses from the central Pomeranian region in the northern part of Poland (Strzelinko village, N 54º 30' 48.0" E 16º 57' 44.9"), aged 8.9 ±1.3 years old, including 6 Hucul ponies, 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*. Before sampling, all horses were thoroughly examined clinically by a veterinarian and screened for hematological, biochemical, and vital parameters, which were within reference ranges. The females were non-pregnant.

Collection of blood samples

Blood samples were collected in the morning, 90 minutes after feeding, while the horses were in the stables (between 8:30 and 10 AM) by jugular venipuncture into tubes with sodium citrate as the anticoagulant and held on the ice until centrifugation at 3,000 rpm for 5 min to remove plasma Blood was stored into 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 equine erythrocytes or plasma. For positive control, 0.1 ml of phosphate buffer added to the equine erythrocytes or plasma was used. After incubation of the mixture at 37 °C for 60 min with continuous stirring, biochemical assays were done. Erythrocytes and plasma aliquots were used in the study.

The 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 2-thiobarbituric acid (TBA) under high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. The nmol per 1 mL was calculated using $1.56 \cdot 10^5$ $mM^{-1}.cm^{-1}$ as the extinction coefficient.

The carbonyl derivatives of oxidative modification of proteins (OMP)

To evaluate the protective effects of the extract against free radical-induced protein damage in equine erythrocytes and plasma, carbonyl derivatives of protein oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the samples 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 derivatives in soluble and insoluble proteins. Carbonyl groups were determined spectrophotometrically from the difference in absorbance at 370 nm (aldehydic derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}).

Measurement of total antioxidant capacity (TAC)

The TAC level in samples 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). The 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 concerning 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 total antioxidant capacity level (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 calculations were performed on separate data from each individual with Statistica 13.3 software (TIBCO Software Inc., Krakow, Poland).

Results and discussion

The TBARS content as a biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, and the total antioxidant capacity (TAC) in the equine erythrocytes after *in vitro* incubation with an extract derived from leaves of *C. brachyptera* was assessed and shown in Figure 2.

Lipid peroxidation can be broadly defined as the process of inserting a hydroperoxy group into a lipid. Polyunsaturated fatty acids present in the phospholipids, indispensable for the normal structure of membranes, are often the targets for peroxidation (Anthonymuthu et al., 2016). The 2-thiobarbituric acid reactive substances (TBARS) assay has been widely used as a generic metric of lipid peroxidation in biological fluids (Aguilar Diaz De Leon et al., 2020). As presented in Figure 2, treatment by extract derived from leaves

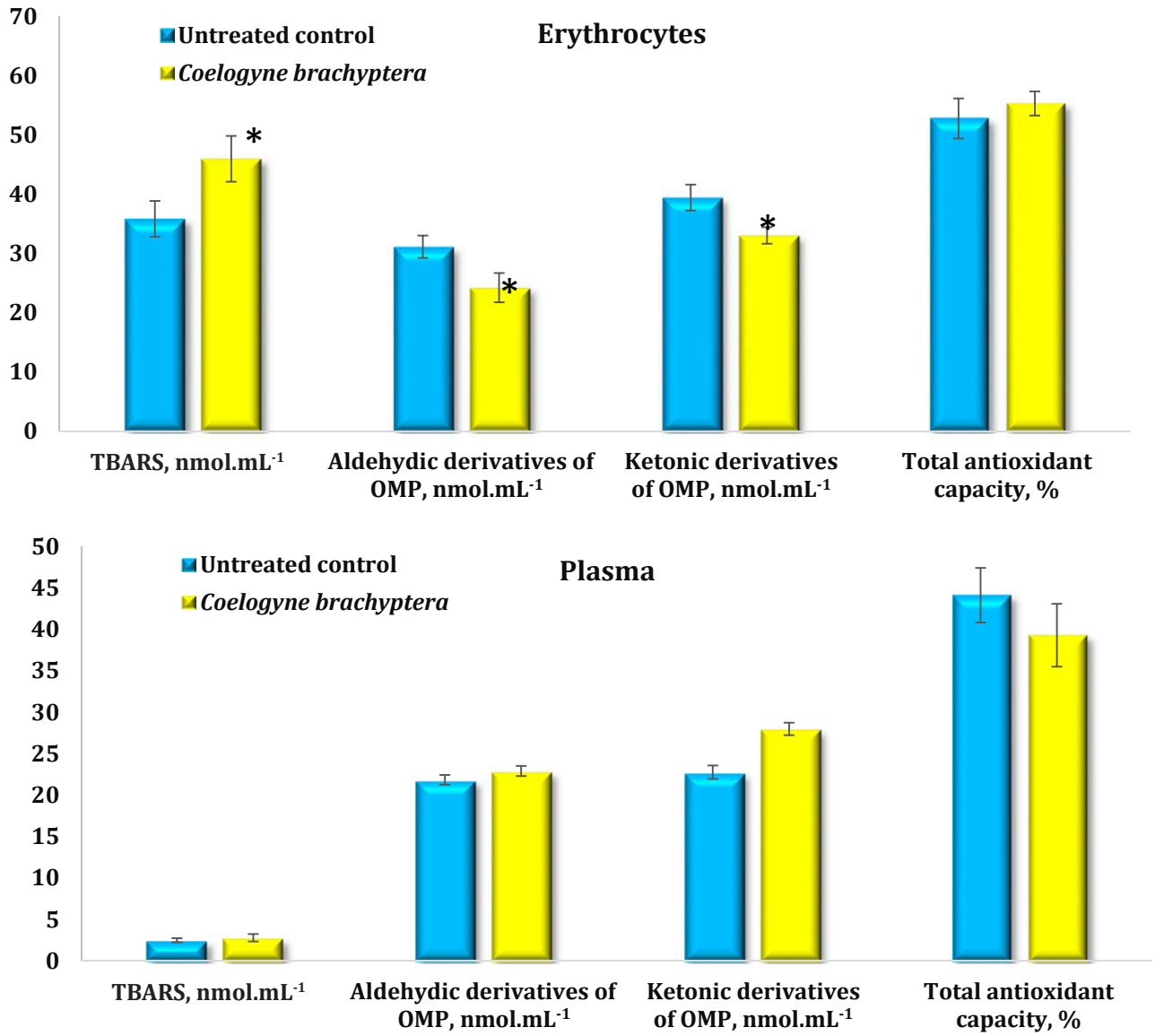

Figure 2 The TBARS content as a biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins, and total antioxidant capacity in the equine erythrocytes and plasma after *in vitro* incubation with an extract - of *Coelogyne brachyptera* Rchb. f. (M ±m, n = 18)
*– statistically significant differences between treated and untreated samples ($p < 0.05$)

of *C. brachyptera* resulted in an increase in erythrocyte TBARS level of (46.02 ±3.86 nmol.mL^{-1}) compared to the untreated samples (35.88 ±3.02 nmol.mL^{-1}). The increase in TBARS level was by 28.3% (p = 0.005). On the other hand, plasma TBARS level after treatment by extract derived from leaves of *C. brachyptera* was at the same level as in untreated controls (2.80 ±0.45 nmol. mL^{-1} vs. 2.49 ±0.25 nmol.mL^{-1}) (Figure 2).

Proteins are major targets for oxidative stress because they are abundant and have rapid rates of reaction with a wide range of radicals and excited state species. Exposure of proteins to free radicals resulted in the loss of the parent amino acid residue, the formation of unstable intermediates, and the generation of stable products (Hawkins et al., 2009; Hawkins and Davies, 2019). The levels of aldehydic and ketonic derivatives of oxidatively modified proteins were also decreased in samples treated with an extract derived from leaves of *C. brachyptera* compared to the untreated samples, and these decreases were statistically significant (p <0.05). When equine erythrocytes were incubated with the extract derived from leaves of *C. brachyptera*, the levels of aldehydic and ketonic derivatives were significantly decreased by 22.1% (24.26 ±2.48 nmol.mL^{-1} vs. 31.16 ±1.89 nmol.mL^{-1}) and 16.2% (p <0.05) (33.07 ±1.40 nmol.mL^{-1} vs. 39.47 ±2.20 nmol.mL^{-1}). On the other hand, aldehydic derivatives of oxidatively modified proteins in the equine plasma after treatment by an extract derived from leaves of *C. brachyptera* were at the same levels as untreated samples (22.91 ±0.62 nmol.mL^{-1} vs. 21.85 ±0.59 nmol.mL^{-1}). Ketonic derivatives of oxidatively modified proteins in the equine plasma after treatment by an extract derived from leaves of *C. brachyptera* were a statistically non-significant increase by 22.9% (p >0.05) (27.99 ±0.75 nmol.mL^{-1} vs. 22.77 ±0.80 nmol.mL^{-1}) (Figure 2). Also, a non-significantly increase in erythrocyte TAC level was observed after incubation with an extract derived from leaves of *C. brachyptera* (by 4.8%, p >0.05) (55.37 ±2.04% vs. 52.83 ±3.38%) (Figure 2). TAC levels in the equine plasma after treatment by an extract derived from leaves of *C. brachyptera* were a statistically non-significant decrease by 11% (p >0.05) (39.28 ±3.79% vs. 44.11 ±3.29%) (Figure 2).

The efficacy of some plants belonging to the *Coelogyne* genus was reported by some researchers using *in vitro* and *in vivo* models. For example, Sharma et al. (2014) have evaluated the bone-forming activity of *Coelogyne cristata* Lindl. extract *in vivo* wherein parameters like trabecular microarchitecture, bone strength, and uterine estrogenicity were studied in the estrogen-deficient female Balb/c mice model. Subsequently, coelogin, a pure compound isolated from ethyl acetate fraction of *C. cristata* alcoholic extract was evaluated in *in vitro* osteoblast cell cultures, alkaline phosphatase activity (a marker of osteoblast differentiation), mineral nodule formation and mRNA levels of osteogenic markers like BMP-2, Type 1 Collagen, and RUNX-2. Their experimental results suggest that both ethanolic extract and Coelogin isolated from *C. cristata* possess significant improvement of trabecular response leading to the restoration of trabecular microarchitecture in both femoral and tibial bones in ovariectomized estrogen-deficient mice along with the biochemical strength. The study by Sharma et al. (2014) also supports the use of *C. cristata* for the treatment of fracture healing as claimed by traditional practitioners. The identified bioactive compound may serve as the starting point for the design and development of pharmaceutical products not only to reduce fracture risk but also for the management of postmenopausal osteoporosis (Sharma et al., 2014). The osteoprotective effect of coelogin was also evaluated on osteopenic adult female Swiss mice in the study by Prakash et al. (2021). Coelogin treatment led to increased osteoblast proliferation, survival, differentiation, and mineralization in osteoblast cells. Coelogin supplementation to Ovx mice promoted new bone formation, prevented Ovx-induced deterioration of bone microarchitecture, and enhanced bone regeneration. In addition, signaling studies revealed that coelogin treatment activates the ER-Erk and Akt-dependent signaling pathways which stimulate osteoblastogenesis in osteoblast cells (Prakash et al., 2021).

The effectiveness of the phenanthrene-rich hydro-alcoholic extract of pseudobulbs of *C. cristata* (CCE) in chronic fatigue syndrome (CFS)-induced behavioral changes in aged animals was done by Mitra et al. (2018). Biochemical estimations were also carried out to establish the antioxidant activity of this plant *in vivo* whereas *Panax ginseng* C.A. Mey. was used as the prototype standard. CCE was found to be non-toxic. CCE-treated aged rats significantly improved the spontaneous locomotor movement concerning control rats, while, decreasing the mobility period or depression score. In CFS, CCE also enhanced the time spent in open arms while reducing the time spent in closed arms as compared to CFS control, indicating lowering anxiety score. Moreover, a marked diminution in lipid peroxidation, nitrite, and superoxide dismutase levels were exhibited after CCE treatment and significantly enhanced catalase level significantly concerning CFS control. *Panax ginseng* also showed similar actions. The results of Mitra et al. (2018) confirmed the potential

therapeutic actions of CCE against experimentally induced CFS in aged rats that might be due to its CNS mediatory antioxidant properties.

Antioxidant and antitumor properties also were revealed for other orchids. For example, data obtained by Zhao et al. (2019) introduces the novel idea that *Dendrobium officinale* Kimura & Migo polysaccharides (DOP) prevent precancerous lesions of gastric cancer (PLGC). These authors demonstrated that DOP are capable of restraining the activity of 8-hydroxy-deoxyguanosine while increasing the nuclear expression of nuclear factor erythroid 2-related factor 2. All told, this activates downstream heme oxygenase-1 and NADPH quinone oxidoreductase-1 expression to improve antioxidant activity and protect gastric mucosal cells from oxidative damage. In addition, DOP can decrease serum levels of alanine aminotransferase, serum uric acid, and blood urea nitrogen, indicating DOP might protect liver and kidney function. These findings show DOP can be considered an effective healthcare product for the treatment of precancerous lesions of gastric cancer (Zhao et al., 2019).

Thus, in the current study, we have undertaken an attempt to investigate the *in vitro* antioxidant activity of an extract derived from the leaves of *C. brachyptera* plants using as a model equine erythrocytes and plasma. The results obtained suggested that an extract derived from the leaves of *C. brachyptera* significantly decrease the protein oxidation in the erythrocyte suspension after *in vitro* treatment. On the other hand, lipid peroxidation was enhanced. In the equine plasma, an extract derived from the leaves of *C. brachyptera* resulted in non-significant alterations in levels of lipid peroxidation, oxidatively modified proteins, and TAC.

Conclusions

In the current study, we investigated the changes in the oxidative stress biomarkers using the model of equine erythrocytes and plasma to evaluate the antioxidant activities of the aqueous extract derived from leaves of *C. brachyptera*. Results of our study revealed that erythrocytes were more sensitive to the action of an extract derived from leaves of *C. brachyptera*. The levels of aldehydic and ketonic derivatives of oxidatively modified proteins in the treated erythrocytes were significantly decreased, while these parameters were no-changed in the equine plasma. The treatment of equine erythrocytes by extract derived from leaves of *C. brachyptera* increased lipid peroxidation. On the other hand, plasma TBARS level after treatment by extract derived from leaves of *C. brachyptera* was at the same level as in untreated controls. The level of total antioxidant capacity was not-significantly changed after treatment both in equine plasma and erythrocytes. Studies concerning the antioxidant properties of orchids have continued in our laboratory. The next step in our further investigation will be HPLC profiling of the plant extract to find new bioactive compounds from a natural source.

Conflicts of interest

The authors declare no conflict of interest.

Ethical statement

This article doesn't contain any studies that would require an ethical statement.

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