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Biomarkers of oxidative stress in the equine erythrocytes after *in vitro* treatment with extracts derived from leaves and pseudobulbs of *Coelogyne pandurata* Lindl. (Orchidaceae) plants

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The current study was conducted to investigate the antioxidant properties of extracts derived from leaves and pseudobulbs of *Coelogyne pandurata* Lindl. using biomarkers of oxidative stress (2-thiobarbituric acid reactive substances (TBARS) as biomarkers of lipid peroxidation, aldehydic and ketonic derivatives of oxidative modification of proteins (OMP), total antioxidant capacity TAC)) in the equine erythrocytes after *in vitro* treatment with the extracts. The obtained results demonstrated the prooxidative activity of *C. pandurata* extract used in the studied dose (5 mg.mL^{-1}) on the equine erythrocytes. Our results also showed that extract derived from the leaves and pseudobulbs of *C. pandurata* after incubation with erythrocyte samples caused to remaining the TAC level at a high level as compared to the group treated by phosphate buffer (controls), while levels of aldehydic and ketonic derivatives of OMP were unchanged. Our results also revealed that extracts derived from the leaves and pseudobulbs of *C. pandurata* after incubation with equine erythrocyte samples caused to increase in the TBARS level compared to untreated samples. Future studies will be conducted to evaluate dose-dependent changes in the levels of oxidative stress biomarkers after incubation with extracts derived from *C. pandurata* using various cell models. Moreover, the plant compound profile characteristics and antioxidant activity of different *Coelogyne* plants may encourage the wider use of these orchids in the development of new medicinal substances in medicine and veterinary.

Keywords: *Coelogyne pandurata*, leaf extract, equine erythrocytes, biomarkers, lipid peroxidation, oxidatively modified proteins, total antioxidant capacity

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Introduction

Orchids constitute one of the largest and most highly developed families of angiosperms and form an extremely peculiar group of flowering plants that are of great value for ornamental, medicinal, conservation, and evolutionary research (Zhang et al., 2018; Wang et al., 2019). Epiphytic orchids are often characterized by succulent leaves with thick cell walls, cuticles, and depressed stomata, while terrestrial orchids have rhizomes, corms, or tubers (Zhang et al., 2018). Various orchids are available through traditional breeding and micropropagation as they are valuable as pot plants and cut flowers in horticultural markets (Wang et al., 2019). Experiments with alkaloids, terpenes, stilbenoids, bibenzyls, phenanthrenes, flavonoids, and polysaccharides isolated from Orchidaceae Juss. have shown their potential medicinal utility (Sut et al., 2017). To date, several classes of phytocomponents have been isolated from therapeutically used orchids, demonstrating great chemical diversity (Sut et al., 2017). Among them, phenol derivatives have been studied for their biological activity, especially anticancer properties (Wang et al., 2021; Śliwiński et al., 2022), anti-inflammation (Jiang et al., 2019; Zhang et al., 2021), and anti-neurodegeneration properties (Li et al., 2017; Zhang et al., 2022).

Coelogyne Lindl. is a genus of about 200 species, distributed from Southeast Asia to the southwestern Pacific Islands (Aung et al., 2017; Zhou et al., 2018; Jiang et al., 2020). *Coelogyne* plants are characterized by a free, never-saccate lip, with high lateral lobes over the entire length of the hypochile and smooth, papillose, toothed, or warty keels on the epichile (Zhou et al., 2018). Most species grow in tropical montane and lowland forest areas (Jiang et al., 2020). The *Coelogyne* genus belongs to the group of orchids that possesses medical properties (Pérez Gutiérrez, 2010; Pant, 2013).

The interesting species within the genus *Coelogyne*, comprising considerable interest for screening of biological activity of various parts of the plants, is *Coelogyne pandurata* Lindl. *Coelogyne pandurata* is found in Malaysia, Sumatra, Borneo, and the Philippines as a large-sized, hot-growing epiphyte found on large trees near rivers or terrestrial with well-spaced, strongly compressed, oblong or suborbicular, sulcate pseudobulb carrying 2, apical, plicate, elliptic-lanceolate, leaves with a stout petiole that blooms in late spring-summer out of the center of newly emerging growths with up to 15 flowers on a terminal, arched to pendant, 15 to 30 cm long, racemose inflorescence. The simultaneously opening flowers are highly fragrant of honey to cinnamon but are short-lived (http://www.orchidspecies.com/).

The current study was conducted to investigate the antioxidant properties of extracts derived from leaves and pseudobulbs of *C. pandurata* using biomarkers of oxidative stress [2-thiobarbituric acid reactive substances (TBARS) as biomarkers of lipid peroxidation, aldehydic and ketonic derivatives of oxidative modification of proteins (OMP), total antioxidant capacity TAC)] in the equine erythrocytes after *in vitro* treatment with the extracts.

Material and methodology

Collection of plant materials and preparation of plant extracts

The leaves and pseudobulbs of *C. pandurata* plants (Figure 1) cultivated under glasshouse conditions were sampled at M.M. Gryshko National Botanic Garden (NBG, Kyiv, Ukraine). 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). Freshly collected leaves and pseudobulbs 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. The extract was stored at -25 °C until use.

Our current scientific project was undertaken in the frame of the cooperation program between the Institute of Biology and Earth Sciences (Pomeranian University in Słupsk, Poland) and M.M. Gryshko National Botanic Gardens of the National Academy of Sciences of Ukraine, directed to assessment of medicinal properties of tropical plants has encompassed some tropical mega-diverse genera, including Orchidaceae.

Horses and collection of blood samples

Eighteen healthy adult horses from the central Pomeranian region in Poland (village Strzelinko, N 54° 30’ 48.0” E 16° 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*

Figure 1 Vegetative shoot with inflorescence of *Coelogyne pandurata* Lindl. plant, cultivated at NBG's glasshouses (Kyiv, Ukraine)
Photo: Lyudmyla Buyun

libitum. All horses were thoroughly examined clinically and screened for hematological, biochemical, and vital parameters, which were within reference ranges. The females were non-pregnant.

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 samples were processed for analysis less than 12 h after blood withdrawal. 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 erythrocytes was resuspended in 4 mM phosphate buffer (pH 7.4). A volume of 0.1 ml of the plant extracts was added to 1.9 ml of equine erythrocytes. For positive control, incubation of equine erythrocytes with 4 mM phosphate buffer (pH 7.4) was used. After incubating the mixture at 37 °C for 60 min with continuous stirring, it was centrifuged at 3000 rpm for 5 min. Erythrocyte aliquots were used in the current 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 of MDA per mL was calculated using $1.56 \cdot 10^5$ $mM^{-1}.cm^{-1}$ as the extinction coefficient.

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

To evaluate the protective effects of the extracts derived from leaves and pseudobulbs of *C. pandurata* against free radical-induced protein damage in equine plasma, a carbonyl derivatives content of protein

oxidative modification (OMP) assay based on the spectrophotometric measurement of aldehydic and ketonic derivatives in the erythrocyte suspension and plasma 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. The carbonyl content was calculated from the absorbance measurement at 370 nm and 430 nm, and an absorption coefficient of 22000 $M^{-1}\cdot cm^{-1}$. 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 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). 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

Statistical analysis of the data obtained was performed by employing the mean ± S.E.M. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test ($p > 0.05$). The significance of differences between the OMP level (significance level, $p < 0.05$) was examined using the Kruskal-Wallis one-way analysis of variance (Zar, 1999). The data were analyzed using a one-way analysis of variance (ANOVA) using Statistica v. 13.3 software (TIBCO Software Inc., Krakow, Poland) (Zar, 1999).

Results and discussion

Levels of TBARS, aldehydic and ketonic derivatives of OMP, and total antioxidant capacity (TAC) in the equine erythrocytes after *in vitro* incubation with an extract derived from leaves of *Coelogyne* pandurate were presented in Figure 2.

Our results revealed that extract derived from the leaves of *C. pandurata* after incubation with equine erythrocyte samples caused to increase in the TBARS level (55.20 ±6.68 $nmol\cdot mL^{-1}$) (by 53.8%, $p < 0.05$) compared to untreated samples (35.88 ±3.02 $nmol\cdot mL^{-1}$). On the other hand, the content of aldehydic derivatives of OMP in the erythrocyte samples after incubation with an extract derived from the leaves of *C. pandurata* was not altered (31.27 ±1.56 $nmol\cdot mL^{-1}$) compared to the untreated samples (31.16 ±1.89 $nmol\cdot mL^{-1}$). Moreover, the content of ketonic derivatives of OMP in the erythrocyte samples after incubation with extracts derived from the leaves of *C. pandurata* was non-significantly decreased (by 2.2%, $p > 0.05$). A non-significant increase in the TAC level of the tested samples incubated with an extract derived from the leaves of *C. pandurata* was observed (58.55 ±2.75%

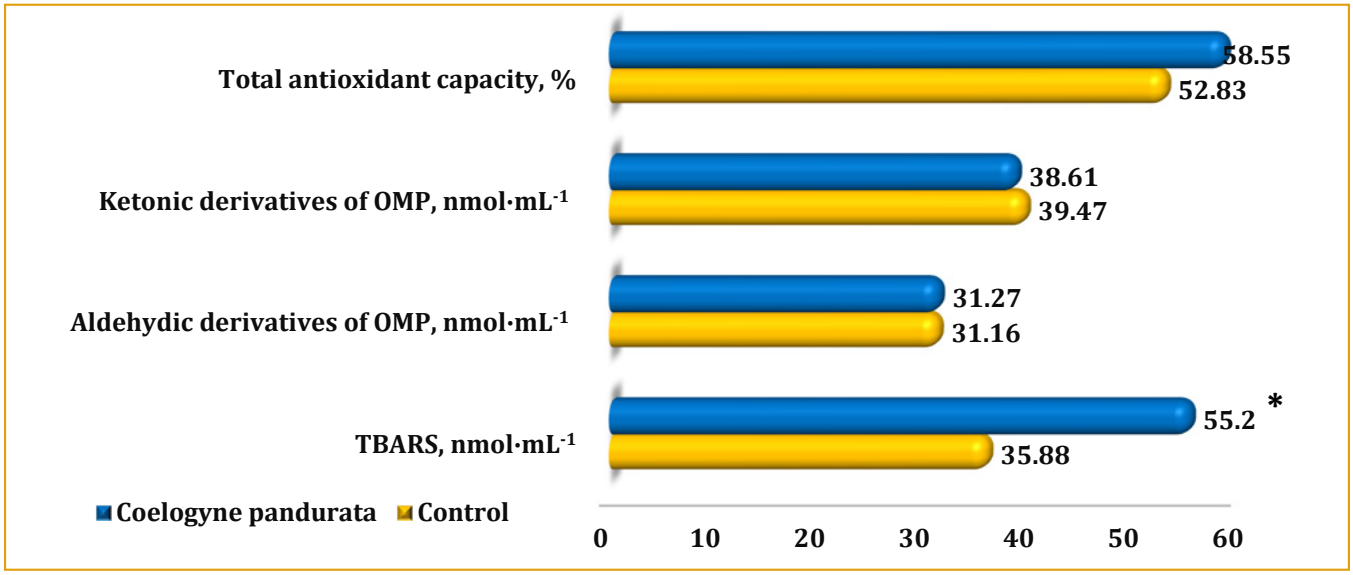

Figure 2 Levels of TBARS, aldehydic and ketonic derivatives of OMP, total antioxidant capacity in the equine erythrocytes after *in vitro* incubation with an extract derived from leaves of *Coelogyne pandurata* Lindl.
*– changes were statistically significant ($p < 0.05$) compared to untreated control

compared to the untreated samples 52.83 ±3.38%). A percentage of non-significant increase was 10.8% (p >0.05) (Figure 2).

Levels of TBARS, aldehydic and ketonic derivatives of OMP, and total antioxidant capacity (TAC) in the equine erythrocytes after *in vitro* incubation with an extract derived from pseudobulbs of *Coelogyne* pandurate were presented in Figure 3.

Our results revealed that extract derived from the pseudobulbs of *C. pandurata* after incubation with erythrocyte samples caused to increase in the TBARS level (45.05 ±4.74 nmol.mL^{-1}) (by 25.6%, p <0.05) compared to untreated samples (35.88 ±3.02 nmol.mL^{-1}). On the other hand, the content of aldehydic derivatives of OMP in the erythrocyte samples after incubation with an extract derived from the pseudobulbs of *C. pandurata* was not altered (30.97 ±1.23 nmol.mL^{-1}) compared to the untreated samples (31.16 ±1.89 nmol·mL^{-1}). Moreover, the content of ketonic derivatives of OMP in the erythrocyte samples after incubation with extracts derived from the pseudobulbs of *C. pandurata* was non-significantly decreased (by 10.9%, p >0.05). A non-significant increase in the TAC level of the tested samples incubated with an extract derived from the pseudobulbs of *C. pandurata* was observed (54.68 ±2.69% compared to the untreated samples 52.83 ±3.38%) (Figure 3).

The antioxidant efficacy of some orchids was reported by some researchers using *in vitro* and *in vivo* models. For example, *in vitro* free radical scavenging, LCMS-based metabolic profiling, and anti-inflammatory activity of *Dendrobium macrostachyum* Lindl. were studied by Sukumaran and Yadav (2016). The results showed a relatively high concentration of phenolics, high scavenger activity, and high anti-inflammatory activity of the stem extract compared to the leaf extract (Sukumaran and Yadav, 2016). Paudel et al. (2019) assessed of antioxidant and cytotoxic activities of stem extracts of *Dendrobium crepidatum* Lindl. & Paxton. The above extracts showed antioxidant and cytotoxic properties using the DPPH (2,2-diphenyl-1-picrylhydrazyl) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays, due to the presence of tetracosane, triacontane, stigmasterol, and some phenol derivatives (2-methoxy-4-vinylphenol, 2-methoxy-5-(1-propenyl)-phenol, *p*-mesyloxyphenol, and 2,6-dimethoxy-4-(2-propenyl)-phenol) (Paudel et al., 2019). Also, *Dendrobium moniliforme* (L.) Sw. extracts contain a number of bioactive compounds which exhibit both antioxidant and cytotoxic activities against free radicals and cancer cell lines respectively (Paudel et al., 2018). The volatile fractions from fresh inflorescences of naturally growing orchids *Anacamptis coriophora* (L.) R. M. Bateman, Pridgeon & M. W. Chase subsp. fragrans (Pollini), *Anacamptis pyrimidalis* (L.) R., *Ophrys holosericea* (Burm.) Greuter and *Serapias vomeracea* (Burm. f.) B. were isolated and analyzed in the study of Robustelli Della Cuna et al. (2019). These volatile compounds may represent a particular feature of these plant species, playing a critical role in the interaction with pollinators. DPPH assay evaluating the antioxidant activity of the essential oils was carried

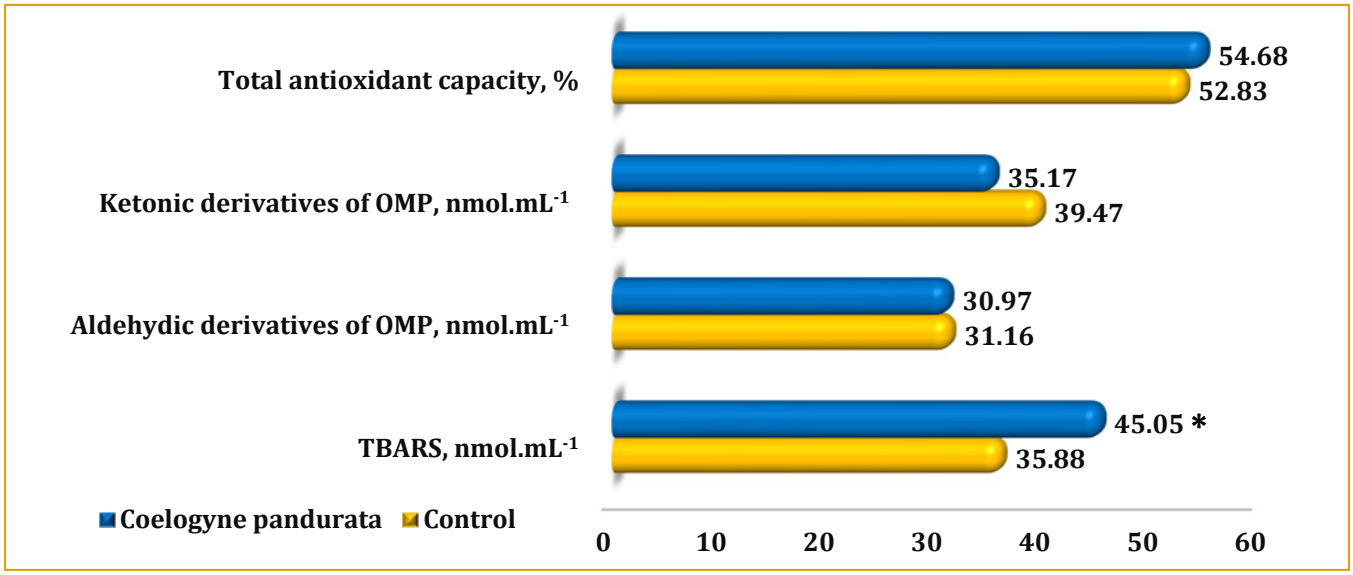

Figure 3 Levels of TBARS, aldehydic and ketonic derivatives of OMP, total antioxidant capacity in the equine erythrocytes after *in vitro* incubation with an extract derived from pseudobulbs of *Coelogyne pandurata* Lindl.
*– changes were statistically significant (p <0.05) compared to untreated control

out, showing a dose-dependent antioxidant activity (Robustelli Della Cuna et al., 2019).

The antioxidative properties of flowers and the aboveground part of *Anacamptis pyrimidalis* L. from Vojvodina were studied by Stajner et al. (2010). Activities of antioxidant enzymes (superoxide dismutase, catalase, peroxidase, and glutathione peroxidase), quantities of malondialdehyde, superoxide, hydroxyl radicals, and reduced glutathione and also the contents of chlorophylls a and b, carotenoids and soluble proteins were determined. The results of these researchers indicated that the aboveground part of the plant exhibited higher antioxidant activity due to low MDA and lipofuscin pigment accumulation, higher scavenging activity, and antioxidant capacity (Stajner et al., 2010). *In vitro* antidiabetic, antioxidant activities, and GC-MS analysis of *Rhynchostylis retusa* (L.) Blume and *Euphorbia neriifolia* L. leaf extracts were revealed by Kumar et al. (2021). This study revealed significant inhibition of α-amylase activity and retardation in glucose diffusion with *E. neriifolia* and *R. retusa* extract in a dose-dependent manner, depending on the extraction solvent. In addition, GC–MS analysis of methanolic, aqueous, and petroleum ether extracts suggested the presence of diverse phytochemical entities with known anti-inflammatory, and antioxidant properties, possibly implicated for use in diabetic conditions (Kumar et al., 2021).

We also 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 *Coelogyne brachyptera* Rchb.f. (Buyun et al., 2022). 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 (Buyun et al., 2022).

Conclusions

The aim of the current study was to investigate the antioxidant properties of extracts derived from leaves and pseudobulbs of *C. pandurata* using biomarkers of oxidative stress in the equine erythrocytes after *in vitro* treatment with the extracts. The obtained results demonstrated the prooxidative activity of *C. pandurata* extract used in the studied dose (5 $mg.mL^{-1}$) on the equine erythrocytes. Extracts derived from the leaves and pseudobulbs of *C. pandurata* after incubation with equine erythrocyte samples caused to increase in the TBARS level compared to untreated samples remaining with the TAC level at a high level as compared to the group treated by phosphate buffer (controls), while levels of aldehydic and ketonic derivatives of OMP were unchanged. Future studies will be conducted to evaluate dose-dependent changes in the levels of oxidative stress biomarkers after incubation with extracts derived from *C. pandurata* using various cell models. Moreover, the plant compound profile characteristics and antioxidant activity of different *Coelogyne* plants may encourage the wider use of these orchids in the development of new medicinal substances in medicine and veterinary.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical statement

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

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