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Lipid peroxidation and total antioxidant capacity in the muscle tissue of atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus* Mitchill) after *in vitro* treatment by extracts derived from various species of *Dracaena* genus (Asparagaceae Juss.)

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Water extracts from selected *Dracaena* plants cultivated in greenhouse conditions were evaluated for antioxidant properties by *in vitro* methods using the muscle tissue of Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus* Mitchill). The level of 2-thiobarbituric acid reactive substances (TBARS) and total antioxidant capacity (TAC) in the muscle tissue of Atlantic sturgeon after treatment *in vitro* by extracts derived from leaves of selected *Dracaena* plants (in final concentration 10 mg.mL^{-1}) were assessed. When muscle tissue was incubated with leaf extracts of various species belonging to the *Dracaena* genus, the TBARS level was significantly increased for the sixteen extracts studied. Moreover, all extracts (except *D. singularis* extract) increase the formation of TBARS in the extracts-treated muscle tissue, and these results were statistically significant. Treatment of muscle tissue by extracts derived from various species from the *Dracaena* genus revealed also increase the TAC level. When homogenates were incubated with leaf extracts derived from various species from the *Dracaena* genus, the TAC level was significantly increased for the fifteen extracts studied. Moreover, all extracts (except *D. hyacinthoides* and *D. roxburghiana* extracts) induced the TAC increase in the extracts-treated muscle tissue of Atlantic sturgeon, and these increases were statistically significant. It can be supposed that secondary plant metabolites, i.e. polyphenolic compounds, alkaloids, flavonoids, saponins, glycosides, terpenoids, tannins, etc., in extracts derived from the leaves of various species belonging to the *Dracaena* genus, may contribute to their antioxidant activity. Further detailed studies on the effect of extracts derived from leaves of selected *Dracaena* plants on long time intervals, antioxidant, and molecular aspects are necessary to understand the mechanism of action of extracts in other fish and animals.

Keywords: 2-thiobarbituric acid reactive substances, total antioxidant capacity, Atlantic sturgeon, muscle tissue, *in vitro*

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Fish and fish products are the most valuable agricultural commodity traded internationally. Their annual sales are nearly US$ 80 billion and increasing each year (FAO-FishStat, 2006). The diseases in aquaculture are the most serious constraint that causes damage to the livelihood of farmers, loss of jobs, reduced incomes, and food insecurity (Assefa and Abunna, 2018). In recent years, to develop alternative practices for disease management in aquaculture, attention was diverted to finding the use of medicinal plant products as potential therapeutic measures for modulating the immune response and, specifically, the use of herbs to prevent and control fish diseases (Galina et al., 2009). Plant product application in aquaculture to combat microbial and parasitic diseases is considered an alternative approach for sustainable aquaculture, which is one of the promising alternatives to antibiotics (Dawood et al., 2021; Su et al., 2021).

The use of herbal therapy within animal production, as well as in the diet of commercial fish has shown promise, in that it is natural and biodegradable and has antimicrobial activity against various pathogens, including those relating to fish (Valladão et al., 2015). The herbals having the characteristics of immunostimulants have been able to increase survival and reduce the pathogenic load against pathogenic challenges by improving the immune system in fish (Anusha et al., 2014). However, applying a new component as a therapeutic drug in the fish diet requires more research on the effects on the physiological condition, biochemical changes in the cells, as well as health welfare of animals. Certainly, a healthy diet and safe feed are important factors in the prevention of widespread various diseases in aquaculture. Therefore, the study of diet components such as dietary supplements, particularly drugs, is an essential approach in aquaculture and fishery (Banaee et al., 2011).

In this study, attention was focused on *Dracaena*, a genus with diverse ethnobotanical uses in its geographical distribution range, which occupies an important place among plant genera applied for the treatment of a broad spectrum of diseases and disorders (Khalumba et al., 2005; Staples and Herbst, 2005; Kiringe, 2006; Owuor and Kisangau, 2006; Takawira-Nyenya et al., 2014). This is a historically recognized genus of flowering plants, now included in the genus *Dracaena* on the basis of molecular phylogenetic studies (Archibald et al., 2015).

The genus *Dracaena* consists of more than 100 accepted species which are mainly distributed in the tropics and subtropics, especially in Africa, Australia, and Southern Asia (Thu et al., 2020, 2021). They are mainly succulent shrubs and trees, and a few are commonly grown as shrubby houseplants, especially the variegated forms (Thu et al., 2020). Leaves and rhizomes of these plants are used in folk medicine for treating asthma, cough, sexual weakness, hypertension, diarrhea, hemorrhoids, abdominal pains, colics, eczema, piles, edema, jaundice, anuria, palpitations, viral hepatitis, malaria, snake- and insect bites, etc. (Andhare et al., 2012; Kpodar et al., 2016; Giovannini and Howes, 2017; Thu et al., 2021). Besides the medicinal aspects, several *Dracaena* species have great horticultural importance and are commercialized for use in landscaping and as indoor ornamental plants (Lekawatana and Suwannamek, 2017). Moreover, it has been reported that *Dracaena* species can be used as bioindicators for the control of increasing air pollution in urban cities (Saxena and Ghosh, 2013).

Our previous study (Buyun et al., 2016, Tkachenko et al., 2017a) has highlighted the antibacterial capacity of ten species of *Sansevieria* genus against *Staphylococcus aureus*. These plants have been screened in order to validate scientifically the inhibitory activity for microbial growth attributed to their popular use and to propose new sources of antimicrobial agents. Our results proved that the zones of inhibition ranged from 16 to 34 mm. Extracts from the leaves of *S. fischeri* and *S. francisii* were particularly active against the tested organisms (inhibition zones comprise up to 34 mm in diameter). This was followed by the activities of extracts from the *S. parva*, *S. kirkii*, *S. aethiopica*, *S. caulescens*, *S. metallica* leaves (diameters of inhibition zones ranged from 25 to 31 mm). The ethanolic extracts of *S. canaliculata* and *S. trifasciata* showed less antimicrobial activity (diameters of inhibition zones ranged between 16 and 16.5 mm). The results proved that the ethanolic extracts from *S. fischeri*, *S. francisii*, *S. parva*, *S. kirkii*, *S. aethiopica*, *S. caulescens*, *S. metallica* exhibit a favorable antibacterial activity against *S. aureus* (Buyun et al., 2016; Tkachenko et al., 2017a).

Although antimicrobial and antioxidant activities of extracts derived from leaves of various species belonging to the *Dracaena* genus were investigated (Al-Fatimi et al., 2007; Buyun et al., 2016, 2017; Maryniuk et al., 2017, 2018, 2019; Tkachenko et al., 2017a, 2017b, 2018, 2019a, 2019b), studies regarding their protective effects against free radical-induced oxidative stress in the muscle tissue of Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus* Mitchill) have not yet been undertaken.

Therefore, the aim of the current study was to evaluate *in vitro* the effect of buffer extracts derived from leaves of selected species belonging to the *Dracaena* genus (in final concentration 10 mg.mL^{-1}) against lipid peroxidation and protein damage using 2-thiobarbituric acid reactive substances and total antioxidant capacity.

Materials and methodology

Collection of plant materials and preparation of plant extracts

The leaves of plants, cultivated at glasshouse conditions, were sampled at M.M. Gryshko National Botanical Garden (NBG), National Academy of Science of Ukraine. The leaves of *Dracaena aethiopica* (Thunb.) Byng & Christenh., *D. canaliculata* (Carrière) Byng & Christenh, *D. caulescens* (N.E.Br.) Byng & Christenh, *D. angolensis* (Welw. ex Carrière) Byng & Christenh, *D. dooneri* (N.E.Br.) Byng & Christenh, *D. singularis* (N.E.Br.) Byng & Christenh, *D. francisii* (Chahin.) Byng & Christenh, *D. forscaliana* (Schult. & Schult. f.) Byng & Christenh, *D. serpenta* Byng & Christenh, *D. hyacinthoides* (L.) Mabb., *D. volkensii* (Gürke) Byng & Christenh, *D. pethera* Byng & Christenh, *D. zebra* Byng & Christenh, *D. parva* (N.E.Br.) Byng & Christenh, *D. roxburghiana* (Schult. Schult.f.) Byng & Christenh, *D. suffruticosa* (N.E.Br.) Byng & Christenh, *D. trifasciata* (Prain) Mabb. were sampled for study. Freshly collected leaves were washed, weighed, crushed, and homogenized in 0.1 M sterile phosphate buffer solution (pH 7.4) (in proportion 1 : 19, w/w) at room temperature. The extracts were filtered and investigated for their antioxidant activity.

Tissue samples and experimental design

Clinically healthy Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus* Mitchill) with a mean body mass of 450–500 g were used in the experiments obtained from the Department of Salmonid Research, Stanislaw Sakowicz Inland Fisheries Institute (Rutki, Poland). The muscle tissue samples derived from fish were homogenized in ice-cold buffer (100 mM Tris-HCl, pH 7.2) using a glass homogenizer immersed in an ice water bath. Homogenates were centrifuged at 3000 rpm for 15 min at 4 °C. After centrifugation, the supernatants were collected and frozen at -25 °C until analyzed. All enzymatic assays were carried out at 21 ±0.5 °C using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany) in duplicate. The reactions were started by adding the tissue supernatants.

The supernatant of the muscle tissue was used to incubate with extracts of various species of *Dracaena* genus (in a ratio of 9 : 1) at room temperature. The control group (muscle tissue) was incubated with 100 mM Tris-HCl buffer (pH 7.2) (in a ratio of 9 : 1). The incubation time was 2 h. Biomarkers of lipid peroxidation and total antioxidant capacity, were studied in the incubated homogenates (control group and in samples with extracts of various species of *Dracaena* genus).

Determination of 2-thiobarbituric acid reactive substances (TBARS)

The level of lipid peroxidation was determined by quantifying the concentration of TBARS by Kamyshnikov (2004) for determining the malonic dialdehyde (MDA) concentration. Briefly, 2.1 mL of sample homogenate was added to 1 mL of 20% of trichloroacetic acid (TCA), and 1 mL of 0.8% of 2-thiobarbituric acid (TBA). The mixture was heated in a boiling water bath for 10 min. After cooling, the mixture was centrifuged at 3,000 rpm for 10 min. The absorbance of the supernatant was measured at 540 nm. The concentration of MDA (nmol. mg^{-1} of protein) was calculated using $1.56.10^5$ mM^{-1}. cm^{-1} as the extinction coefficient.

Measurement of total antioxidant capacity (TAC)

The TAC level in the 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). Sample inhibits the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. 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 samples.

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 (significance level, $p < 0.05$) was examined using the Mann-Whitney *U* test (Zar, 1999). All statistical calculation was performed on separate data from each individual with STATISTICA 8.0 software (StatSoft Polska Sp. z o.o., Krakow, Poland).

Results and discussion

A progressive accumulation of oxidative-induced damage to important cellular molecules resulting in oxidative stress and lipid peroxidation and protein damage is involved in various and numerous physiological and pathological states, i.e. senescence, inflammation, atherosclerosis, neurodegenerative diseases, cancer, etc. (Praticò, 2002; Guéraud et al., 2010). Oxidative damage occurs when free radicals produced within an organism are not completely destroyed by the appropriate endogenous defense systems. Because lipids are a major component of living organisms and probably the first easy target of free radicals once they are produced, lipid peroxidation might play an important role in initiating and/or mediating some aspects of the pathological processes (Praticò, 2002).

The first stage of our study was the assessment of 2-thiobarbituric acid reactive substances in the muscle tissue of Atlantic sturgeon after treatment *in vitro* by extracts derived from leaves of selected *Dracaena* plants.

When muscle tissue was incubated with leaf extracts of various species belonging to the *Dracaena* genus, the TBARS level was significantly increased ($p < 0.05$) for sixteen extracts studied (Figure 1). Moreover, all extracts (except *D. singularis* extract) increase the formation of TBARS in the extracts-treated muscle tissue, and these results were statistically significant. The most potent effect was demonstrated for the extracts derived from *D. angolensis* (TBARS increased by 154.9%, $p < 0.05$), *D. francisii* (by 143.7%, $p < 0.05$), *D. hyacinthoides* (by 136.2%, $p < 0.05$), *D. canaliculata* (by 128.7%, $p < 0.05$), *D. roxburghiana* (by 116.6%, $p < 0.05$), *D. aethiopica* (by 109.1%, $p < 0.05$). Also, extracts derived from *D. caulescens* (by 48.9%, $p < 0.05$), *D. suffruticosa* (by 30.1%, $p < 0.05$), *D. zebra* (by 81.3%, $p < 0.05$), *D. pethera* (by 63.9%, $p < 0.05$), *D. trifasciata* (by 45.9%, $p < 0.05$), *D. forscaliana* (by 49.7%, $p < 0.05$), *D. singularis* (by 10.6%, $p < 0.05$), *D. dooneri* (by 80.5%, $p < 0.05$), *D. volkensii* (by 54.2%, $p < 0.05$), *D. serpenta* (by 45.2%, $p < 0.05$), and *D. parva* (by 96.3%, $p < 0.05$) exhibited increase in the TBARS level in the muscle

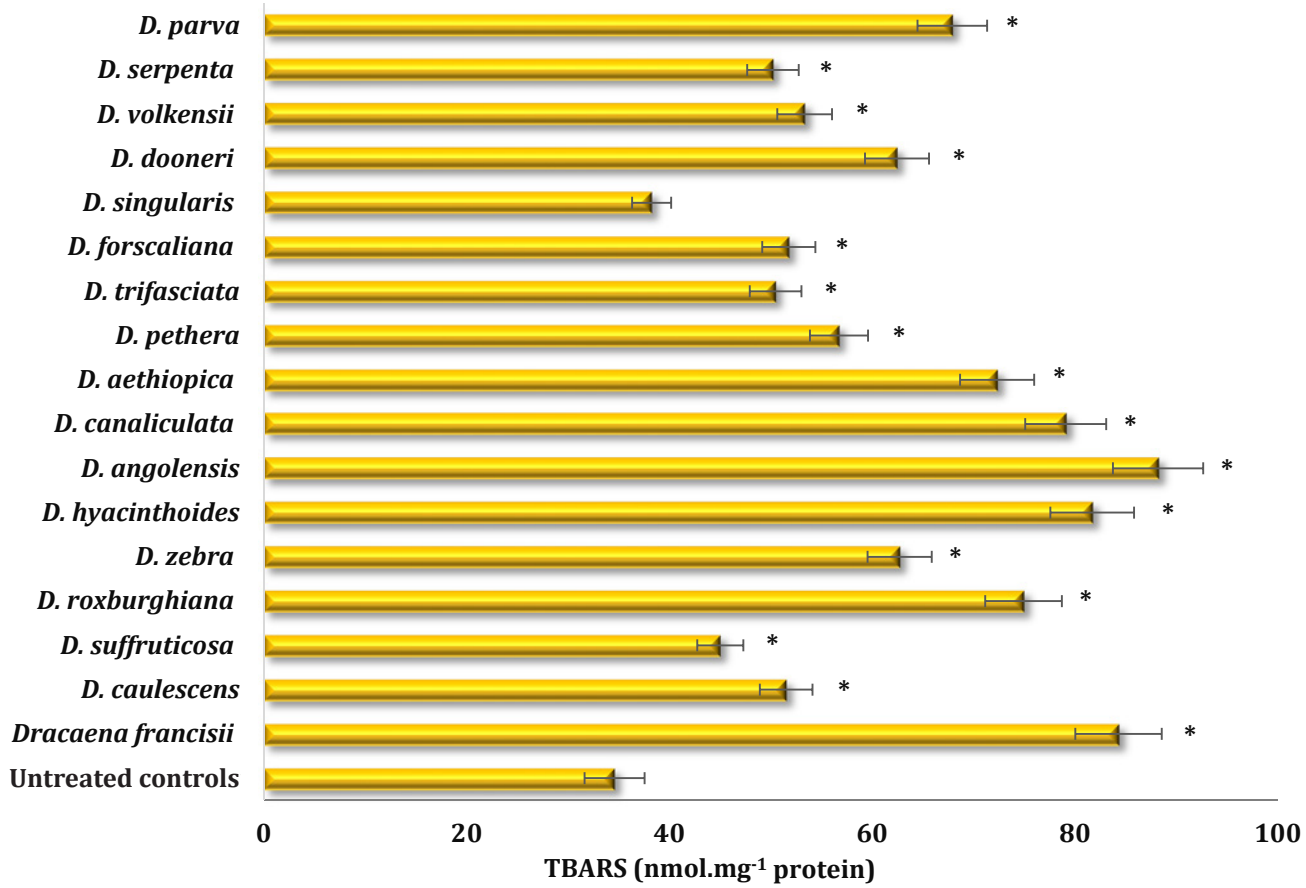

Figure 1 The level of 2-thiobarbituric acid reactive substances (TBARS) in the muscle tissue of Atlantic sturgeon after treatment *in vitro* by extracts derived from leaves of selected *Dracaena* plants (M ±m, n = 6)
Results are presented as the mean (M) ± the standard error of the mean (S.E.M.)
* the changes are statistically significant ($p < 0.05$) compared to the untreated control group

tissue compared to phosphate buffer as a control samples (Figure 1).

One of the strategies most commonly used to assess a free radical-antioxidant balance in chemical and biological systems is the determination of the total antioxidant capacity (Fraga et al., 2014). The measure of antioxidant capacity considers the cumulative action of all antioxidants present in plasma and body fluids, thus providing an integrated parameter rather than the simple sum of measurable antioxidants. The capacity of known and unknown antioxidants and their synergistic interaction is therefore assessed, thus giving an insight into the delicate balance *in vivo* between oxidants and antioxidants (Ghiselli et al., 2000). Thus, the next step of our study was the evaluation of the total antioxidant capacity in the muscle tissue of Atlantic sturgeon after treatment *in vitro* by extracts derived from leaves of selected *Dracaena* plants.

Treatment of muscle tissue by extracts derived from various species from the *Dracaena* genus revealed also increase the TAC level. When homogenates were incubated with leaf extracts derived from various species from the *Dracaena* genus, the TAC level was significantly increased ($p < 0.05$) for fifteen extracts studied. Moreover, all extracts (except *D. hyacinthoides* and *D. roxburghiana* extracts) induced the TAC increase in the extracts-treated muscle tissue of Atlantic sturgeon, and these increases were statistically significant (Figure 2).

The most potent effects were demonstrated for leaf extracts derived from various species belonging to the *Dracaena* genus, i.e. *D. singularis* (by 78%, $p < 0.05$), *D. serpenta* (by 78%, $p < 0.05$), *D. zebra* (by 71%, $p < 0.05$), *D. volkensii* (by 68%, $p < 0.05$), *D. caulescens* (by 69.5%, $p < 0.05$), *D. forscaliana* (by 61.1%, $p < 0.05$), *D. trifasciata* (by 58.8%, $p < 0.05$), *D. pethera* (by 57.3%, $p < 0.05$), and *D. suffruticosa* (by 53.4%, $p < 0.05$) compared to the control samples. Also, leaf extracts of *D. francisii* (by 41.1%, $p < 0.05$), *D. dooneri* (by 39.6%, $p < 0.05$), *D. angolensis* (by 30.4%, $p < 0.05$), *D. aethiopica* (by 18.9%, $p < 0.05$), *D. canaliculata* (by

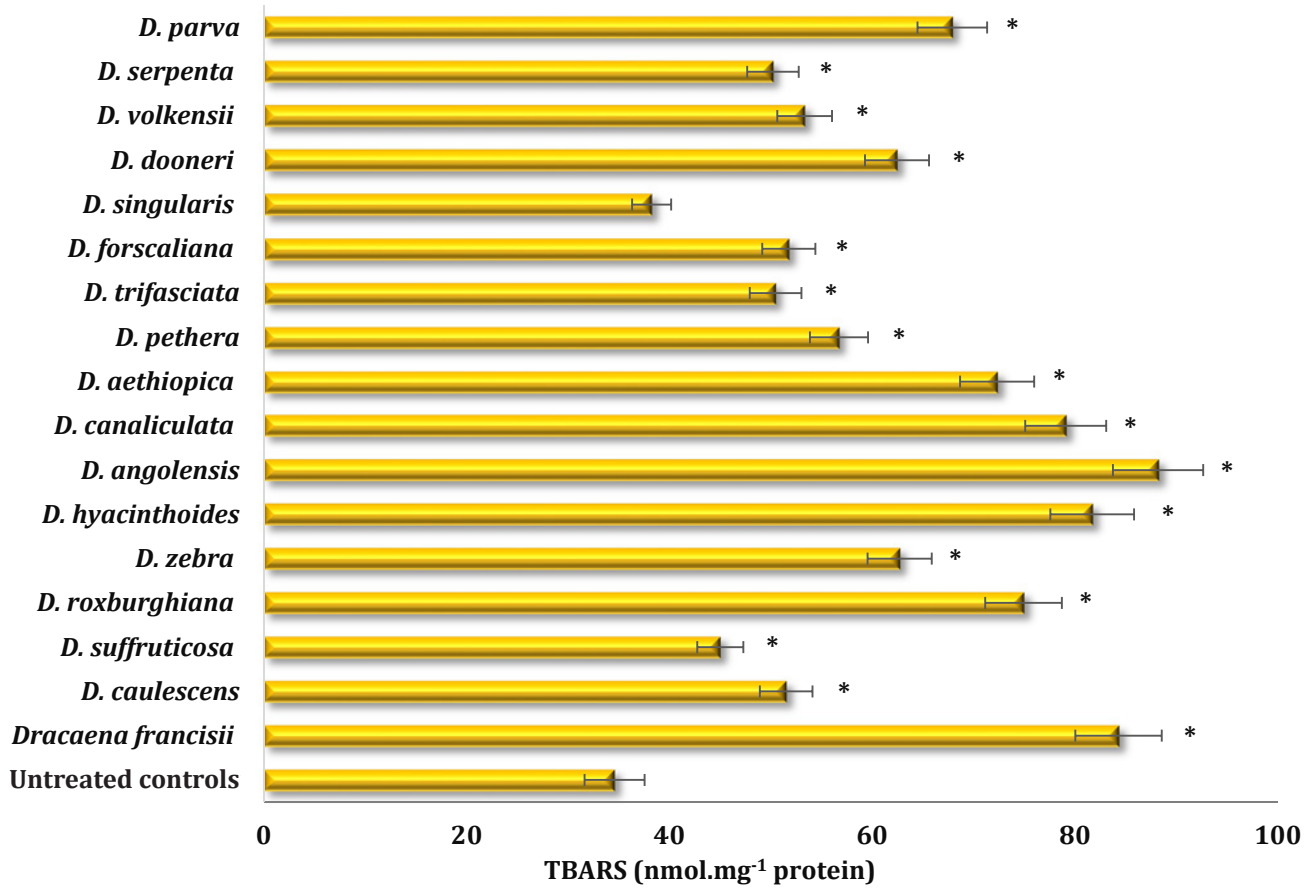

Figure 2 The level of total antioxidant capacity (TAC) in the muscle tissue of Atlantic sturgeon after treatment *in vitro* by extracts derived from leaves of selected *Dracaena* plants (M ±m, n = 6)
Results are presented as the mean (M) ± the standard error of the mean (S.E.M.)
* the changes are statistically significant ($p < 0.05$) compared to the untreated control group

14.3%, p <0.05), *D. roxburghiana* (by 13.5%, p <0.05), and *D. hyacinthoides* (by 12.8%, p <0.05) also increase the TAC level in the muscle tissue of Atlantic sturgeon compared to control samples (Figure 2).

In our previous study, we also studied the antioxidant activity of extracts obtained from leaves of selected species from *Sansevieria* species against oxidative stress using equine erythrocyte suspension (Tkachenko et al., 2017b). When erythrocytes were incubated with leaf extracts of various species from the *Sansevieria* genus, the aldehydic derivatives level was significantly reduced by 13.6% (p <0.05) for *S. forskaliana* extract. Moreover, all extracts (except *S. francisii* extract) reduced the formation of intracellular aldehydic derivatives of oxidatively modified proteins in the extracts-treated erythrocytes, but these results were non-significant. Treatment by extracts of various *Sansevieria* species reduced the concentration of ketonic derivatives of OMP when compared to untreated erythrocytes. The most potent effect was demonstrated by the *S. canaliculata*, *S. forskaliana*, *S. aethiopica*, *S. cylindrica*, *S. metallica*, *S. hyacinthoides*, and *S. kirkii* compared to control samples (phosphate buffer) (16.1%, 14.7%, 13.4%, 12.9%, 12.9%, 12.7%, 12.1%, respectively). However, there were no significant changes in other extracts. The experimental evidence obtained in our previous study indicated that various species of *Sansevieria* genus are a rich source of compounds that manifest antioxidant activity and can effectively protect erythrocytes against oxidative-induced damage. Thus, *S. canaliculata*, *S. forskaliana*, *S. aethiopica*, *S. cylindrica*, *S. metallica*, *S. hyacinthoides*, and *S. kirkii* may be a valuable source of natural antioxidants that may potentially be recommended for applications in medicine and veterinary practice. According to the above-mentioned antioxidant mechanisms, extracts of various species from the *Sansevieria* genus may inhibit the formation of protein carbonyl by scavenging free radicals formed *in vitro*. According to many supporting documents, it can be assumed that secondary plant metabolites, i.e. polyphenolic compounds in extracts of various species from *Sansevieria* genus extract may contribute to their antioxidant activity (Tkachenko et al., 2017b).

Really, the study of *D. roxburghiana* and *D. trifasciata* has revealed the presence of important compounds which were separated by thin layer chromatography (Kingsley et al., 2013). Preliminary phytochemical screening of the extracts of *D. trifasciata* plant showed the presence of alkaloids, flavonoids, saponins, glycosides, terpenoids, tannins, proteins and carbohydrates (Anbu et al., 2009). Additionally, the methanolic extract of the whole plant of *D. trifasciata* has yielded 12 steroidal saponins, 10 of which are new constituents (Mimaki et al., 1996). Phytochemical analysis of the whole plant of *D. trifasciata* has resulted in the isolation of four new pregnane glycosides (Mimaki et al., 1997). Gas chromatographic analysis of the leaves revealed the presence of alkaloids, allicins, glycosides, and saponins (Ikewuchi et al., 2011). *Dracaena* and *Sansevieria* species are rich sources of steroidal saponins and have intriguing structures and interesting biological properties, including high cell antiproliferative/cytotoxic and anti-inflammatory activities. Several bioactive saponins from *Dracaena* and *Sansevieria* have the potential to become lead compounds for the development of anticancer therapeutic agents (Thu et al., 2021).

We suggested that the high TAC value in the muscle tissue of Atlantic sturgeon after treatment *in vitro* by extracts derived from leaves of selected *Dracaena* plants is the result of the high content of by-products, i.e. alkaloids, flavonoids, saponins, glycosides, terpenoids, tannins, proteins, carbohydrates, etc. in the plant extracts. Thu et al. (2021) have reviewed the literature of about 180 steroidal saponins, isolated from *Dracaena* and *Sansevieria* species, as a basis for further studies. Saponins are among the most characteristic metabolites isolated from the two genera. They show a great variety in structural motifs and a wide range of biological activities, including anti-inflammatory, anti-microbial, anti-proliferative effects and, in most cases, remarkable cytotoxic properties (Thu et al., 2021). Saponins, an important group of bioactive plant natural products, are glycosides of triterpenoid or steroidal aglycones. Accumulated evidence suggests that saponins have significant neuroprotective effects on attenuation of central nervous system disorders, such as stroke, Alzheimer‘s disease, Parkinson‘s disease, and Huntington‘s disease. The proposed mechanisms of their neuroprotective function include antioxidant, modulation of neurotransmitters, anti-apoptosis, anti-inflammation, attenuating Ca^{2+} influx, modulating neurotrophic factors, inhibiting tau phosphorylation, and regeneration of neural networks (Sun et al., 2015).

The chemical structures of flavonoids support their capacity to scavenge free radicals and chelate redox-active metals. The thermodynamic analysis predicts that both, scavenging of oxygen-derived radicals and the sequestration of redox-active metals are energetically favored. Lipid-flavonoid and protein-flavonoid interactions can indirectly mediate a decrease in oxidant (free radical) production and/or oxidative damage to both cell and extracellular

components (Galleano et al., 2010). Also, biologically active isoquinoline alkaloids exhibit a broad range of bioactivities, including antitumor, antidiabetic and its complications, antibacterial, antifungal, antiviral, antiparasitic, insecticidal, anti-inflammatory, antioxidant, neuroprotective, and other activities (Shang et al., 2020). Moreover, many sesquiterpene's biological activities (anti-inflammatory, antiparasitic and anti-carcinogenic activities) are based on antioxidant or pro-oxidant actions of sesquiterpenes. Structure, concentration, metabolism as well as the type of cells determine if sesquiterpene acts as an antioxidant or prooxidant (Bartikova et al., 2014). On the other hand, the natural flavones, as well as some of their synthetic derivatives, have been shown to exhibit several biological activities, including antioxidant, anti-inflammatory, antitumor, anti-allergic, neuroprotective, cardioprotective, and antimicrobial (Catarino et al., 2015). Also, flavonoids are found to influence several mammalian enzymes like protein kinases that regulate multiple cells signaling pathways and alterations in multiple cellular signaling pathways are frequently found in many diseases (Singh et al., 2014). Some flavones interfere in distinct oxidative-stress related events by directly reducing the levels of intracellular free radicals (hydroxyl, superoxide, and nitric oxide) and/or of reactive species (e.g. hydrogen peroxide, peroxynitrite, and hypochlorous acid) thus preventing their amplification and the consequent damage of other biomolecules such as lipids, proteins, and DNA (Catarino et al., 2015). Flavones and flavonols re-establish the redox regulation of proteins, transcription factors and signaling cascades that are otherwise inhibited by elevated oxidative stress (Dajas et al., 2013). Flavones can also hinder the activity of central free radical-producing enzymes, such as xanthine oxidase and nicotinamide adenine dinucleotide phosphate oxidase (NADPH-oxidase) or inducible nitric oxide synthase (iNOS) and can even modulate the intracellular levels of pro-oxidant and/or antioxidant enzymes (Catarino et al., 2015).

Conclusions

The present findings revealed that the extracts derived from various species belonging to the *Dracaena* genus have exhibited remarkable antioxidant potential in increasing the total antioxidant capacity in the muscle tissue of Atlantic sturgeon after *in vitro* treatment by extracts in a final concentration of 10 $mg.mL^{-1}$. On the other hand, according to the above-mentioned antioxidant mechanisms, extracts of various species from the *Dracaena* genus resulted in increasing the formation of lipid peroxidation using the measurement of TBARS as biomarkers of these processes. Thus, future studies are needed to assess the dose- and time-dependent changes in the antioxidant capacity in the different cell models after treatment by extracts derived from various species belonging to the *Dracaena* genus. It can be supposed that secondary plant metabolites, i.e. polyphenolic compounds, alkaloids, flavonoids, saponins, glycosides, terpenoids, tannins, etc., in extracts derived from the leaves of various species belonging to the *Dracaena* genus, may contribute to their antioxidant activity.

Conflicts of interest

The authors declare no conflict of interest.

Ethical statement

This article does not contain any studies that would require an ethical statement.

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