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IN VITRO ANTIOXIDANT RESPONSE OF MUSCLE TISSUE OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM) TREATED WITH EXTRACT FROM LEAVES OF EUROPEAN MISTLETOE (*VISCUM ALBUM* L.)

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Many studies, based on the understanding of the relationship between the generation of reactive oxygen species and antioxidant defences, propose a link with the recent use of mistletoe based on its antioxidant properties, supported by phytochemical and pharmacological data. The uniqueness of mistletoe metabolism, a direct consequence of its hemiparasitism, is used as a key interpretive element to explain its biological properties and guide its consequent therapeutic use.The aim of the present study was to determine the antioxidant activity of extracts derived from the leaves of Viscum album L. using biomarkers of oxidative stress [2-thiobarbituric acid reactive substances (TBARS) as a biomarker of lipid peroxidation, carbonyl derivatives of oxidative modification of proteins, total antioxidant capacity (TAC)] in the muscle tissue of rainbow trout (Oncorhynchus mykissWalbaum) after in vitro treatment with extracts at two final concentrations (5 and 2.5 mg/mL). Our study revealed that treatment with V. album leaf extracts resulted in non-significant changes in TBARS levels in the muscle tissue after in vitro incubation with V. album leaf extracts at final concentrations of 5 and 2.5 mg/mL.The levels of aldehydic derivatives of oxidatively modified proteins in rainbow trout muscle tissue after treatment with V. album leaf extracts at final concentrations of 5 mg/mL were at the same levels as in untreated controls. When muscle tissue was incubated with V. album leaf extracts, the levels of ketonic derivatives were significantly reduced after treatment with extracts at a final concentration of 5 and 2.5 mg/mL compared to untreated samples. TAC levels in rainbow trout muscle tissue were increased after in vitro incubation with V. album leaf extracts (at final concentrations of 5 and 2.5 mg/mL) compared to untreated samples. The results of the current study demonstrated the antioxidant properties of extracts from the leaves of V. album at two final concentrations (5 and 2.5 mg/mL) after incubation with rainbow trout muscle tissue.Further studies should focus on the antioxidant effect of extracts derived from the leaves of Viscum album using other cellular models.

Keywords: *European mistletoe (Viscum album L.), rainbow trout (Oncorhynchus mykiss Walbaum), extracts, 2-thiobarbituric acid reactive substances (TBARS), carbonylderivatives of oxidative modification of proteins, total antioxidant capacity (TAC).*

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ВІДПОВІДЬ АНТИОКСИДАНТНОЇ СИСТЕМИ М'ЯЗОВОЇ ТКАНИНИ ФОРЕЛІ (*ONCORHYNCHUS MYKISS* WALBAUM), ОБРОБЛЕНОЇ ЕКСТРАКТОМ З ЛИСТЯ ОМЕЛИ ЄВРОПЕЙСЬКОЇ (*VISCUM ALBUM* L.) *IN VITRO*

Багато досліджень, які базуються на з'ясуванні зв'язку між утворенням активних
форм кисню (АФК) і антиоксидантним захистом, підтверджують використання омели,

базуючись на її антиоксидантних властивостях, підтверджених фітохімічними та фармакологічними результатами.Унікальність метаболізму омели білої, прямий наслідок її геміпаразитизму, використовується як ключовий елемент для пояснення її біологічних властивостей і для її подальшого терапевтичного використання. Метою даного дослідження було визначення антиоксидантної активності екстрактів, отриманих із листя Viscum album L., з використанням біомаркерів окиснювального стресу [речовини, які взаємодіють з 2-тіобарбітуровою кислотою (TBARS) як біомаркери перекисного окиснення ліпідів, карбонільні похідні окиснювальної модифікації. білків, загальна антиоксидантна активність (TAC)] у м'язовій тканині райдужної форелі (Oncorhynchus mykiss Walbaum) після інкубації in vitro з екстрактами у двох кінцевих концентраціях (5 та 2,5 мг/мл). Наше дослідження показало, що інкубація м'язової тканини з екстрактами листя V. album призвело до незначних змін рівнів TBARS у м'язовій тканині після інкубації in vitro з екстрактами листя V. album у кінцевих концентраціях 5 і 2,5 мг/мл. Рівні альдегідних похідних окиснювально модифікованих білків у м'язовій тканині райдужної форелі після інкубації з екстрактами листя V. album у кінцевій концентрації 5 мг/мл були на однаковому рівні, що й у необроблених контрольних зразках. Коли м'язову тканину інкубували з екстрактами листя V. album, рівні кетонових похідних були значно знижені після обробки екстрактами в кінцевій концентрації як 5, так і 2,5 мг/мл порівняно з необробленими зразками. Рівні TAC у м'язовій тканині райдужної форелі були підвищені після інкубації in vitro з екстрактами листя V. album (у кінцевих концентраціях 5 і 2,5 мг/мл) порівняно з необробленими зразками. Результати цього дослідження продемонстрували антиоксидантні властивості екстрактів з листя V. album у двох кінцевих концентраціях (5 та 2,5 мг/мл) після інкубації з м'язовою тканиною райдужної форелі. Подальші дослідження повинні бути зосереджені на вивченні антиоксидантних ефектів екстрактів, отриманих з листя Viscum album, з використанням інших клітинних моделей.

Ключові слова: *омела звичайна (Viscum album L.), форель райдужна (Oncorhynchus mykiss Walbaum), екстракти, речовини, які взаємодіють з 2-тіобарбітуровою кислотою (TBARS), карбонільні похідні окиснювальної модифікації білків, загальна антиоксидантна активність (TAC).*

INTRODUCTION

Oxidative stress is an imbalance between the production of free radicals and the body's ability to neutralise their effects. It is a major contributor to several chronic human diseases, such as atherosclerosis and cardiovascular disease, mutagenesis and cancer, several neurodegenerative diseases and the ageing process [43]. It is hypothesised that increasing the intake of dietary antioxidants may help maintain an acceptable antioxidant status and aid in disease prevention [29].

Radical scavenging activity and protective effects against oxidative stress caused by free radicals, nitric oxide and superoxide anion have been demonstrated for a number of mistletoe extracts and isolated lectins [22, 24, 39, 47]. Mistletoe (*Viscum album* L.) is an evergreen, perennial, branching shrub that parasitises the branches of trees. It is usually globular. Mistletoe leaves are pale green, leathery and thick. The flowers are yellowish-green and the berries are white and translucent when ripe. Inside are sticky black seeds. Mistletoe roots penetrate under the bark and into the wood, drawing moisture and minerals from the trees, but also synthesising organic substances themselves, which is why they are called semi-parasites. Depending on the tree on which the mistletoe grows, the plant has different properties, which are sometimes taken into account in the treatment [39, 41, 63]. Mistletoe parasitises many deciduous trees, i.e. poplars, limes, maples,

birches, willows, oaks, walnuts and sometimes fruit trees. Mistletoe is less common on conifers. The spread of mistletoe is disastrous because it dries out the tops of the trees on which it grows. When mistletoe infects fruit trees, it reduces their yield [5, 30]. The most popular species for clinical use are mistletoe parasites on fir, maple, almond, birch, hawthorn, ash, apple, pine, poplar, oak, willow, lime and elm [21].

Mistletoe contains a number of biologically active substances such as proteins commonly classified as viscotoxins and mistletoe lectins, nitrogenous and organic acids, flavonoids, phenolic acids, sterols, lignans, terpenoids, phenylpropanoids, gums, saponins and alkaloids [50, 51]. The latter are considered to be poisonous, so care must be taken when using the plant [9, 42, 46, 56].When used correctly, mistletoe soothes inflammation, tones and strengthens the body, calms the central nervous system, relieves irritation, strengthens the heart, and dilates and cleanses the blood vessels [15, 44].Extracts of *V. album* are widely used in complementary medicine for the treatment of cancer. In many preclinical and clinical studies, *V. album* extracts have been shown to exert immunomodulatory functions [4, 13, 57].

In folk medicine, mistletoe has long been used for epilepsy and hysteria because of its known ability to relieve convulsions [51]. Externally, mistletoe preparations have been used to treat rheumatism, gout and various abscesses [50]. Mistletoe decoctions and tinctures are also used to treat headaches [51], bleeding (haemorrhoids, pulmonary bleeding, gastrointestinal bleeding, uterine bleeding, prolonged menstruation) [50], helminth infections [54], rheumatism, gout, [59], wounds [11], abscesses, psoriasis [35], enteritis, gastritis, colitis [58], inflammation of the gastrointestinal mucosa [33], and nervous system disorders including epilepsy, hysteria, nervousness, hysterical psychosis, dizziness and headache [12, 14, 26, 32].

Mistletoe-based preparations improve cardiac function, dilate blood vessels, lower blood pressure, reduce excitability of the central nervous system and have an astringent, haemostatic effect [20, 31, 36, 52, 53].Mistletoe preparations are used to treat conditions such as hypertension, intestinal atony, rare forms of neuralgia and angina pectoris [18, 45, 49]. They are effective against staphylococcal and streptococcal infections and viral diseases [16, 19, 34, 55]. In dermatology, they are used as antipruritic, antimicrobial and antiseptic agents [50].

The aim of the present study was to determine the antioxidant activity of extracts from the leaves of *Viscum album*. For this purpose, biomarkers of oxidative stress [2-thiobarbituric acid reactive substances (TBARS) as a biomarker of lipid peroxidation, carbonyl derivatives of oxidative modification of proteins, total antioxidant capacity (TAC)] were used in the muscle tissue of rainbow trout (*Oncorhynchus mykiss*Walbaum) after *in vitro* treatment with extracts derived from the leaves of *Viscum album* at two final doses (5 and 2.5 mg/mL).

MATERIALS AND METHODOLOGY

Collection of Plant Materials. The plant material was collected from Dubno (50°23′35″N 25°44′06″E), a city and municipality located on the Ikva River in

Rivne Oblast (province) of western Ukraine. It serves as the administrative center of DubnoRaion (district). Freshly collected leaves were washed, weighed, crushed, and homogenized 0.1M phosphate buffer (pH 7.4) (in proportion 1:19, w/w) at room temperature, and centrifuged at 3,000 rpm for 5 minutes. The extracts were then filtered and used for analysis. Supernatants were stored at -20°C in bottles protected with laminated paper until required.This work was supported by the Pomeranian University in Słupsk (Poland) in cooperation with H.S. SkovorodaKharkiv National Pedagogical University (Kharkiv, Ukraine) and Ivan Franko National University in Lviv (Lviv, Ukraine). The authors acknowledge and are grateful for the support of the International Visegrad Fund.

Experimental fish.Clinically healthy rainbow trout (*Oncorhynchus mykiss*Walbaum) with an average body weight of 80-125 g were used in the experiments. Fish for the experiments were sampled at the Department of Salmonid Research, Stanislaw Sakowicz Institute of Inland Fisheries in Olsztyn (Rutki, Poland). The water temperature was 14.5 ± 0.5°C and the pH was 7.2-7.4. The dissolved oxygen level was approximately 9 ppm with supplemental oxygen supply, with a water flow of 25 L/min and a photoperiod of 12 h per day. The same experimental conditions were used throughout the study. Water parameters were continuously monitored. Fish were housed in square tanks (150 fish per tank) and fed commercial pelleted diets.

Muscle tissue samples.Trout muscle tissue samples were homogenised in ice-cold buffer (100 mMTris-HCl, pH 7.2). Minced muscle tissue was rinsed of blood with cold isolation buffer and homogenised on ice in an H500 homogeniser using a motorised pestle. The homogenates were centrifuged at 3,000 rpm for 15 min at 4°C. After centrifugation, the supernatant was collected and frozen at -25°C until analysis. Protein content was determined by the method described by Bradford (1976) using bovine serum albumin as standard [3]. The absorbance was recorded at 595 nm. All enzymatic assays were performed at 22 ± 0.5°C (n = 8). Biochemical reactions were initiated by the addition of tissue supernatant.

Experimental design.The muscle tissue supernatant was incubated with extracts from *V. album* leaves (at final concentrations of 5 and 2.5 mg/ml) at room temperature. The control group (trout muscle tissue) was incubated with 100 mMTris-HCl buffer (pH 7.2) (in a 19:1 ratio). The incubation time was 2 hours. Biomarkers of oxidative stress were investigated in the incubated homogenate (control group and samples with extracts of *V. album* leaves).

The 2-thiobarbituric acid reactive substances (TBARS) assay. Lipid peroxidation was assessed by the production of 2-thiobarbituric acid reactive substances (TBARS). An aliquot of the homogenate was used to determine the lipid peroxidation status of the sample by measuring the concentration of 2-thiobarbituric acid reactive substances (TBARS) according to the method of Kamyshnikov (2004). The absorbance of the supernatant was measured at 540 nm. TBARS values were expressed as nmoles malonic dialdehyde (MDA) per mg protein [17].

Carbonyl groups of the oxidatively modified proteins assay. Carbonyl groups were measured as an indication of oxidative damage to proteins according to the method of Levine and co-workers (1990) as modified by Dubinina and co-workers (1998). Carbonyl content was measured spectrophotometrically at 370 nm (aldehydic derivatives, OMP_{370}) and 430 nm (ketonic derivatives, OMP_{430}) (molar extinction coefficient 22,000 $M^{-1} \cdot cm^{-1}$) and expressed as nmol per mg protein [7, 27].

Total antioxidant capacity (TAC) assay. The level of TAC in the sample was estimated by measuring the level of TBARS after oxidation of Tween 80. This level was determined spectrophotometrically at 532 nm [10]. The sample inhibits the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. The content of TAC in the sample (%) was calculated with reference to the absorbance of the blank.

Statistical analysis. The mean ± S.E.M. values were calculated for each group to determine the significance of the difference between the groups. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors tests ($p > 0.05$). The significance of differences between the levels of oxidative stress biomarkers (significance level, $p < 0.05$) was tested using the Kruskal–Wallis one-way analysis of variance [61]. All statistical calculations were performed on separate data from each individual using STATISTICA 13.3 software (TIBCO Software Inc., USA).

RESULTS AND DISCUSSION

2-Thiobarbituric acid reactive substances (TBARS) are formed as a by-product of lipid peroxidation (i.e. fat breakdown products) and can be detected by the TBARS assay using thiobarbituric acid as the reagent. Because reactive oxygen species (ROS) have an extremely short half-life, they are difficult to measure directly. Instead, several products of oxidative stress damage, such as TBARS, can be measured. The TBARS assay measures malondialdehyde (MDA) present in a sample as well as malondialdehyde formed from lipid hydroperoxides under hydrolytic reaction conditions. MDA is one of several low-molecular-weight end products formed during the decomposition of various primary and secondary products of lipid peroxidation [1].

The levels of TBARS as a biomarker of lipid peroxidation, aldehydic and ketonic derivatives of oxidatively modified proteins (OMP), and total antioxidant capacity (TAC) in rainbow trout muscle tissue after *in vitro* incubation with extracts derived from *V. album* leaves (at two final concentrations of5 and 2.5 mg/mL) were assessed and shown in Figures 1-3.

As shown in Figure 1, treatment with *V. album* leaf extracts resulted in non-significant changes in TBARS levels of (61.61 ± 5.49 nmol/mg protein) and (52.19 ± 4.62 nmol/mg protein) in the muscle tissue after *in vitro* incubation with *V. album* leaf extracts at final concentrations of 5 and 2.5 mg/mL, respectively, compared to untreated samples (56.33 ± 6.41 nmol/mg protein). A statistically non-significant

increase in the TBARS levels (by 9.4% $p > 0.05$) was observed for the extract at final concentrations of 5 mg/mL. On the other hand, a statistically non-significant decrease in the TBARS level (by 7.4% $p > 0.05$) was observed for the extract at final concentrations of 2.5 mg/mL (Fig. 1).

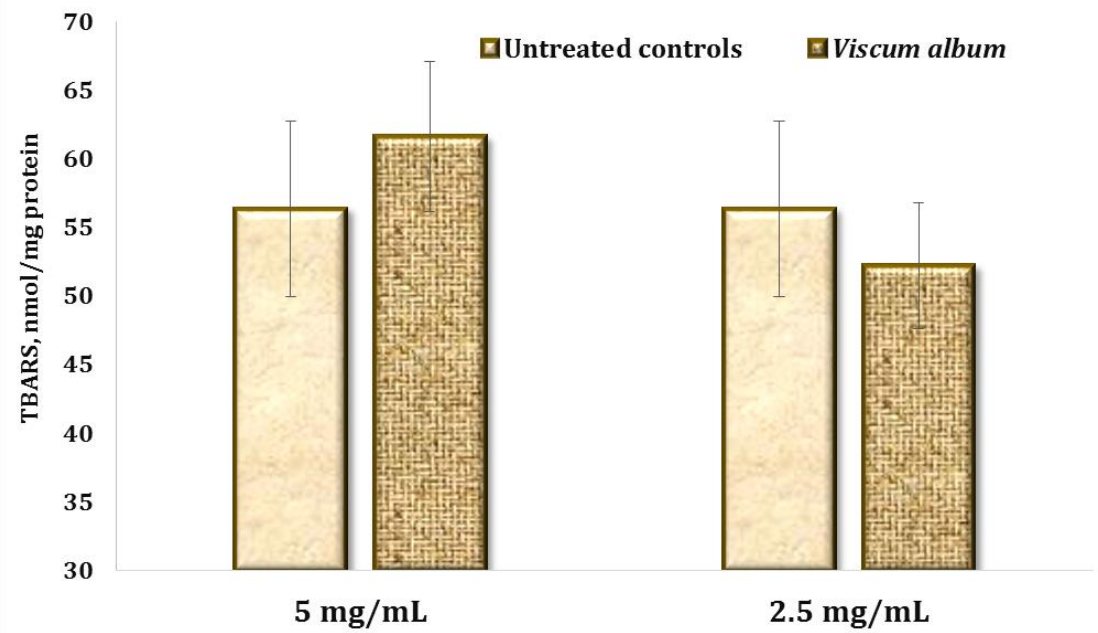

Fig.1.TBARS content, as a biomarker of lipid peroxidation, in rainbow trout muscle tissue after *in vitro* treatment with extracts derived from the leaves of *Viscum album* at two final concentrations (5 and 2.5 mg/mL) (M ± m, n = 8)

*– statistically significant differences between treated and untreated samples ($p < 0.05$)

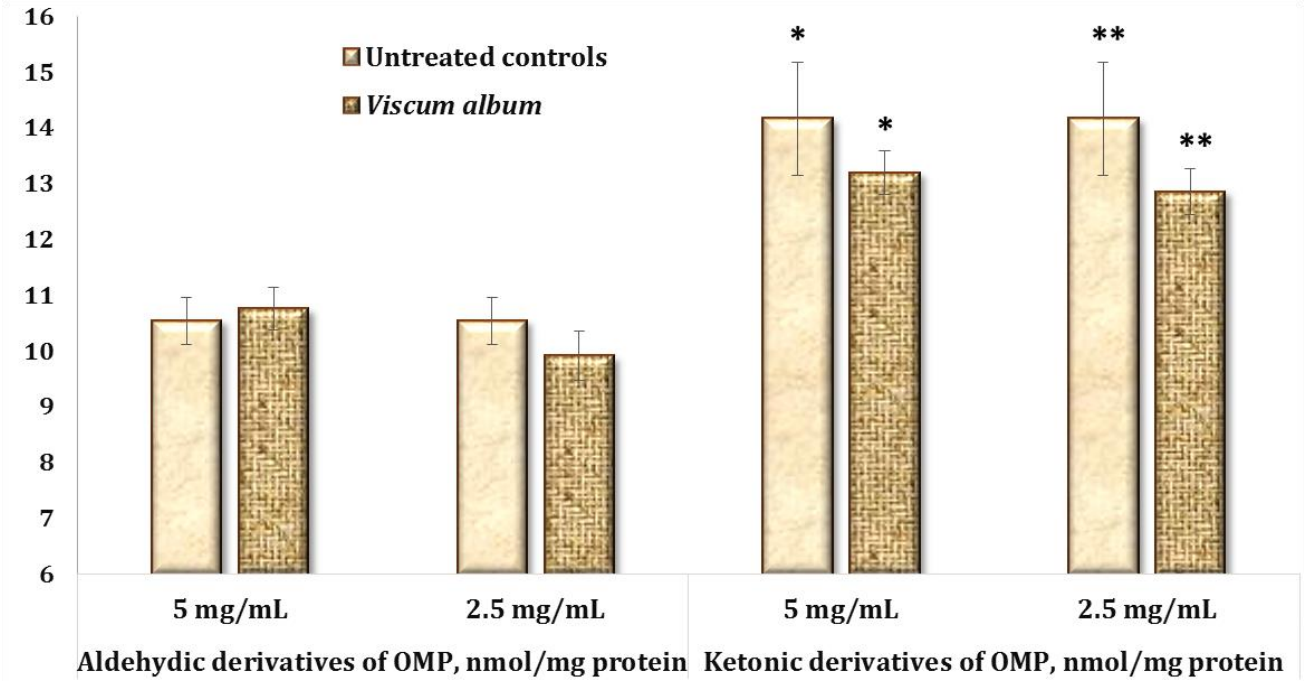

Fig. 2. Levels of aldehydic and ketonic derivatives of oxidatively modified proteins in rainbow trout muscle tissue after *in vitro* treatment with extracts derived from the leaves of *Viscum album* at two final concentrations (5 and 2.5 mg/mL) (M ± m, n = 8)

*– statistically significant differences between treated and untreated samples ($p < 0.05$)

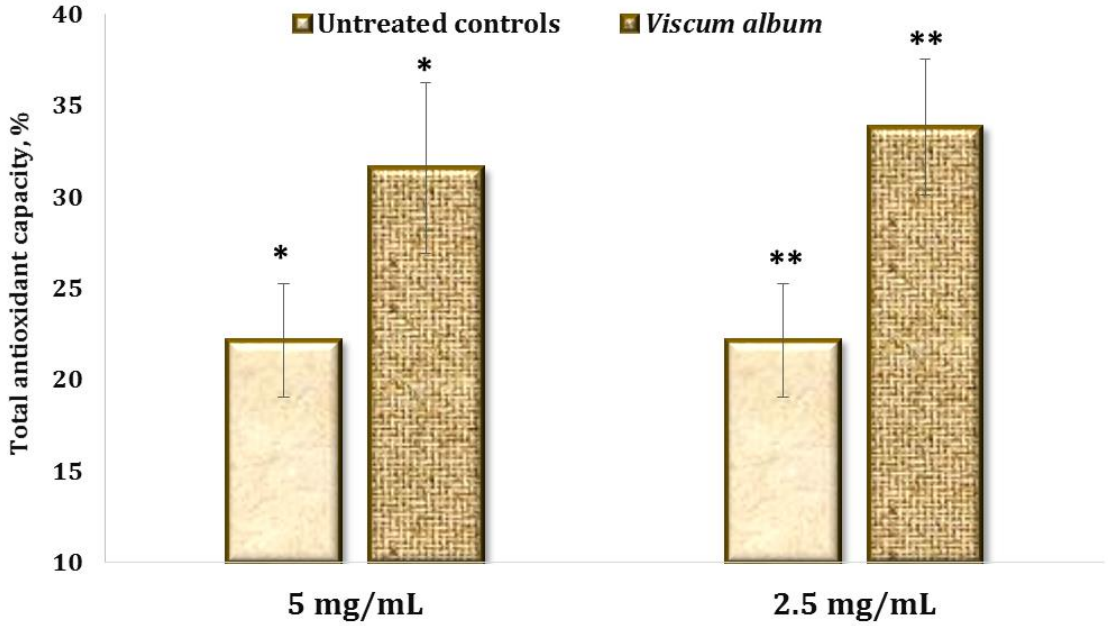

Fig. 3. Total antioxidant capacity (TAC) levels in rainbow trout muscle tissue after *in vitro* treatment with extracts derived from the leaves of *Viscum album* at two final concentrations (5 and 2.5 mg/mL) (M ± m, n = 8)
*– statistically significant differences between treated and untreated samples ($p < 0.05$)

Under the influence of ROS, the native conformation of proteins is disrupted with the formation of large protein aggregates or fragmentation of the protein molecule. Carbonyl derivatives of proteins are stable products formed with the participation of amino acid residues of proline, arginine, lysine, threonine with the formation of Michael adducts [48]. Carbonyl derivatives of proteins can also be formed with the participation of amino acid residues of lysine, cysteine and histidine with the products of lipid peroxidation. According to a number of researchers, carbonyl derivatives are formed during metal-catalysed oxidation of proteins. Oxidative modification of proteins causes at least three types of changes in the physicochemical properties of the protein molecule: fragmentation, aggregation and susceptibility to proteolysis [28]. The result is either the formation of products with high functional activity or the inactivation of the active centres of enzymes or the modification of protein molecules, which contributes to the exacerbation of the clinical picture of the underlying pathological condition [62].

The levels of aldehydic and ketonic derivatives of oxidatively modified proteins in rainbow trout muscle tissue after *in vitro* treatment with extracts derived from the leaves of *Viscum album* at two final concentrations (5 and 2.5 mg/mL) are shown in Figure 2.

The levels of aldehydic derivatives of oxidatively modified proteins were reduced in samples treated with *V. album* leaf extracts at final concentrations of 5 mg/mL (10.75 ± 0.38 nmol/mg protein) compared to untreated samples (10.54 ± 0.42 nmol/mg protein), but these reductions were not statistically significant ($p > 0.05$). Treatment of muscle tissue with *V. album* leaf extracts at final concentrations

of 2.5 mg/mL resulted in a statistically non-significant decrease in the levels of aldehydic derivatives of oxidatively modified proteins to (9.91 ± 0.45 nmol/mg protein) compared to untreated samples (10.54 ± 0.42 nmol/mg protein). The decrease was 5.9% ($p > 0.05$) (Fig. 2).

When muscle tissue was incubated with *V. album* leaf extracts, the levels of ketonic derivatives (13.19 ± 0.39 nmol/mg protein) were significantly reduced by 6.9% ($p < 0.05$) compared to untreated samples (14.16 ± 1.02 nmol/mg protein). Treatment of muscle tissue with *V. album* leaf extracts at final concentrations of 2.5 mg/ml resulted in a statistically significant decrease in the levels of ketonic derivatives of oxidatively modified proteins to (12.85 ± 0.41 nmol/mg protein) compared to untreated samples (14.16 ± 1.02 nmol/mg protein). The reduction was 9.3% ($p < 0.05$) (Fig. 2).

Total antioxidant capacity is an indicator of the body's antioxidant system, which protects the body from the toxic effects of a range of oxygen compounds produced in the body such as oxygen ions, peroxides and free radicals [2].

Total antioxidant capacity (TAC) levels in rainbow trout muscle tissue after *in vitro* treatment with extracts derived from the leaves of *Viscum album* at two final concentrations (5 and 2.5 mg/mL) are shown in Figure 3.

In the current study, TAC levels in rainbow trout muscle tissue were increased to (31.56 ± 4.68 %) after *in vitro* incubation with *V. album* leaf extracts (final concentration 5 mg/ml) compared to untreated samples (22.13 ± 3.12 %). This represented a 42.6% ($p < 0.05$) increase in TAC levels compared to untreated samples. Treatment of muscle tissue with *V. album* leaf extracts at a final concentration of 2.5 mg/ml also resulted in a statistically significant increase in TAC levels to (33.81 ± 3.71%) compared to untreated samples (22.13 ± 3.12%). The decrease was 52.8% ($p < 0.05$) (Fig. 3).

The results of the current study demonstrated the antioxidant properties of *V. album* leaf extracts at two final concentrations (5 and 2.5 mg/ml) after incubation with rainbow trout muscle tissue. The results of the current study are similar to those of other researchers who have demonstrated the antioxidant capacity of numerous mistletoe extracts and isolated lectins. For example, Sengul and co-workers (2009) showed that *Viscum album* and *Crocus sativus* L. had the highest antioxidant (82.23%) and total phenolic content (42.29 mg GAE/g dw) [47]. The radical scavenging effects and protective activities against oxidative stress of Korean mistletoe (*Viscum album coloratum*) lectin were investigated *in vitro* and with a cellular system using LLC-PK1 renal epithelial cells by Kim and co-workers (2010). The Korean mistletoe lectin (KML) showed 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity with an IC_{50} value of 42.6 μg/mL. It also exhibited nitric oxide (NO), superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical scavenging activities in a concentration-dependent manner. These results suggest that KML is a promising antioxidant by scavenging free radicals. Furthermore, in the LLC-PK1 cellular model, cells showed a decrease in viability and an increase in lipid peroxidation due to oxidative stress induced by sodium nitroprusside (SNP) and pyrogallol,

generators of NO and $O_2^{\cdot-}$, respectively. However, KML significantly inhibited cell cytotoxicity and lipid peroxidation in a dose-dependent manner. Furthermore, 3-morpholinosydnonimnie (SIN-1), a generator of peroxynitrite ($ONOO^-$) formed by simultaneous release of NO and $O_2^{\cdot-}$, caused cytotoxicity, lipid peroxidation and NO overproduction in LLC-PK1 cells, whereas KML ameliorated $ONOO^-$-induced oxidative damage. Overexpression of cyclooxygenase-2 and inducible NO synthase induced by SIN-1 was also observed, but KML downregulated the expression levels of both genes. KML also reduced SIN-1-induced nuclear factor-kappa B expression and inhibitor-kappa B alpha phosphorylation in LLC-PK1 cells. The results obtained by Kim and co-workers (2010) suggest that KML has protective effects against free radical-induced oxidative damage [47].

Korean mistletoe lectin (KML) is one of the major active constituents of *Viscum album* var. (*coloratum*), which exhibits various biological effects such as anti-tumour and anti-metastatic activities [25]. Cytotoxic lectins (KML-C) were isolated from an extract of Korean mistletoe [*Viscum album* C. (*coloratum*)] by affinity chromatography on a hydrolysedSepharose 4B column, and the chemical and biological properties of KML-C were investigated by Yoon and co-workers (1999), partly by comparison with a lectin (EML-1) from European mistletoe [*Viscum album* L. (*loranthaceae*)] [60]. The isolated lectins exhibited potent cytotoxicity against various human and murine tumour cells, and the cytotoxic activity of KML-C was higher than that of EML-1. Tumour cells treated with KML-C exhibited typical patterns of apoptotic cell death, such as obvious morphological changes and DNA fragmentation, and its apoptosis-inducing activity was blocked in a dose-dependent manner by the addition of Zn^{2+}, an inhibitor of Ca^{2+}/Mg^{2+}-dependent endonucleases. The results of Yoon and co-workers (1999) suggest that KML-C is a novel lectin associated with the cytotoxicity of Korean mistletoe and that its cytotoxic activity against tumour cells is due to apoptosis mediated by Ca^{2+}/Mg^{2+}-dependent endonucleases [60].

KML may be involved in the regulation of various macrophage-mediated innate and adaptive responses by binding to surface proteins with D-galactose, and that some of these may be involved in the therapeutic activities of KML, such as anti-tumour and anti-microbial effects. Lee and co-workers (2007) demonstrated the regulatory role of KML on macrophage-mediated immune responses [25]. KML clearly blocked lipopolysaccharide-LPS-induced events [interleukin IL-10 expression, nitric oxide (NO) production and phagocytic uptake] and suppressed the normal expression levels of IL-10 (at 2 ng/ml) and tumour necrosis factor (NF-α at 10 ng/ml). In contrast, the expression of cytokine (TNF-α) and the generation of ROS induced by LPS were significantly upregulated by co-treatment with KML. In addition, KML itself increased IL-3 and IL-23 mRNA levels; phagocytic uptake; surface levels of co-stimulatory molecules (CD80 and CD86), pattern recognition receptors (PRRs) [such as dectin-1 and toll-like receptor (TLR)-2] and adhesion molecules [β1-integrins (CD29) and CD43]; and CD29-mediated cell adhesion events. Finally, co-treatment of D-galactose with KML under LPS-induced NO

production conditions suggests that KML inhibition is mediated by binding to proteins with D-galactose [25].

The enhanced antioxidant activity of fermented Korean mistletoe is due to an increase in the levels of caffeic acid and lyoniresinol, as demonstrated by Kim and co-workers (2016). The KM extract showed enhanced antioxidant activity in 2,2-diphenyl-1-picrylhydrazyl, Trolox equivalent antioxidant capacity and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester assays after fermentation with a crude enzyme extract from a soybean paste fungus, *Aspergillus kawachii*. High-performance liquid chromatography analysis revealed four elevated peaks in the enzyme-treated KM. The elevated peaks were isolated and identified as caffeic acid (1), hesperetin (2), syringaldehyde (3) and lyoniresinol (4). Of the four compounds, only 1 and 4 showed strong antioxidant activity. Therefore, fermentation increased the content of 1 and 4, which consequently increased the antioxidant activity of KM [23].

Methanolic extracts of *Viscum album* ssp. *album* (mistletoe) grown on different host trees were investigated by Onay-Uçar and co-workers (2006) for their potential antioxidant activity [37]. The scavenging activity was tested by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method and the inhibitory effect on lipid peroxidation was tested by the ferric thiocyanate and 2-thiobarbituric acid methods. The extract from mistletoe grown on lime trees in summer showed the highest activity. It was found that the antioxidant capacity of the plant varied according to the time of harvest and the host tree [37]. Also, Pietrzak and co-workers (2017) used a new rapid, sensitive and selective liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS) method to determine the content of flavonoid aglycones and phenolic acids in mistletoe (*Viscum album* L.) berries harvested from six different Polish host trees. In addition, total phenolic content (TPC) and total flavonoid content (TFC), as well as antioxidant and antiproliferative activity were evaluated for the first time. LC-ESI-MS/MS analysis revealed the highest content of phenolic acids in mistletoe berries from *Populusnigra* 'Italica' L. and flavonoid aglycones in mistletoe berries from *Tiliacordata* Mill. (354.45 μg and 5.955 μg per g dry extract, respectively). The moderate antioxidant activity of the extracts studied was maintained. The studies of these researchers showed that the studied extracts decreased the proliferation of the human colon adenocarcinoma cell line LS180 in a dose-dependent manner, without cytotoxicity in the human colon epithelial cell line CCD 841 CoTr. Moreover, the results of Pietrzak and co-workers (2017) suggest a significant influence of polyphenols on the anticancer activity of these extracts [41].

Mistletoe extracts could also be used as a promising immunostimulant in aquaculture diets. Choi and co-workers (2008) investigated the immunostimulatory effects of Korean mistletoe extract (KM-110; *Viscum album Coloratum*) on non-specific immune response and protection against *Aeromonashydrophila* infection in Japanese eel (*Anguilla japonica*) [6]. Eels were fed under 4 regimes, 0%, 0.1%, 0.5% and 1.0% KM-110 mixed diet. On day 14 post-feeding, 15 fish from each group were injected i.p. with live *A. hydrophila* (3×10^6 CFU) and the remaining

uninfected fish from each group were used to study the innate immune response. At 14 days post infection, overall survival rates were 26.6% in the control group and 33.3%, 66.6% and 80% in the 0.1%, 0.5% and 1% KM-110 treated groups, respectively. Maximum lysozyme activity was observed in the group treated with 1% KM-110. There was no significant difference in lysozyme activity between the 0.1% and 0.5% KM-110 groups. Superoxide anion ($O_2^{\cdot-}$) production was significantly increased in the 0.5% and 1% KM-110 groups compared to the control and 0.1% KM-110 groups. No significant difference in $O_2^{\cdot-}$ production was found between the 0.5% and 1% KM-110 groups. Similarly, there was a significant increase in phagocytic activity in the 0.5% KM-110 group compared to the 0.1% group, but no significant difference between the 0.5% and 1% KM-110 groups, indicating that the 0.5% KM-110 concentration is suitable for stimulating maximum phagocytic activity resulting in a high amount of ROS production [6].

The immunostimulating effects of dietary intake of various medicinal plant extracts on fish, rainbow trout (*Oncorhynchus mykiss*), were investigated by Dügenci and co-workers (2003). The fish were fed diets containing aqueous extracts of mistletoe (*Viscum album*), stinging nettle (*Urticadioica* L.) and ginger (*Zingiberofficinale* Roscoe). Diets containing lyophilised extracts of these plants at 0.1 and 1% were fed at a rate of 2% of body weight per day for three weeks. At the end of the experimental period, various parameters of non-specific defence mechanisms were examined, including extracellular and intracellular respiratory burst activities, phagocytosis in blood leukocytes and total plasma protein levels. All plant extracts added to the fish diets increased total plasma protein levels, with the exception of 0.1% ginger. The highest plasma protein levels were observed in the group fed the diet containing 1% ginger extract [8].

Park and Choi (2012) evaluated the effect of dietary mistletoe extract on non-specific immune response and disease resistance in Nile tilapia, *Oreochromisniloticus*, against *Aeromonashydrophila* infection [40]. Tilapia fingerlings were fed diets containing 0 mg, 10 mg, 50 mg and 200 mg mistletoe powder per kg dry diet as control for 80 days. Immunological parameters, respiratory burst activity, lysozyme activity, alternative complement haemolysis activity and phagocytic activity of the fish were assessed after 20, 40 and 80 days of feeding. Fish were challenged with *A. hydrophila* at 80 days post feeding and mortality was monitored for 10 days post infection. The results showed that fish fed mistletoe extract showed increased activity in all immunological parameters compared to the control group, depending on the feeding period and dose of mistletoe. After challenge with *A. hydrophila*, the survival rate in the control group was 42% lower than in the other experimental diet groups. The highest survival rate (83%) was observed in the group fed 50 mg mistletoe per kg diet. The results of Park and Choi (2012) suggest that mistletoe may enable tilapia to boost immunity and be more resistant to *A. hydrophila* infection [40].

CONCLUSIONS

The aim of the present study was to determine the antioxidant activity of extracts from the leaves of *Viscum album* using biomarkers of oxidative stress [2-thiobarbituric acid reactive substances (TBARS) as a biomarker of lipid peroxidation, carbonyl derivatives of oxidative modification of proteins, total antioxidant capacity (TAC)] in the muscle tissue of rainbow trout (*Oncorhynchus mykiss*Walbaum) after *in vitro* treatment with extracts at two final concentrations (5 and 2.5 mg/ml). Our study showed that treatment with *V. album* leaf extracts resulted in non-significant changes in TBARS levels in muscle tissue after in vitro incubation with *V. album* leaf extracts at final concentrations of 5 and 2.5 mg/mL. The levels of aldehydic derivatives of oxidatively modified proteins in rainbow trout muscle tissue after treatment with *V. album* leaf extracts at final concentrations of 5 mg/ml were at the same levels as in untreated controls. When muscle tissue was incubated with *V. album* leaf extracts, the levels of ketone derivatives were significantly reduced after treatment with extracts at a final concentration of 5 and 2.5 mg/ml compared to untreated samples. TAC levels in rainbow trout muscle tissue were increased after *in vitro* incubation with *V. album* leaf extracts (at final concentrations of 5 and 2.5 mg/ml) compared to untreated samples. Further studies should focus on the antioxidant effect of *Viscum album* leaf extracts using other cellular models.

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