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LIPID PEROXIDATION LEVEL IN THE MUSCLE TISSUE OF THE RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM) UNDER *IN VITRO* INCUBATION WITH EXTRACTS FROM LEAVES OF VARIOUS CULTIVARS OF *CAMELLIA JAPONICA* L. (*THEACEAE*) (OVERVIEW)

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The aim of this study was to evaluate the lipid peroxidation level (2-thiobarbituric acid reactive substances (TBARS) as biomarker) in the muscle tissue of rainbow trout after incubation with extracts obtained from leaves of various Camellia japonica L. cultivars. The leaves of various Camellia japonica cultivars (C. japonica 'Kramer's Supreme', C. japonica 'C.M.Wilson', C. japonica 'La Pace', C. japonica 'Mrs. Lyman Clarke', C. japonica cv. 3, C. japonica cv. 15), cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanical Garden (NBG), National Academy of Sciences of Ukraine. Specifically.

Results obtained in this study showed that there is a possibility of using extracts derived from leaves of various Camellia japonica cultivars in intensive aquaculture farms. Leaf extracts of Camellia japonica may be used as an antioxidant agent in aquaculture. Furthermore, the use of such plants products as antioxidants and immunostimulants in aquaculture systems may also have environmental value because of their biodegradability.

Keywords: **rainbow trout (*Oncorhynchus mykiss* Walbaum), aquaculture, lipid peroxidation, 2-thiobarbituric acid reactive substances, *Camellia japonica* L., cultivars.**

Herbs are currently used in commercial aquaculture as growth-promoting substances, antimicrobial agents, nutrients to prevent and control fish diseases as well as many other applications (Galina et al. 2009). The growing interest of using herbs in aquaculture has increased world-wide because they are easy to prepare, cheap, and they contain natural organic compounds such as several phenolic, polyphenolic, alkaloid, quinone, terpenoid, lectine, and polypeptide compounds, many of which shown to be very effective alternatives to antibiotics, chemicals or synthetic compounds and vaccines in aquaculture [3, 34, 37]. Plants (fruits, vegetables, medicinal herbs) may contain a wide variety of free radical scavenging molecules such as phenolic compounds (Phenolic acids, flavonoids, quinons, coumarins, lignans, stilbenes, tannins etc.), nitrogen compounds (alkaloids, amines, betalains etc.), vitamins, terpenoids (including carote-

noids) and some other endogenous metabolites which are rich in antioxidant activity [39].

They also facilitate growth, anti-stress, environmentally friendly and antimicrobial properties in finfish and shrimp [5]. Modulation of the immune response using medicinal plant products as a possible therapeutic measure has become the focus of extensive scientific investigation [8].

In this study extracts derived from leaves of various cultivars of *Camellia japonica* plants were chosen because of their recorded potential medical significance with antimicrobial [14], antioxidant [24, 28], anti-allergic [16, 29], antiviral [1, 31] and skin healing properties [11]. Therefore, it would be reasonable to expect that plant extract obtained from *Camellia japonica* plants can express the antioxidant properties.

The genus *Camellia* L. (*Theaceae* D. Don) comprises more than 200 woody evergreen species. These plants are native to East Asia – China, Taiwan, Japan and South Korea [23]. Some species possess great economic value, particularly *Camellia sinensis* (L.) Kuntze (the tea plant) which is grown commercially mainly in tropical and subtropical regions. Other species such as *Camellia japonica* L., *Camellia reticulata* Lindl. and *Camellia sasanqua* Thunb. are cultivated in subtropical regions worldwide as ornamentals. The seeds of *Camellia* species can be pressed to obtain high quality oils, some of which have been used for years in Asian cultures. The oil from *Camellia oleifera* C.Abel. (widely known as tea seed oil or tea oil) is used extensively in the Hunan Province in China for cooking, and *C. japonica* oil has a long history of traditional cosmetic usage in Japan as a protectant to maintain the health of skin and hair [11]. Leaves and shoots of *C. sasanqua* is a source of eugenol, and of the fruits and seeds extracted oil. *Camellia* spp. have been used also in Oriental ethnomedicine and appear to be very promising leads for possible pharmaceutical exploitation since modern science has made it possible to specify potential medical significance of the genus *Camellia*.

Camellia japonica is the most well-known species of the genus *Camellia*. *C. japonica* (Japanese name of ‘‘tsubaki’’) has traditionally been a popular tree as both a garden ornamental plant and as the source of oil material and folk medicine in Japan. An edible oil known as 'tsubaki oil’ is obtained from the seed [7, 18, 40]. Dried flowers are used as a vegetable cooked or mixed with gelatinous rice to make a Japanese food called ’mochi’ [7] or used as a flower tea [18]. The leaves serve as a substitute for tea and tobacco [7, 17, 36].

The flowers are astringent, anti-haemorrhagic, haemostatic, salve and tonic in folkloric traditional medicine [6, 36]. When mixed with sesame oil, they are used in the treatment of burns and scalds. The flower has been prescribed in Chinese traditional preparations for the treatment of haematemesis (vomiting of blood) and oketsu syndrome (blood stagnation), and the seed is used as stomachic and anti-inflammatory in Japanese folk medicine [18, 42]. *C. japonica* oil has been used traditionally in East Asia to nourish and soothe the skin as well as help restore the elasticity of skin and on all types of bleeding instances [15].

The leaf extract of *C. japonica* exhibited the most potent effect on degranulation in antigen-stimulated rodent and human mast cells of 100 Korean plants screened [20]. Aqueous extract from *C. japonica* leaf have antioxidant and neuronal cell protective effects. It including phenolics may be useful in the natural antioxidant substance and reduce the risk of neurodegenerative disease such as Alzheimer’s disease [10]. Among eight *Camellia* species, *C. sasanqua* showed potent anticancer activities in prostate cancer PC3 cells. In addition to catechins, the major component, eugenyl β-primeveroside was detected in *C. sasanqua* [41]. The *Camellia* spp. extract possesses a wide range of

pharmacological activities including anti-inflammatory, anti-cancer, anti-microbial, and antioxidant activities [1, 26, 29, 43].

Although pharmacological activities of extracts from various parts of *Camellia* species were well investigated, studies regarding its protective effects against oxidative damage have not yet been undertaken. The aim of this study was to evaluate the lipid peroxidation (2-thiobarbituric acid reactive substances (TBARS) as biomarker) level in the muscle tissue of rainbow trout after incubation with extracts obtained from leaves of various *Camellia japonica* cultivars. The lipid peroxidation product, malondialdehyde (MDA), is commonly used as a biomarker of the oxidative stress in cells. This aldehyde is a highly toxic molecule and it should be considered as more than just a marker of lipid peroxidation. Its interaction with the DNA and proteins has often been referred to as potentially mutagenic and atherogenic [33].

Materials and methods. *Collection of Plant Materials.* The leaves of various *Camellia japonica* L. cultivars (*C. japonica 'Kramer's Supreme'*, *C. japonica 'C.M.Wilson'*, *C. japonica 'La Pace'*, *C. japonica 'Mrs. Lyman Clarke'*, *C. japonica* cv. 3, *C. japonica* cv. 15*)*, cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanical Garden (NBG), National Academy of Science of Ukraine. Specifically, fully expanded new leaves of these cultivars were selected for study.

Preparation of Plant Extracts. Freshly collected leaves were washed, weighted, crushed, and homogenized in 100 mM sterile phosphate buffer saline solution (pH 7.2) (in proportion 1:19, w/w) at room temperature. The extracts were then filtered and investigated. All extracts were stored at -20°C until use.

Experimental fish. Clinically healthy rainbow trout with a mean body mass of 80-120 g were used in the experiments. The experiments were performed in water at 14.5 ± 0.5°C and pH 7.2-7.4. The dissolved oxygen level was about 9 ppm with additional oxygen supply, with a water flow of 25 L/min, and a photoperiod of 12 h per day. The same experimental conditions were used during the whole research. The water parameters were maintained under constant surveillance. The fish were held in square tanks (150 fish per tank) and fed commercial pelleted diet.

Collection of muscle tissue samples. The muscle tissue samples were homogenized in ice-cold buffer (100 mM Tris-HCl, pH 7.2) using a glass homogenizer immersed in ice water bath. Homogenates were centrifuged at 3000 g for 15 min at 4°C. After centrifugation, the supernatant was collected and frozen at −20°C until analyzed. Protein contents were determined with the method described by Bradford [4] with bovine serum albumin as a standard. Absorbance was recorded at 595 nm. All enzymatic assays were carried out at 22 ± 0.5°C using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany). The enzymatic reactions were started by adding the tissue supernatant.

Experimental design. The supernatant of the muscle tissue was used to incubate with extracts of various cultivars of *C. japonica* (in a ratio 19:1) at room temperature. The positive control group (trout muscle tissue) was incubated with 100 mM Tris-HCl buffer (pH 7.2) (in a ratio 19:1). The incubation time was 2 hours. Lipid peroxidation biomarker was evaluated in the incubated homogenate (control group and in samples with extracts of various cultivars of *C. japonica*).

Determination of 2-thiobarbituric acid reactive substances (TBARS). The level of lipid peroxidation was estimated by assessing the levels of 2-thiobarbituric acid reactive substances (TBARS) by Kamyshnikov [13]. The TBARS assay was performed by using the malonic dialdehyde (MDA) equivalents. The method involved heating of incubated homogenates with the trichloroacetic acid (TCA) and 2-thiobarbituric acid (TBA). MDA was identified as a product of lipid peroxidation which reacted with TBA to give a pink colored samples that gave an absorbance at 540 nm. Briefly, 2.1 mL of

homogenate sample (in tris-HCl buffer, 100 mM, pH 7.2) was added to 1 mL of 20 % TCA, and 1 mL of 0.8 % TBA. The mixture was heated in a boiling water bath for 10 min. After cooling, the mixture was centrifuged at 3,000 g for 10 min. The absorbance of the supernatant was measured at 540 nm. The concentration of MDA (nmol/mg of protein) was calculated using $1.56\ 10^5\ mM^{-1}\ cm^{-1}$ as the extinction coefficient.

Statistical analysis. The mean ± S.E.M. values was calculated for each group to determine the significance of intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test ($p>0.05$). Significance of differences between the total antioxidant capacity level (significance level, $p<0.05$) was examined using Mann-Whitney *U* test [44]. All statistical calculation was performed on separate data from each individual with STATISTICA 8.0 software (StatSoft, Krakow, Poland).

Results and discussion. When muscle tissue was incubated with leaf extracts of various *C. japonica* cultivars, the TBARS level ranged to value of control group. Moreover, all extracts (except *C. japonica* cv. 3 and cv. 15) reduced the TBARS level in the extracts-treated muscle tissue, but these differences were non statistically significant (Fig. 1).

The obtained results showed that lipid peroxidation level in the muscle tissue of rainbow trout after incubation with extracts from leaves of various *C. japonica* cultivars was not changed. Therefore, our research could only weakly support the previous studies which were conducted for assessment of biological activities of plant extracts derived from various parts of *C. japonica* plants.

Several researchers investigated the efficiency of plant extracts and their effective compounds in different parts of the *C. japonica* plant as antioxidant agents. In particular, previous studies have indicated that the extracts of different parts of the *C. japonica* plant have various biological activities. It has been reported that extracts of *C. japonica* flowers possess antioxidant effects *via* scavenging of reactive oxygen species (ROS) and induction of antioxidant enzymes. Piao and co-workers [32] have investigate the antioxidant properties of the ethanol extract of the flower of *C. japonica* (*Camellia* extract). *Camellia* extract exhibited 1,1-diphenyl-2-picrylhydrazyl radical and intracellular ROS scavenging activity in human HaCaT keratinocytes. In addition, *Camellia* extract scavenged superoxide anion generated by xanthine/xanthine oxidase and hydroxyl radical generated by the Fenton reaction ($FeSO_4 + H_2O_2$) in a cell-free system, which was detected by electron spin resonance spectrometry. Furthermore, *Camellia* extract increased the protein expressions and activity of cellular antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase. It was shown that *Camellia* extract contained quercetin, quercetin-3-O-glucoside, quercitrin and kaempferol, which are antioxidant compounds [32].

Extracts of *C. japonica* leaves have shown antihuman HIV activity by inhibiting the HIV-protease enzyme, which is required for cleaving newly synthesized polyproteins essential for virion maturity. To identify substances with anti-human immunodeficiency virus (HIV) activity in traditional medicines, 101 extracts of Korean medicinal plants were screened for their inhibitory effects on HIV type 1 protease (PR) by Park and co-workers [30]. Of the extracts tested, strong inhibitory effects were observed in the acetone extracts of the pericarp and leaves of *Camellia japonica*, the water extract of the leaves of *Sageretia thea* (syn. *S. theezans*) and the methanol extract of the aerial part of *Sophora flavescens*. Camelliatannin H from the pericarp of *C. japonica*, showed a potent inhibitory activity on HIV-1 PR with IC_{50} of 0.9 microM [30].

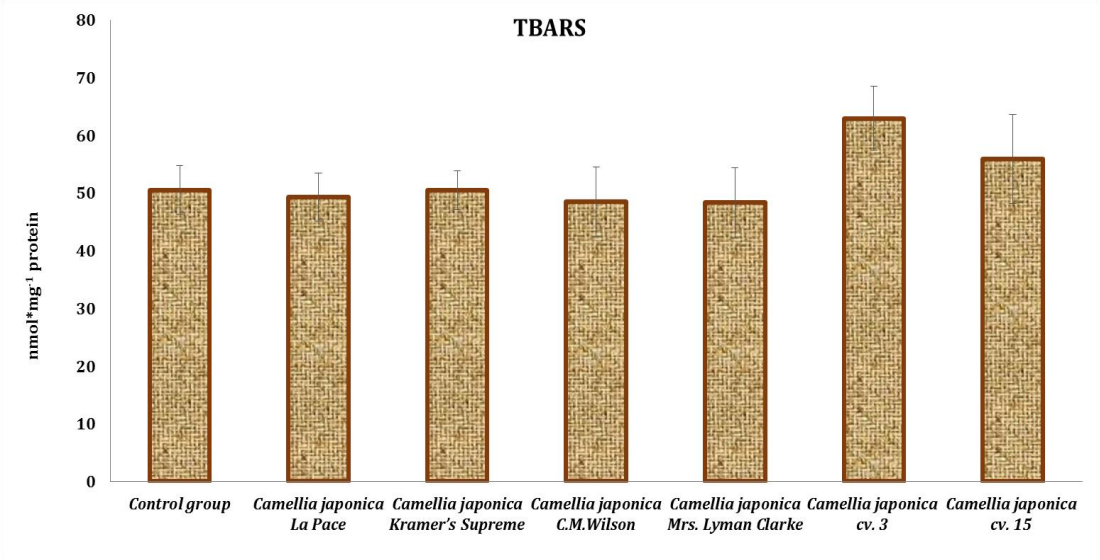

Fig. 1. The lipid peroxidation level measured by 2-thiobarbituric acid reactive substances (TBARS) level in the muscle tissue of rainbow trout after incubation with extracts from leaves of various. *Camellia japonica* cultivars (M±m, n=6).

In addition, it exhibits antioxidant activity *via* free radical scavenging potency mainly due to the presence of tannins and polyphenolic compounds, antiphotoaging capability by reducing the carbonylation of tape-stripped stratum corneum after ultraviolet B (UVB) irradiation, and decreasing intracellular ROS generation in HaCaT keratinocytes. In study of Mizutani and Masaki [24], both mature leaves of *C. japonica* (CJML) and green leaves (JGL) were extracted with 50 % 1,3-butylene glycol (1,3-BG) and used for investigations. In a chemical examination, we compared both extracts in terms of scavenging activities against hydrogen peroxide and hydroxyl radicals. CJGL exhibited higher scavenging activities against both types of ROSs compared with CJML. In a biological study, the intra-cellular ROS level of HaCaT keratinocytes precultured with CJGL for 24 h was significantly lower than that of the control cells. Furthermore, cell damage induced by H_2O_2 exposure was attenuated by 24 h precultivation with CJGL but not by 2 h precultivation. The results of examinations indicate that CJGL possess properties that reduce oxidative stress. In addition, the result of 2 h precultivation with CJGL suggests that CJGL might affect the status of intra-cellular antioxidants [24].

Furthermore, its hypotriglyceridemic activity is mediated by decreasing serum and hepatic triglyceride level as well as lowering lipogenic enzymes activity in the liver. Tamaru and co-workers [38] have investigated hypotriglyceridemic potential of the mixed tea in rats. Fermented mixed tea made with third-crop green tea leaves and camellia leaves by a tea-rolling process has been developed. The mixed tea contained theasinensins and theaflavins. Rats fed the mixed tea extract at the level of 1% exerted significantly lower body weight and adipose tissue weight compared to animals fed third-crop green tea or camellia tea extract alone for 4 weeks. Serum and hepatic triglyceride was significantly and dose-dependently decreased by the mixed tea. This decrease was associated with lowered lipogenic enzyme activities in the liver. Furthermore, an oral administration of 4 or 8% of the mixed tea extract followed by fat emulsion suppressed the increment of serum triglyceride level. The mixed tea has hypotriglyceridemic action, partially via delaying triglyceride absorption in the small intestine and repressing hepatic lipogenic enzymes [38].

The findings of Lee and co-workers [19] have suggested that the fruit of *C. japonica* has strong cardiovascular protective effects and could be a good potential candidate for development as a natural-based medicine for the prevention and treatment of cardiovascular diseases. Despite these beneficial effects of the fruit, there is very little information available on its constituents compared with the seeds and leaves. The studies of the biological activities of *C. japonica* have been focused on the seeds, flowers, and leaves, but not the fruits. The constituents of *C. japonica* such as saponins in the seeds, flavonol glycosides in the leaves, and triterpenes, several hydrolyzable tannins, acylated anthocyanins, and purine alkaloids in the flowers have been reported [21, 25, 28, 31, 45].

Many study suggests that green tea feed may effectively enhance the antioxidant system and immune system in rainbow trout. Kakoolaki and co-workers [12] have evaluated the effect of dietary supplementation of *Camellia sinensis* leaf-extract on non-specific immune responses and disease resistance of *Mugil cephalus* fingerling against *Photobacterium damselae*. Fish were fed with 0 (unsupplemented), 50, 100 and 200 mg/kg of green tea extract (GTE) supplemented diets. Results indicated that GTE decreased mortality in *M. cephalus* in a dose-dependent manner after challenge with *P. damselae*. Haematological parameters containing erythrocytes, hematocrit, hemoglobin and leukocytes and growth performance (weight gain) showed remarkable changes in comparison with control group. Lysozyme statistically increased in GTE supplemented fish. Overall, results of Kakoolaki and co-workers [12] indicated that incorporation of *C. sinensis* supplemented diet at 100 and 200 mg/kg doses significantly enhanced the immune responses in *M. cephalus* and that the mortality percentage could be remarkably reduced after challenging the fish against *P. damselae*.

The efficacy of green tea (*Camellia sinensis*) on growth performance, immune and antioxidant systems and cytokine gene expression in rainbow trout tissues have elucidated by Nootash and co-workers [27]. Green tea was supplemented at 20, 100, and 500 mg kg^{-1} diet and fed to fish (average weight: 23.5 g) for 35 days. No remarkable changes in growth performance were observed among all test groups. Lower lipid peroxidation product and higher superoxide dismutase activity were noted in fish received the medium dose of green tea. Significant increase in serum bactericidal activity and total protein were recorded in all treatment groups. All doses of green tea up-regulated Interleukin-1β transcription in the spleen, while Interleukin-1β mRNA level decreased significantly in the kidney of low dose of green tea. Interleukin-6 mRNA level was up-regulated in the spleen of high dose of green tea and liver of middle and high doses of green tea. High dose and medium dose of green tea up-regulated the interleukin-8 transcription in the kidney and liver, respectively. Meanwhile, green tea inhibited the production of interleukin-10 in all treatment groups compared with control group. Medium dose of green tea up-regulated tumor necrosis factor-α transcription in all fish tissues, while high dose and low dose of green tea enhanced tumor necrosis factor-α mRNA levels in the kidney and spleen, respectively [27].

The decaffeinated green tea in lower doses of administration could be optimum to enhance the immunity of rainbow trout. In order to study the immunomodulatory effects of decaffeinated green tea extract on rainbow trout, a study with a 30-day feeding trial was conducted by Sheikhzadeh and co-workers (2011). Results of the this study showed that the inclusion of 20 mg kg^{-1} green tea (T1) in fish diet enhanced the serum bactericidal activity against *Yersinia ruckeri*, while significant elevation of lysozyme activity was shown in T2 group. Anti-trypsin activity due to α1-antiprotease was significantly higher in T1 and T2 groups while peroxidase content showed significant increase in all treatment groups compared to control group. Hemagglutination antibody

titer against C-RBC was significantly higher in fish administered with 100 mg kg^{-1} green tea (T2) [35].

Magcwebeba and co-workers [22] have investigated the relationship between polyphenol constituents, antioxidant properties of aqueous and methanol extracts of green tea (*Camellia sinensis*), the herbal teas, rooibos (*Aspalathus linearis, Fabaceae*) and honeybush (*Cyclopia* spp.), against skin cell viability *in vitro*. Phenolic composition, particularly high levels of potent antioxidants, of rooibos and green tea methanol extracts were associated with a strong reduction in cell viability specifically targeting premalignant cells. In contrast, the aqueous extracts of *Cyclopia* spp. were more effective in reducing cell viability. This correlated with a relatively high flavanol/proanthocyanidin content and ABTS radical cation scavenging capacity. The major green tea flavanol (epigallocatechin gallate) and rooibos dihydrochalcone (aspalathin) exhibited differential effects against cell viability, while the major honeybush xanthone (mangiferin) and flavanone (hesperidin) lacked any effect presumably due to a cytoprotective effect [22].

It should be noted that, MDA measurement is very important in pathological states, but it has also a large significance on the toxicological effects of pollutants such as metals, solvents and xenobiotics in humans and animals [9]. Nevertheless, in order to avoid any misinterpretation of the results obtained in this study, another alternative test for evaluating lipid peroxidation level in the muscle tissue of rainbow trout and assessing free radical scavenging potency of leaf extracts of *C. japonica* cultivar could be employed.

Conclusions. Based on our results, we suggested that there is a possibility of using extracts from leaves of various *C. japonica* cultivars in intensive aquaculture farms. Some discrepancy in scavenging potential of the *C. japonica* leaf extracts screened in this study and bibliographic data reporting profound biological activities of extracts derived from various part of this plant species may be due to variation in the percentage of phytoconstituents extracted in various solvents or to different cultivation conditions as well. To conclude, *Camellia japonica* may be used as an antioxidant agent in aquaculture as it can be easily obtained and is not expensive. Furthermore, the use of such plants products as antioxidants and immunostimulants in aquaculture systems may also have environmental value because of their biodegradability.

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УРОВЕНЬ ПЕРЕКИСНОГО ОКИСЛЕНИЯ ЛИПИДОВ В МЫШЕЧНОЙ ТКАНИ РАДУЖНОЙ ФОРЕЛИ (*ONCORHYNCHUS MYKISS WALBAUM*) ПРИ ИНКУБАЦИИ С ЭКСТРАКТАМИ ИЗ ЛИСТЬЕВ РАЗЛИЧНЫХ СОРТОВ *CAMELLIA JAPONICA L. (THEACEAE) (ОБЗОРНАЯ)*

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Целью исследования была оценка уровня перекисного окисления липидов (реактивные вещества 2-тиобарбитуровой кислоты (TBARS) как биомаркера) в мышечной ткани радужной форели после инкубации с экстрактами из листьев Camellia japonica. Исследовали экстракты листьев различных сортов камелии японской (C. japonica 'Kramer's Supreme', C. japonica 'C.M.Wilson', C. japonica 'La Pace', C. japonica 'Mrs. Lyman Clarke', C. japonica cv. 3, C. japonica cv. 15), выра-

щенных в условиях теплицы Национального ботанического сада им. М. М. Гришко НАН Украины.

Результаты исследования показали, что в интенсивных аквакультурных хозяйствах существует возможность использования экстрактов, полученных из листьев различных сортов камелии японской. Экстракт листьев камелии японской может быть использован в качестве антиоксиданта в аквакультуре. Кроме того, использование таких растительных продуктов, как антиоксиданты и иммуностимуляторы, в системах аквакультуры может также иметь экологическую ценность из-за их биоразлагаемости.

Ключевые слова: радужная форель (Oncorhynchus mykiss Walbaum), аквакультура, перекисное окисление липидов, реактивные вещества 2-тиобарбитуровой кислоты, Camellia japonica L., сорта растений.

РІВЕНЬ ПЕРЕКИСНОГО ОКИСЛЕННЯ ЛІПІДІВ У М'ЯЗОВІЙ ТКАНИНІ РАЙДУЖНОЇ ФОРЕЛІ (ONCORHYNCHUS MYKISS WALBAUM) ПРИ ІНКУБАЦІЇ З ЕКСТРАКТАМИ З ЛИСТЯ РІЗНИХ СОРТІВ CAMELLIA JAPONICA L. (THEACEAE) (ОГЛЯДОВА)

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Метою дослідження була оцінка рівня перекисного окислення ліпідів (реактивні речовини 2-тиобарбитуровой кислоти (TBARS) як биомаркера) в м'язовій тканині райдужної форелі після інкубації з екстрактами з листя Camellia japonica. Досліджували екстракти листя різних сортів камелії японської (C. japonica 'Kramer's Supreme', C. japonica 'C. M. Wilson', C. japonica 'La Pace', C. japonica 'Mrs. Lyman Clarke', C. japonica cv. 3, C. japonica cv. 15), вирощених в умовах теплиці Національного ботанічного саду ім. М. М. Гришка НАН України.

Результати дослідження показали, що в інтенсивних аквакультурних господарствах є можливість використання екстрактів, отриманих з листя різних сортів камелії японської. Екстракт листя камелії японської може бути використаний в якості антиоксиданту в аквакультурі. Крім того, використання таких рослинних продуктів, як антиоксиданти та імуностимулятори, в системах аквакультури можуть також мати екологічну цінність через їх біорозкладності.

Ключові слова: райдужна форель (Oncorhynchus mykiss Walbaum), аквакультура, перекисне окислення ліпідів, реактивні речовини 2-тиобарбитуровой кислоти, Camellia japonica L., сорти рослин.