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STUDY OF ANTIOXIDANT PROPERTIES OF GRAPE MARC EXTRACTS AS PERSPECTIVE PHARMACEUTICAL AND COSMETIC PRODUCTS

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The antioxidant activity (AA) of various extracts of grape marc (Vitis vinifera L.) was studied. Radical absorption activity (RAA) was calculated in reactions with $DPPH^+$ and ABTS•+ radicals and AA in rat liver homogenate by two markers of oxidative stress (OS): contents of thiobarbiturative products and carbonyl groups of proteins. All the extracts showed antioxidant activity. Water-based extract and 96 % ethanol extract obtained via heat bath method were inhibitors of the formation of free radical oxidation of lipids and proteins. 96% ethanol extract is promising for the development of pharmaceutical and cosmetic products.

Key words: extracts of grape marc, radical absorption activity, antioxidant properties.

Introduction

Viticulture is one of the most promising sectors in agriculture. More than half of the total grape harvest is used in winemaking, juice and special functional drinks production. These processes lead to a significant amount of waste, grape marc (other names: pomace, meal, cake, pulp), which amount is estimated from 10 to 30% of crushed grapes weight [1].

Grape marc (GM) is commonly used to make grape brandy and grape seed oil, also as a food for cattle. Much of this marc is composted for re-application to vineyards to complete the carbon cycle. New advanced GM processing technologies, such as thermochemical and biological treatment for energy recovery, environmental programs and technologies for the extraction of valuable components, are in considerable commercial interest. Grape contain more than 150 biologically active substances (BAS), much of it remains in the pomace. Due to the growing consumer demand for the use of eco-organic products, there is a wide range of applications for these BAS: functional foods and supplements, biosurfactants, pharmaceuticals and cosmetics, biomedical products and others [2,3]. The market potential of individual product lines based on grape pomace as a guide is hundred millions of dollars [4].

GM also contains a large number of BAS, the main are flavonoids. It is known that red grape polyphenols, in particular resveratrol, have antioxidant activity. These substances are able to eliminate or inhibit the free radical oxidation (FRO) of organic substances with oxygen, accept free radicals and inhibit the lipid peroxidation (LPO) of proteins and other compounds [5].

One of the adverse effects LPO considered of formation malonic dialdehyde (MDA) as a result of free radical-induced rupture of polyunsaturated fatty acids at the double bond site. This aldehyde forms Schiff bases with protein amino groups, acting as a “crosslinking” agent. Insoluble lipid-protein complexes (so-called wear pigments) are formed as a result of crosslinking. The concentration of MDA in the serum reflects the activity of LPO-processes in the patient's body, and it is used as a marker of the degree of endogenous intoxication [6].

Oxidation of lipids leads to disruption of the normal packaging of the membrane bilayer, which can cause damage to membrane-bound proteins [7]. For example, LPO can lead to inactivation of membrane receptors, also enzymes such as glucose-6-phosphatase and Na/K-ATPase, which is directly involved in maintaining cell ionic homeostasis [8].

However, LPO is not an exclusively destructive process. Peroxidation of lipids is important for the renewal of biological membranes, rotation of their protein and lipid components, regulation of physicochemical properties of cell membranes and subcellular structures [9]. Lipid peroxides and low molecular weight degradation products of oxidized lipids can participate in signal transduction, which determines the possibility of cell survival or death in

stressful situations [10]. Antioxidants are essential components of all tissues and body cells and support the physiological norms of free radical auto-oxidative processes. GM extracts are becoming increasingly popular in medical practice as antio-xidants in lipid-containing systems for the treatment of FRO-induced pathologies [11–12].

Obtaining extracts from plant raw materials is a multifactorial technological process. Technological parameters largely determine the extract properties from GM, such as: chemical composition, anti-radical and antioxidant properties, a restorative power. Therefore, the question of choosing the optimal drying conditions and storage of secondary grape raw materials, methods and conditions of extraction, equipment, as well as storage of finished extracts pay attention of many scientists and manufacturers [13–15].

One of the most important factors is the nature of the extraction solvent,because since the dependence of the antiradical properties of the extract on the extractant is known [13]. There are enough publications devoted to the study of the extraction conditions and the search for the optimal solvent, but there is no consensus on this issue. According to publications [14, 15], 96% ethanol can be considered the optimal extractant for extraction from grape seeds, however, the authors of some publications suggest using other solvents for the extraction of fat-soluble substances. According to [16], water is the best extractant. Temperature modes are also offered in different ways: from 36 to 105 °C (when receiving water extracts). Temperature in the range of 75–79 °C is optimal for obtaining extracts of pulp and skin of grapes with the maximum content of biologically active substances [15].

The **goal** of this work was to establish the best extract from the grape marc of red varieties in in terms of indicators of "optimal technology – antioxidant properties".

The following tasks were set: select, based on the analysis of information sources, the most accessible and easily reproducible technological processes for obtaining extracts from GM of Isabella and Moldova species; laboratory production of extracts and the study of their antioxidant properties.

Materials and methods of research

Objects of research: water, ethanol and chloroform extracts obtained from the marc of grapes of Isabella and Moldova species.

The results GM extracts were pre-tested for their radical absorption activity (RAA) to assess the antioxidant power. This method was based on the reaction with the relatively stable radical 2,2-diphenyl-1-picrylhydrazil (DPPH) and on the reaction with the establishment of the total antioxidant activity by neutralizing the cation radical 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$). Known substances with antioxidant properties – ascorbic acid and quercetin were selected as standards of comparison.

After identifying RAA GM-extracts, their antioxidant properties *in vitro* investigation were provided on the rat liver hepatocytes in free-radical oxidation conditions. Results identified by two oxidative stress markers: thiobarbituric acid active (TBA-active) products and carbonyl groups (CG) of proteins.

The method principle is based on the activation of lipid peroxidation (LPO) and oxidative modification of proteins (OMP) by ferrous ions to a level that is recorded spectrophotometrically. To do this, thawed tissues were homogenized in potassium phosphate buffer in a weight:volume ratio (1:10). Determination both indicators of oxidative stress were carried out in one sample. The amount of protein in the samples was determined by the Lowry method (Lowry, 1951).

Control solutions were solvents corresponding to the extracts. The study data were statistically processed taking into account the arithmetic mean M and the standard error of the arithmetic mean m in the form (M±m) at n=5. Differences between experi-mental data were determined using the Tukey's test of one-way analysis (ANOVA), where the differences were considered significant at $P<0.05$ [17].

Methods of obtaining extracts

Based on the information sources analysis [13–15], the following technological modes of the process of drying secondary grape raw materials were selected: convective drying at 50–52 °C for 24 hours. The dried raw materials were stored in a cool (14–18 °C) room with sufficient ventilation.

Aqueous, ethanol and chloroform extracts were obtained for the determination of the antio-xidant powers of the grape pomace of Isabella and Moldova varieties. Extracts in organic solvents were obtained in a Soxhlet extractor. Extraction of grape meal was carried out with a low-boiling solvent in the vapor phase, followed by its distillation and return to the stage of extraction of a new batch of raw materials. The extraction was performed until complete depletion of the raw material.

Aqueous extracts were obtained by two methods: 1) by steaming GM with water at 85 °C and infusing for 2 hours in the ratio mass:volume (1:5); 2) by extraction for 45 minutes in a water bath in the ratio of mass: volume (1:5) and infusion for 2 hours.

The obtained extracts were investigated for RAA in reactions with DPPH and $ABTS^{\bullet+}$ and for the non-enzymatic link of oxidative stress, namely, the content of MDA as secondary products of lipid peroxidation and the content of carbonyl groups of proteins as a result of OMP.

Methods of studying the indicators of radical-absorbing activity of DPPH.

Freshly prepared DPPH solution has a deep purple color. Antioxidant molecules can reduce DPPH free radicals and convert them to a colorless or slightly yellow product (2,2-diphenyl-1-picrylhydrazil) due to their proton-donor properties, which leads to a decrease in optical density at 517 nm (Fig. 1).

This property allows to observe the reaction and calculate the number of primary radicals by changing the optical density [18]. Current data were evaluated using the parameter IC_{50} – substrate concentration, which provides neutralization of DPPH by 50%. Ethanolic solution of ascorbic acid with a concentration of $1.76 \cdot 10^{-4}$ M and ethanolic solution of quercetin with a concentration of $3.02 \cdot 10^{-4}$ M. As reference standards used 10^{-4} M in a ratio 1:3 of DPPH with a concentration of $3.94 \cdot 10^{-4}$ M, because as this ratio of reagents corresponds to the IC_{50}.

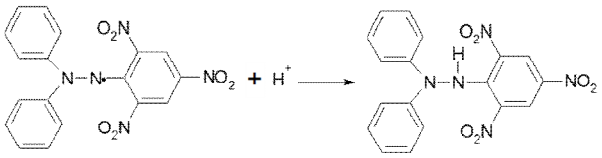

Fig. 1 Interaction scheme of DPPH with antioxidant

To 0.5 ml of test extracts was added 1.5 ml of a solution of DPPH in ethanol. The mixture was stirred vigorously and left for 30 minutes. After that, the solution was introduced into the cuvette of the ULAB 108UV spectrophotometer and its optical density was determined. The RAA of the compounds was calculated by the formula:

$$[RAA] = \frac{A_{DPPH} - A_{subst.}}{A_{DPPH}} \cdot 100\%, \qquad (1)$$

where A_{DPPH} – optical density of DPPH free radical solution (1.5 ml, $3.94 \cdot 10^{-4}$ M + 0.5 ml of ethanol); $A_{subst.}$ – the optical density of the DPPH solution with the test substance.

Methods of research of indicators of radical-absorbing activity of $ABTS^{\bullet+}$.

$ABTS^{\bullet+}$, 2,2'-azino-bis(3-ethylenebenzthiazoline-6-sulfonic acid) diammonium salt, is a stable cation radical used in the method of detecting the antioxidant properties of substances. $ABTS^{\bullet+}$ cation radical is obtained by incubating a mixture (1:1), wich containing 7 mmol $ABTS^{\bullet+}$ and 2.45 mmol $K_2S_2O_8$, under the following conditions: temperature – 20–25 °C, in the dark, for 20 hours (until the appearance of the characteristic dark green or blue-green color $ABTS^{\bullet+}$). Colored $ABTS^{\bullet+}$ is restored to its original colorless form in antioxidants presence (Fig. 2).

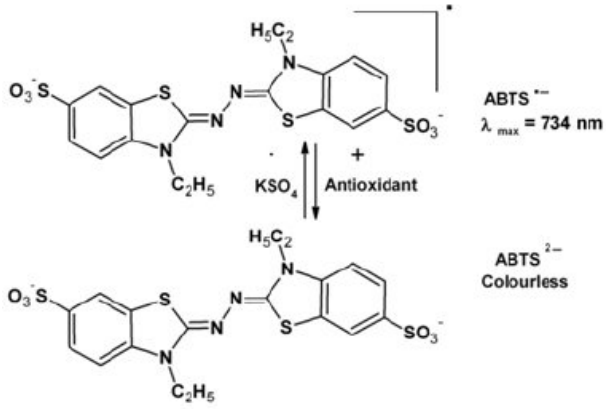

Fig. 2 Color change scheme of $ABTS^{\bullet+}$ with antioxydant

The reaction mixture consisted of 1 ml $ABTS^{\bullet+}$ and 100 μl test extracts diluted with phosphate buffer (pH=7.4) in a ratio of 1:20. A sample of 100 μl $ABTS^{\bullet+}$-free test extract with the addition of 1 ml distilled water was used as a control. A mixture of distillate and test extract in a ratio of 1:1 was poured into the comparison cuvette. Optical density was measured at a wavelength of 734nm after holding for 6 min in a dark place [18]. Antiradical activity was calculated by the formula:

$$\textbf{\% inhibition} = \frac{A_0 - A_v}{A_0} \cdot 100\%, \qquad (2)$$

where A_0– solution value; $A_v = A_g - A_k$; A_g – the value of the experiment; A_k – control value.

Methods research markers of non-enzymatic link oxidative stress in rat liver homogenate: lipid peroxidation (LPO) and oxidative modification of proteins (OMP)

5 ml of potassium phosphate buffer was added to 0.5 g of thawed and crushed rat liver tissue. 0.3 ml of the studied grape meal extracts was added to 0.3 ml of the obtained homogenate, and the corresponding solvents were added as a control. To induce LPO, 0.3 ml of 2.8% $FeSO_4$ solution was added and after 10 minutes. 0.3 ml of 4% H_2O_2 solution and incubated for 2 hours. The reaction was stopped with 1.2 ml of 40% trichloroacetic acid, which simultaneously precipitated proteins, followed by centrifugation for 10 minutes at 5000g. Determination of both indicators of oxidative stress was carried out in one sample – the content of TBA-active products was determined in the supernatant, and CG – in the sediment according to the method of VI Lushchak [9].

Determining methods TBA-active products

The content of TBA-active LPO products was determined in the selected samples by the reaction of MDA with thiobarbituric acid (TBA). At high temperatures in an acidic environment, MDA reacts with TBA, forming a colored trimethine complex with an absorption maximum λ= 532 nm. To 2 ml of the supernatant was added 1.5 ml of a 0.8% solution of TBA in 0.1 M HCl (pH=2.5) and incubated in a water bath at a temperature of 95–100 °C for 60 min. After cooling, 3 ml of butanol was added and centrifuged for 10 minutes at 5000g. Extinction measurements were performed in the upper butanol layer at λ=532 nm. The amount of protein in the samples was determined by the Lowry method (Lowry, 1951). The calculation was performed according to the formula:

$$[\boldsymbol{TBAAP}] = \frac{E \cdot V_1 \cdot V_2}{\varepsilon \cdot V \cdot C} \mu mol/mg \text{ protein}, \quad (3)$$

where E – extinction of the test sample; ε – millimolar extinction coefficient (ε=156 $cm^2/\mu mol$); V_1 – volume of butanol, ml; V_2 – sample volume, ml; V – volume of supernatant, ml; C – protein concentration in the supernatant, μmol.

Methods for determining the content of CG proteins

The degree of OMB was determined by the number of formed additional carbonyl groups in the side chains of amino acids, the content of which was determined by reaction with 2,4-dinitrophenylhydrazine (DNFH). For determination the CG of protein, 1 ml of a 1% solution of DNFH in 2M HCl was added to the precipitates after centrifugation of the homogenates. Mixture was triturated and incubated for 1 hour. at room temperature, then centrifuged for 10 min. at 5000 g. The precipitate was washed three times with 1 ml of a mixture of ethanol and ethyl acetate (1:1) and centrifuged in the previous mode. Washed precipitate was dissolved within 45 minutes in 3 ml of 50% urea solution. Undissolved material was separated by centrifugation in the previous mode. In the supernatants, the content of CG proteins was determined on a ULAB 108UV spectrophotometer at a wavelength λ=370 nm (light absorption by 2,4-diphenylhydrazones). Calculated the content of CG according to the formula:

$$[\boldsymbol{CG}] = \frac{\Delta D \cdot V_{samples}}{E_{370} \cdot C} nmol/mg \ protein, \quad (4)$$

where ΔD is the value of the difference between the optical densities of the experimental and control samples ($\Delta D = D_{research} - D_{control}$); $V_{samples}$ – sample volume (3 ml); E_{370} – molar extinction coefficient of DNFH (22000 $mol^{-1}cm^{-1}$); C – concentration of total protein, mg/ml.

Research results and their discussion

As a result of the study extracts, which were obtained from the grape pomace of Isabella and Moldova varieties convective drying at 50–52 °C for 24h. storage at 14–18 °C in the dark place.

As substances that inhibit the formation of products of free radical oxidation, the following conclusions can be drawn. Extracts in organic solvents were obtained in a Soxclet apparatus. Aqueous extracts were obtained by two methods: 1) steaming the HS with water at 85 ° C and infusion for 2 hours. in the ratio of mass: volume (1: 5); 2) extraction for 45 minutes in a water bath in the ratio of mass: volume (1: 5) and infusion for 2 hours.

All studied GM extracts can be considered as substances with antioxidant properties, as their action was manifested in the reduction markers of RAA and non-enzymatic link of oxidative stress.

The following conclusions can be drawn from the influence of extracts from red grape meal as substances that inhibit the formation of products of free radical oxidation

Research results of free radical level recovery DPPH and $ABTS^{\bullet+}$ for GM extracts in comparison with ascorbic acid and quercetin are presented in Fig. 3.

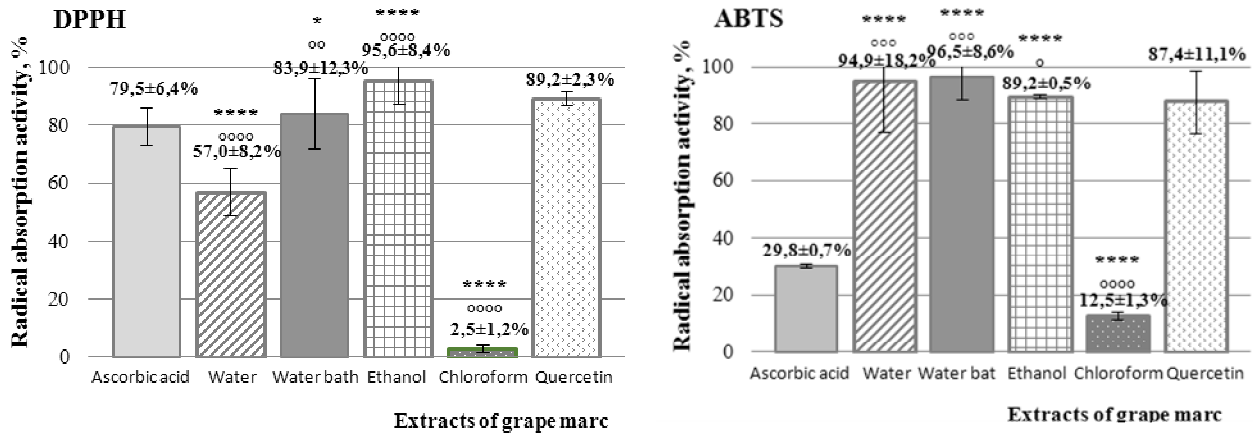

*Fig. 3. Radical absorption activity of extracts in reactions with DPPH and ABTS$^{\bullet+}$ (***– p≤0,005, ****– p≤0,001 compared to ascorbic acid; ^{o}– p≤0,05, ^{oo}– p≤0,01, ^{oooo} – p≤0,001 compared to quercetin; M±m; n=5)*

All extracts, except chloroform, showed high radical-absorbing activity. In terms of the ability to capture DPPH free radicals, the most effective, with a high degree of reliability was ethanol extract, the RAA of which was higher than the RAA of ascorbic acid and quercetin and was (95.6±8.4)%.,Higher RAA ,of the two aqueous extracts, has an aqueous extract (water bath method), which is 26.9% more able to capture DPPH free radicals. The aqueous extract (by steaming method) showed (57±8.2)% RAA and in comparison with ascorbic acid and quercetin, respectively, 22.5% and 32.2% less captures free radicals DPPH. Also, the above extracts, in terms of the ability to capture free radicals ABTS$^{\bullet+}$ showed higher than the standards of comparison RAA. This difference in antioxidant activity, determined by two methods, can be explained by the different content in the constituent extracts of hydrophilic and lipophilic antioxidants.

Research results of LPO on rat hepatocytes, namely, the content of TBA-active products under the action of the studied extracts of grape meal are presented in Fig. 4, and the results of OMP are represented by the content of CG shown in Fig. 5.

During the analysis of the obtained data it was found that according to two indicators of oxidative stress among the studied extracts the most effective was ethanol extract, which reduced the level of TBA-active products by 74.9% and 84.8% the level of CG in the proteine. significant reduction of LPO and OMP processes in comparison with control.

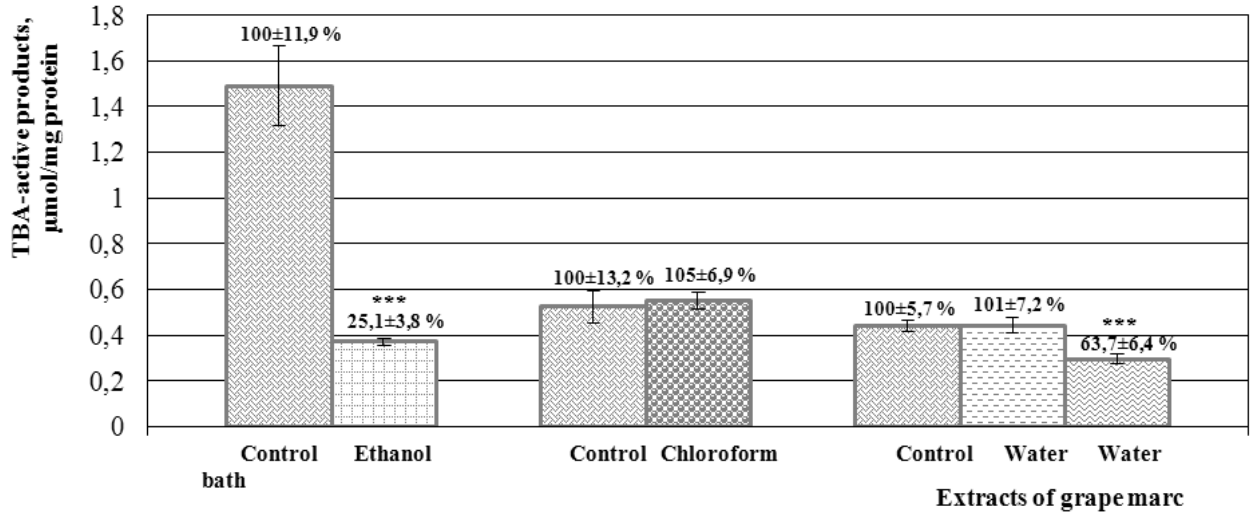

*Fig. 4. Amount of TBA-active products in rat liver homogenate influenced by red grape marc extracts(***- p≤0,005; M±m; n=5)*

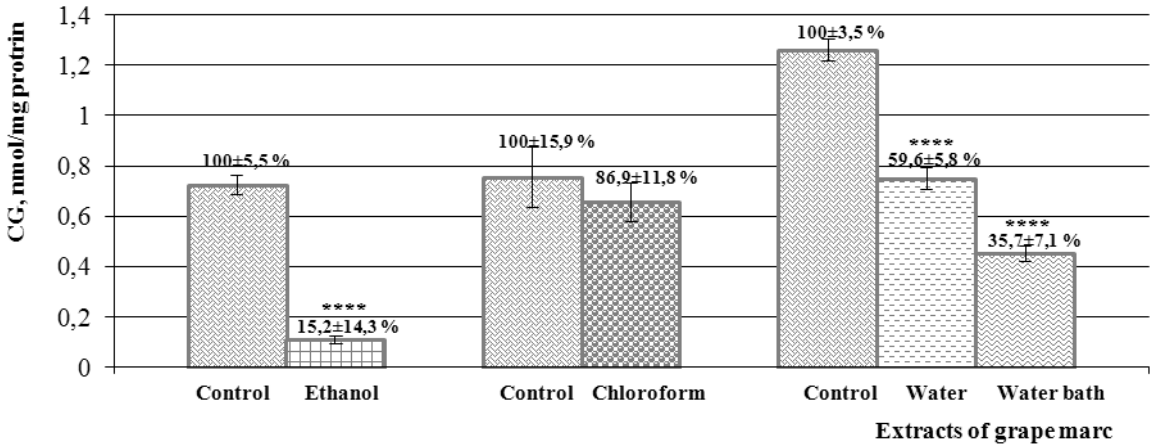

*Fig. 5. Amount of CG of proteins in rat liver homogenate influenced by red grape marc extracts (***- p≤0,005, ****- p≤0,001; M±m; n=5)*

Aqueous extract (water bath method) with a high degree of reliability reduced the level of lipoperoxidation by 36.3% and the content of CG in proteins by 64.3% relative to control. Chloroform and aqueous extracts, in turn, showed action within control.

The results of the influence of the studied extracts on the processes of OMP are presented in Fig. 5. The diagram shows that their best effect was manifested in the neutralization of free radical processes in proteins due to a significant reduction in the intensity of CG formation compared to control, possibly due to the activation of enzymatic systems of antioxidant protection.

In particular, with the highest degree of reliability relative to control, ethanol extract by 84.8%, aqueous extract (obtained by extraction in a hot bath) – by 64.3 % and aqueous extract (obtained by steaming) – by 40.4% reduced the content of CG in proteins. Chloroform extract also showed some antioxidant effect, which reduced the level of OMP by 13.1% compared to the control.

Conclusions

The GM extract in 96% ethanol had the best antioxidant properties. Its presence reduced the amount of MDA by 74.9% and the formation of CG by 84.8% compared to the control.

The aqueous extract obtained by extraction in a water bath reduced the amount of MDA by 36.3% and the formation of CG by 64.3%.

The results of studing radical-absorbing action of above-mentioned studied extracts in reactions with stable radicals DPPH and $ABTS^{\bullet+}$ correlate with the results of their antioxidant properties in the processes of LPO and OMP.

The chloroform extract had a stronger effect in OMP processes, as the level CG in comparison with the control decreased by 13.1%, and in the processes of lipoperoxidation – within the control.

For further use in the development of antioxidant prodacts, the most promising are the extract in 96% ethanol.

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