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THE IMPACT OF GRAPE POMACE EXTRACT RICH IN NATURAL COMPLEX OF POLYPHENOLS ON MORPHO-FUNCTIONAL STATE OF LEUKOCYTES UNDER EXPERIMENTAL DIABETES MELLITUS

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Background. Diabetes mellitus is one of the most common diseases in the world. Under this pathology all organs and systems of an organism are damaged, including the immune system. Peripheral blood leukocytes are an important element of this system that suffer damage under diabetes mellitus due to the influence of reactive oxygen species and reactive nitrogen species, the number of which increases fast and leads to the development of oxidative-nitrative stress. Thus, the discovery of new diabetes-correcting drugs that possess hypoglycemic, antioxidant and immunomodulatory properties is one of the principal tasks. Such properties are inherent in polyphenolic compounds, a large amount of which is contained in the grape pomace. That is why the study of grape pomace extract, rich in a natural complex of polyphenols, is important to evaluate the possibility of further use of these substances as a basis for drugs that can be used in the complex therapy of diabetes mellitus.

Materials and Methods. The research used peripheral blood leukocytes of the control rats, the control animals that were treated with grape pomace extract for 14 days, animals with streptozotocin-induced diabetes mellitus and rats with experimental diabetes mellitus that were treated with grape pomace extract for 14 days. To evaluate the corrective effect of the grape pomace extract rich in a natural complex of polyphenols on the state of the antioxidant defense and the L-arginine/NO systems, the activity of antioxidant enzymes, the level of oxidative modification products of proteins and lipids, the activity of NO-synthase, the content of nitrites and nitrates, 3'-nitrotyrosine-modified proteins were studied. The total number of leukocytes, the white blood cell differential and the phagocytic activity, and the average cytochemical coefficients of

cationic proteins and NADPH-oxidase activity were indicated to study the effect of the grape pomace extract on the functional state of leukocytes.

Results and Discussion. The study revealed normalization of the total number of leukocytes and the white blood cell differential, the activity of NADPH-oxidase, superoxide dismutase, catalase and glutathione peroxidase, and the levels of the oxidative modification products of proteins and lipids, nitrites, nitrates and 3'-nitrotyrosine-modified proteins, as well as an increase in the average cytochemical coefficient of cationic proteins, and a decrease of the inducible NO-synthase activity after grape pomace extract administration for 14 days to animals with streptozotocin-induced diabetes mellitus.

Conclusion. The obtained results confirm the antioxidant and immunomodulatory effects of the studied extract and justify the feasibility of using grape pomace complex of polyphenolic compounds as a basis for new drugs that will be used in the complex therapy of diabetes mellitus.

Keywords: grape pomace, diabetes mellitus, leukocytes, oxidative stress, nitrative stress, polyphenols

INTRODUCTION

According to the World Health Organisation, diabetes mellitus (DM) is a chronic metabolic disease characterized by an elevated level of glucose in the blood (Diabetes, n.d.).

Changes in the intracellular metabolism, intensification of glycation processes and the development of oxidative-nitrative stress against the long-term hyperglycemia background are the main factors that induce pathological changes in the structural components of cells and affect their functional state under conditions of DM (Giri *et al.*, 2018) tissues and organ systems. Hyperglycemia can induce oxidative stress, upsurge polyol pathway, activate protein kinase C (PKC, in particular peripheral blood leukocytes. It is worth noting that an increase in the glucose level in the blood causes damage to blood cells, which, in turn, leads to chronic inflammation, and defects in the chemotactic, phagocytic and bactericidal abilities of immunocompetent cells (Pettersson *et al.*, 2011).

Natural compounds with hypoglycemic, antioxidant, immunomodulatory and antibacterial properties attract attention of researchers because these compounds are a potential basis for new drugs. Polyphenols occupy an important place among such natural compounds. A large amount of polyphenols is found in grapes, and the main part of these compounds is concentrated in the seeds and skin (Elejalde *et al.*, 2021; Sabadashka *et al.*, 2021). Thus, it is advisable to obtain an extract from grape pomace, which is a by-product of winemaking and contains a lot of polyphenols.

The aim of our research was to study the effect and mechanisms of action of the grape pomace extract, rich in a natural complex of polyphenols (NCP extract), in leukocytes under DM, to evaluate the prospects of further use of such a natural complex of polyphenols as a basis for new diabetes-correcting drugs.

MATERIALS AND METHODS

The experiments were performed on male Wistar rats weighing 100–180 g. The research was conducted according to “General Principles of Experiments on Animals”, approved by the First National Congress on Bioethics (Kyiv, Ukraine, 2001) and agreed with the guidelines from the Directive 2010/63/EU of the European

Parliament “On protection of Animals from Cruelty” of February 26, 2006, and also approved by the Ethics Committee of Ivan Franko National University of Lviv, Ukraine (protocol No. 42-03-2024 of March 5, 2024).

Grape pomace for the research was provided by Odesa National University of Technology (Ukraine). The method of obtaining the NCP extract is described in (Skorobahatko *et al.*, 2023). The total content of polyphenols in the NCP extract was detected using the Folin-Ciocalteu reagent (Waterhouse, 2002). Gallic acid was used as a standard. The content of polyphenolic compounds in the obtained concentrate was 80 mg/mL.

Experimental DM was induced by intraabdominal injection of Streptozotocin (Sigma, USA), dissolved in a 10 mM citrate buffer (pH 5.5), at a dose of 60 mg/kg of body weight. The induction of DM was controlled according to blood glucose level, which was measured on the 3rd and 14th days after Streptozotocin injection. Rats with glucose concentrations higher than 12 mmol/L were used in the study. Glucose concentration was detected with a Contour plus glucometer (Bayer, Switzerland).

All experimental rats were divided into 4 groups: group 1 – control animals (C), group 2 – control rats that were treated with the NCP extract for 14 days (C + NCP), group 3 – rats with experimental DM (DM), and group 4 – rats with experimental DM that were orally treated with the NCP extract for 14 days (DM + NCP) (**Fig. 1**).

The obtained extract was administered *per os* with water by gavage at a dose of 45 mg of polyphenols per 1 kg of body weight.

Rats from all experimental groups were decapitated under ether anesthesia on the 29th day of the experiment (**Fig. 1**). Blood sampling was performed using the anticoagulant heparin (final dilution of heparin to whole blood = 1:100).

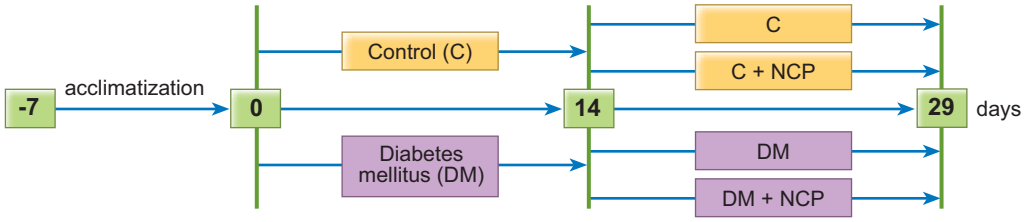

Fig. 1. Scheme of the experimental design

The total number of leukocytes was determined using the hemocytometer (Chala *et al.*, 2024). The white blood cell differential was performed by counting cells on fixed smears of peripheral blood painted by the Romanovic–Gimzia method (Chala *et al.*, 2024).

Separation of leukocytes was performed by centrifugation over a gradient of ficoll-triombrast density (Chala *et al.*, 2024). The study of the functional activity of leukocytes was carried out by determining the phagocytic activity and calculating the average cytochemical coefficient (ACC) of cationic proteins and the nitro-blue tetrazolium reduction test (NBT-test), for the detection of defects in the oxidative metabolism of phagocytes, following (Chala *et al.*, 2024). Non-opsonized yeast was used as an object of phagocytosis.

For further experiments, cells were lysed with 50 mM Na-K-phosphate buffer (pH 7.4) at the rate of 1 million leukocytes in 60 µL of buffer. The studied parameters were determined in the supernatant obtained after centrifugation of lysates for 5 min at 10,000 rpm.

The concentration of proteins was detected by the conventional Lowry method (Lowry *et al.*, 1951). Activities of catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1) and glutathione peroxidase (GPx, EC 1.11.1.9) were detected according to (Sabadashka *et al.*, 2021). The content of proteins oxidative modification products and the content of thiobarbituric acid-reactive substances (TBARS) were detected according to (Sabadashka *et al.*, 2021). Activities of inducible isoform of NO-synthase (iNOS) and constitutive isoforms of NO-synthase (cNOS), the content of nitrite- and nitrate-anions were detected according to (Spryn *et al.*, 2021). The level of 3'-nitrotyrosine-modified proteins was determined by Western blot analysis following (Drel & Sybirna, 2010).

Statistical analysis of the research results was carried out using Microsoft Excel. The calculation of main statistical parameters was performed by direct quantitative data obtained from the research (arithmetic mean – M; standard deviation of the arithmetic mean – SD). The statistical significance of differences between group means was assessed by one-way analysis of variance (ANOVA) with post hoc analysis. The pairwise comparison of data was performed using Tukey's test. The difference was considered significant under the indications of reliability $p \geq 0.95$ (significance level $P < 0.05$).

RESULTS AND DISCUSSION

The immunomodulatory effect of the grape pomace extract. An increase in the total number of peripheral blood leukocytes by 19.66 %, a decrease in the number of segmented neutrophils by 27.32 %, as well as an increase in the number of lymphocytes by 16.67 % under the condition of DM, compared to the control, were detected (**Table 1**). Under the NCP extract administration to control animals, an increase in the number of segmented neutrophils by 12.49 % and a decrease in the number of lymphocytes by 5.83 %, compared to the control, were detected (**Table 1**).

Table 1. **The number of leukocytes and the white blood cell differential of rats' peripheral blood in the norm, under experimental diabetes mellitus and after treatment with the grape pomace extract (M±SD, n = 6–9)**

Parameters	Groups			
	C	C + NCP	DM	DM + NCP
Total leukocytes, 10^3/μL	10.82±0.52	9.88±0.52	12.94±0.82*	10.14±0.42#
Band neutrophils, %	2.44±0.38	2.38±0.42	1.2±0.37	2.63±0.38
Segmented neutrophils, %	35.22±0.68	40.25±1.88*	25.6±1.72**	30.44±1.82#
Eosinophils, %	0.11±0	0.38±0.26	0.4±0.24	0.56±0.24
Basophils, %	0.78±0.22	0.75±0.31	0.8±0.37	0.89±0.31
Monocytes, %	2.22±0.4	1.25±0.56	2.0±0.63	2.78±0.4
Lymphocytes, %	60.0±1.19	56.5±1.54*	70.0±1.61***	61.63±1.36##

Note: **, *** – significant difference compared to the control (p ≥0.99, p ≥0.999); #, ## – significant difference compared to diabetes (p ≥0.95, p ≥0.99)

The administration of the NCP extract to animals with experimental DM contributed to the normalization of the total number of leukocytes, while the number of lymphocytes

decreased by 13.58 %, and the number of segmented neutrophils increased by 18.92 %, compared to diabetes (**Table 1**). The administration of the NCP extract contributed to an increase in the number of neutrophils combined with a decrease in the number of lymphocytes, as well as a decrease in the total number of leukocytes, which indicates the anti-inflammatory properties of polyphenolic compounds (Chala *et al.*, 2024; Magrone *et al.*, 2008). Thus, a decrease in the number of pro-inflammatory cytokines due to the influence of polyphenols can lead to a decrease in the number of immune cells, in particular lymphocytes, which was found in animals with DM (Chala *et al.*, 2024).

Changes in the functional properties of leukocytes under DM are a prerequisite for the immune status violation and the development of infectious and inflammatory processes, which tangles the course of the disease and causes complications (Moradi *et al.*, 2012). Therefore, we studied the ability of peripheral blood leukocytes to phagocytosis, ACC of cationic proteins and the NBT-test.

The number of leukocytes that entered phagocytosis (phagocytic index, PI) in rats with DM, compared to the control, decreased by 6.06 % and 3.65 % after 30 and 120 min of incubation, respectively. In the group of animals with DM, after stimulation cells with N-formylmethionyl-leucyl-phenilalanine (fMLP), PI decreased by 7.59 % and 4.06 % after 30 and 120 min of incubation, respectively, compared to control (**Table 2**). When the NCP extract was administered to control animals, significant changes in PI were not detected, both without and with fMLP stimulation of leukocytes. In animals with DM, in the case of the NCP extract administration, the number of phagocytic cells was normalized after 30 and 120 min of incubation without and with the addition of fMLP (**Table 2**).

Table 2. **The number of phagocytic peripheral blood leukocytes of rats (the phagocytic index, PI) and the average number of microorganisms in the phagocyte (the phagocytic number, PN) in the norm, under experimental diabetes mellitus and after treatment with the grape pomace extract (M±SD, n = 6–9)**

Parameters	Groups			
	C	C + NCP	DM	DM + NCP
PI 30, %	96.6±1.17	97.8±0.80	90.75±1.25**	95.17±1.14$^{\#}$
PI 30 + fMLP, %	97.4±0.68	98.2±0.37	90.0±1.08***	94.83±0.60$^{\#\#\#}$
PI 120, %	96.0±0.55	96.67±0.67	92.5±1.19*	96.67±0.76$^{\#\#}$
PI 120 + fMLP, %	97.2±0.58	98.29±0.57	93.25±0.85***	96.57±0.29$^{\#\#}$
PN 30, yeast cells	2.34±0.04	2.66±0.03***	1.82±0.05***	2.13±0.02$^{\#\#\#}$***
PN 30 + fMLP, yeast cells	2.89±0.01	2.93±0.06	2.17±0.01***	2.31±0.03**
PN 120, yeast cells	2.44±0.02	2.51±0.11	1.79±0.05***	2.05±0.02$^{\#\#, ***}$
PN 120 + fMLP, yeast cells	2.62±0.07	2.87±0.03**	2.22±0.01***	2.35±0.03***

Note: *, **, *** – significant difference compared to the control (p ≥.95, p ≥0.99, p ≥0.999); #, ##, ### – significant difference compared to the diabetes (p ≥0.95, p ≥0.99, p ≥0.999)

To determine the number of absorbed objects, the number of internalized particles inside the phagocyte (phagocytic number, PN) is determined (de Souza Ferreira *et al.*, 2012). A decrease in the number of absorbed microorganisms in one phagocyte in animals with DM by 22.29 % and 26. 27 % was detected after 30 and 120 min of incubation,

respectively, compared to the control. Under the conditions of cells stimulation with fMLP, PN in animals with DM decreases by 24.93 % and 15.46 % after 30 and 120 min of incubation, respectively, compared to the control (**Table 2**). An increase in PN by 12.02 % after 30 min of incubation without fMLP stimulation and by 8.76 % after 120 min of incubation with fMLP stimulation was detected under conditions of the studied extract administration to control animals, compared to the control, which indicates the ability of polyphenols to restore the functional activity of leukocytes. When the NCP extract was administered to animals with the studied pathology, an increase in the number of absorbed objects of phagocytosis by 16.85 % and 14.23 % after 30 and 120 min of incubation, respectively, without fMLP stimulation, and a tendency to increase PN by 6.25 % and 6. 09 % after 30 and 120 min of incubation, respectively, with fMLP stimulation were detected, compared to DM (**Table 2**).

The obtained results can be explained by the fact that long-term hyperglycemia is accompanied by preactivation of neutrophils, which is manifested by defects in chemotaxis, phagocytic and bactericidal abilities of these cells (Giri *et al.*, 2018; Insuela *et al.*, 2019) tissues and organ systems. Hyperglycemia can induce oxidative stress, upsurge polyol pathway, activate protein kinase C (PKC. Polyphenolic compounds can cause a decrease in glucose concentration in rats' blood (Boydens *et al.*, 2016), and, thus, eliminate the negative impact of hyperglycemia on the functional state of immunocompetent cells. In addition, phagocytic activity can be increased due to an increase in the ATP level in leukocytes under conditions of the NCP extract administration (Wood dos Santos *et al.*, 2018).

It was established that under experimental DM the number of cationic proteins in peripheral blood leukocytes of rats decreases, compared to the control, by 20.64 % and 17.36 % without and in the presence of non-opsonized yeast without leukocytes preincubation with fMLP, respectively. Under conditions of the studied cells preincubation with fMLP under DM, the ACC of cationic proteins decreases, compared to the control, by 30.19 % and 25.96 % without and in the presence of non-opsonized yeast, respectively (**Fig. 2**). Indicators of cationic proteins ACC of peripheral blood leukocytes of control rats treated with the NCP extract does not significantly change in any of the experimental groups compared to the indicators of control animals (**Fig. 2**).

It was detected that under administration of the NCP extract to animals with experimental DM, the lysosomal cationic proteins ACC increased by 7.17 % and 13.08 %, compared to DM, without and in the presence of non-opsonized yeast, respectively, without stimulation with fMLP. Under the same conditions, after pre-incubation of leukocytes with the bacterial tripeptide the studied parameter increases by 7.11 % and 7.05 % without and in the presence of non-opsonized yeast, respectively, compared to DM (**Fig. 2**).

A decrease in the cationic proteins level in neutrophils is the evidence of a violation of the cells' bactericidal system which can be associated with a violation of cell signaling, the consequence of which is, in particular, the suppression of the expression of defensins genes (Froy *et al.*, 2007). The NCP extract administration to animals with DM increased the level of cationic proteins in cells without and with stimulation by the bacterial tripeptide fMLP. This effect may be due to the fact that polyphenols, in particular quercetin, catechin and myricetin, can modulate the immune response through their powerful antioxidant and anti-inflammatory effects (Bahadoran *et al.*, 2013). We also assume that cationic proteins, like other proteins, are damaged by reactive oxy-

gen species (ROS), the level of which increases under oxidative stress (Sabadashka *et al.*, 2021). However, polyphenolic compounds, showing antioxidant scavenger effect (Sabadashka *et al.*, 2021), reduce the ROS level under DM and, thus, lead to an increase in the level of cationic proteins.

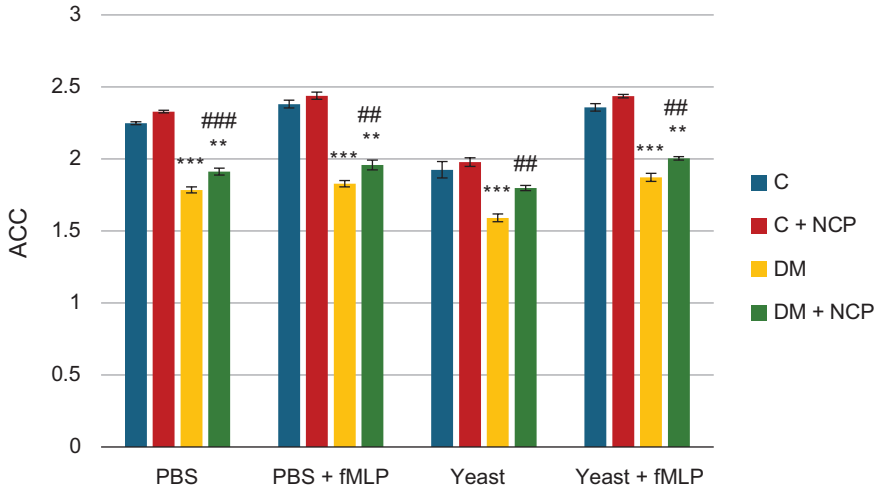

Fig. 2. The average cytochemical coefficient (ACC) of cationic lysosomal proteins of peripheral blood leukocytes of rats in the norm, under experimental diabetes mellitus and after treatment with the grape pomace extract (M±SD, n=6–9)

Note: **, *** – significant difference compared to the control (p ≥0,99, p ≥0,999); ##, ### – significant difference compared to the diabetes (p ≥0,99, p ≥0,999). PBS – phosphate-buffered saline

NADPH-oxidase is the enzyme responsible for the effector potential of phagocytes through the synthesis of ROS, in particular superoxide anion, which contributes to the neutralization of pathogens (Taylor-Fishwick, 2013; Vermot *et al.*, 2021). Therefore, we conducted the NBT-test, which reflects the activity of this enzyme (Baisya *et al.*, 2023). The study was conducted under the conditions of spontaneous (without leukocytes preincubation with fMLP) and induced (with leukocytes preincubation with fMLP) phagocytosis.

It was found that under the conditions of experimental DM, the ACC of the peripheral blood leukocytes NBT-test increased by 18.05 % and 32.38 %, compared to the control, under spontaneous phagocytosis without and in the presence of non-opsonized yeast, respectively. In the case of induced phagocytosis under DM, an increase in the studied parameter by 36.19 % and 21.34 % was found without and in the presence of non-opsonized yeast, respectively, compared to the control (**Fig. 3**). When the NCP extract was administered to the control animals, a tendency to an increase in the studied parameter by 7.12 % and 11.89 % was found under spontaneous phagocytosis without and in the presence of non-opsonized yeast, respectively, compared to the control. Under the induced phagocytosis, the NCP extract administration revealed a tendency to increase the ACC of the peripheral blood leukocytes NBT-test of control animals by 11.05 % without non-opsonized yeast, compared to the control (**Fig. 3**). The NCP extract administration to animals with experimental DM contributed to the normalization of the ACC of peripheral blood leukocytes NBT-test both during spontaneous and induced phagocytosis (**Fig. 3**).

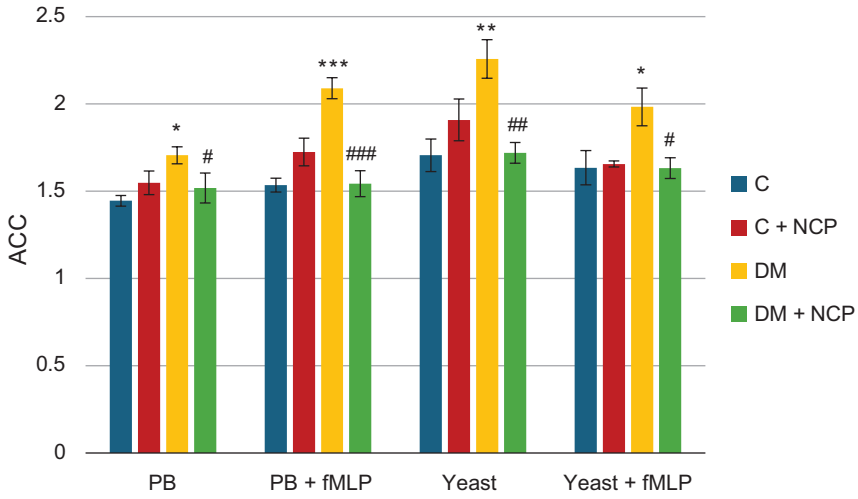

Fig. 3. The average cytochemical coefficient (ACC) of the NBT-test of peripheral blood leukocytes of rats in the norm, under experimental diabetes mellitus and after treatment with the grape pomace extract (M ± SD, n = 6–9)

Note: *, **, *** – significant difference compared to the control (p ≥0.95, p ≥0.99, p ≥0.999); #, ##, ### – significant difference compared to the diabetes (p ≥0.95, p ≥0.99, p ≥0.999). PB – Na/K-phosphate buffer

Pro-inflammatory cytokines, the level of which increases under DM, can influence the activity of NADPH-oxidase (Checa & Aran, 2020; Taylor-Fishwick, 2013). Under these conditions the content of superoxide anion increases, therefore the indicators of the NBT-test are high. The effect of polyphenolic compounds under DM may be explained by their ability to inhibit chronic inflammatory processes, in particular through the inhibition of the pro-inflammatory cytokines release (TNF-α, IL-1β and IL-6) (Zhang *et al.*, 2018).

The antioxidant activity of the grape pomace extract under diabetes-induced oxidative-nitrative stress. It was found that the activity of SOD, CAT and GPx of peripheral blood leukocytes of rats with experimental DM was reduced by 72.91 %, 56.72 % and 34.84 %, respectively, compared to the control (**Table 3**). The activity of these antioxidant enzymes in the peripheral blood leukocytes of the control animals did not significantly change compared to the control after the NCP extract administration. When the studied extract was administered to animals with DM, normalization of the activity of SOD, CAT and GPx in the peripheral blood leukocytes was observed (**Table 3**).

Suppression of the SOD activity under the studied pathology can be caused by its inactivation by hydrogen peroxide (H_2O_2) (Hink *et al.*, 2002). Since H_2O_2 is formed as a result of glucose autoxidation, its level is high in patients with diabetes. In addition, the accumulation of $O_2^{\bullet-}$ and the overproduction of NO leads to the excessive formation of peroxynitrite ($ONOO^-$) under DM, which is an oxidative damage mediator to cells. In turn, $ONOO^-$ can inactivate SOD by nitration at the Tyr34 in the active site of the enzyme (Hertsyk *et al.*, 2021). Also, the inactivation of such enzymes as SOD may be caused by a lack of cofactors, including Zn^{2+} and Cu^{2+}, the homeostasis of which is impaired under DM conditions (Ganesh & Meenakshi, 2023; Promyos *et al.*, 2023). A decreased CAT activity under DM may be associated with gene expression dysregulation. In addition,

under DM, CAT undergoes excessive phosphorylation, which can also inhibit enzyme activity (Hertsyk *et al.*, 2021). It is also known that the formation of NO, which can bind to the Ferrum-porphyrin complex of CAT with the formation of nitroso derivatives, increases under diabetes. The formation of such a complex prevents binding of H_2O_2 in the active site of CAT, which, accordingly, inhibits its splitting (Hertsyk *et al.*, 2021). In addition, the reduced activity of CAT under the studied pathology may be due to the inhibition of this enzyme by excessive amounts of $O_2^{\bullet-}$, which is due to the reduced SOD activity (Altobelli *et al.*, 2020). A decrease in the GPx activity under DM may be related to the fact that, under pathological conditions, glucose metabolism in the sorbitol pathway increases, in which the enzyme aldose reductase uses $NADPHH^+$. Since it is also a cofactor of glutathione reductase, this can lead to a decrease in the level of reduced glutathione, which is the main substrate for GPx (Kanikarla-Marie *et al.*, 2019) and its depletion increases oxidative stress. Diabetes is associated with lower blood levels of lc and GSH. The mechanisms leading to a decrease in lc in diabetes are not entirely known. This study reports a significant decrease in LC in human monocytes exposed to high glucose (HG).

Table 3. **The activity of antioxidant enzymes and the content of protein and lipid oxidative modification products in peripheral blood leukocytes of rats in the norm, under experimental diabetes mellitus and after treatment with the grape pomace extract (M±SD, n=6–9)**

Parameters	Groups			
	C	C + NCP	DM	DM + NCP
The activity of antioxidant enzymes				
Superoxide dismutase, U/μg of protein	72.32±4.98	71.21±5.73	19.59±3.54***	66.80±4.34###
Catalase, nmol of H_2O_2/(min·mg of protein)	37.37±3.54	32.63±3.25	16.17±1.51***	35.19±2.63###
Glutathione peroxidase, μmol G-SH/(min·mg of protein)	1425.17±113.25	1416.74±95.74	928.68±52.25*	1523.09±187.37##
Oxidative modification products of proteins and lipids				
Carbonyls of neutral character, %	100.0±13.79	106.73±8.88	199.02±25.98***	108.37±14.01###
Carbonyls of basic character, %	100.0±14.29	104.09±7.68	180.12±31.05**	117.29±14.59#
TBA-reactive substance, %	100.0±2.08	87.4±1.94*	172.59±7.59***	92.69±2.48###

Note: *, **, *** – significant difference compared to the control (p ≥0.95, p ≥0.99, p ≥0.999); #, ##, ### – significant difference compared to the diabetes (p ≥0.95, p ≥0.99, p ≥0,999)

An increase in the activity of SOD after the NCP extract administration under DM may be caused by an increase in the activity of CAT and GPx, which can neutralize such a product of the SOD reaction as H_2O_2. After all, an excessive amount of ROS causes a decrease in the SOD activity by a negative feedback mechanism. Polyphenolic compounds can increase CAT activity due to the ability of these compounds to suppress

excessive phosphorylation of this enzyme under DM (Hertsyk *et al.*, 2021). In addition, polyphenolic compounds can scavenge ROS and Reactive Nitrogen Species (RNS), which contributes to the CAT activity restoration under DM. Normalization of the GPx activity after the NCP extract administration may be associated with the hypoglycemic effect of polyphenols (Sabadashka *et al.*, 2021). Polyphenolic compounds can show a hypoglycemic effect acting as tyrosine kinase inhibitors, in particular Abelson tyrosine kinase (c-Abl) (Fountas *et al.*, 2015). This can contribute to the inhibition of glucose metabolism in the sorbitol pathway, and accordingly, a sufficient amount of NADPH goes to glutathione reductase, which contributes to the formation of reduced glutathione, necessary for GPx.

In peripheral blood leukocytes of rats under experimental DM, an increase in the content of proteins oxidative modification products of neutral and basis characters by 99 % and 80.1 % was detected, respectively, compared to the control (**Table 3**). No significant changes in the level of proteins oxidative modification products were detected after the NCP extract administration to control animals. When the studied extract was administered to rats with DM, normalization of the level of proteins oxidative modification products in peripheral blood leukocytes was observed (**Table 3**).

The study revealed an increase in the level of TBARS under the conditions of DM in the peripheral blood leukocytes of rats by 72.6 %, compared to the control (**Table 3**). When the NCP extract was administered to the control animals, a decrease in the level of lipid peroxidation products was observed, compared to the control. Normalization of the TBARS level was found in peripheral blood leukocytes of rats with experimental DM, which were treated with the extract (**Table 3**).

The NCP extract administration contributed to the normalization of the oxidative modification products of proteins and lipids under DM both due to the ability of polyphenolic compounds to scavenge ROS and the ability of polyphenols to normalize the activity of antioxidant enzymes (Sabadashka *et al.*, 2021).

The state of the L-arginine/NO system elements was also studied. Under experimental DM in peripheral blood leukocytes, an increase in the activity of the inducible isoform of NO-synthase (iNOS) by 80.8 %, compared to the control was detected, while no significant changes in the activity of constitutive isoforms of NO-synthase (cNOS) were observed (**Table 4**). No significant changes in the activity of the enzyme were detected when the NCP extract was administered to the control animals. In rats with DM, the studied extract contributed to a decrease in iNOS activity in peripheral blood leukocytes by 41.17 %, compared to DM (**Table 4**).

The decrease in the iNOS activity after the NCP extract administration may be due to the ability of polyphenols, in particular gallic acid and quercetin, to inhibit gene expression of this NOS isoform (Serreli & Deiana, 2023).

Under the conditions of experimental DM, an increase in the content of stable NO metabolites: nitrite- and nitrate-anions in peripheral blood leukocytes by 81.03 % and 36.55 %, respectively was detected (**Table 4**). No significant changes in the studied parameters were found when the NCP extract was administered to the control animals. In peripheral blood leukocytes, when the NCP extract was administered to animals with DM, a decrease in the level of nitrite- and nitrate-anions by 102.55 % and 33.39 %, respectively, compared to DM was found (**Table 4**).

The content of 3'-nitrotyrosine is considered an indirect indicator of the $ONOO^-$ level in the cell, as well as an important marker of oxidative-nitrative stress (Sherwani

et al., 2016). An increase in the level of 3'-nitrotyrosine-modified proteins under conditions of DM in peripheral blood leukocytes by 96.54 % was established, compared to the control (**Fig. 4**).

Table 4. **The NO-synthase activity and nitrite- and nitrate-anions content in peripheral blood leukocytes in the norm, under experimental diabetes mellitus and after treatment with the grape pomace extract (M±SD, n = 6–9)**

Indicators	Groups			
	C	C + NCP	DM	DM + NCP
The NO-synthase activity				
iNOS, μmol NO_2^-/(mg of protein·min)	132.57±6.98	140.97±14.32	239.67±19.55***	169.78±14.03##
cNOS, μmol NO_2^-/ (mg of protein·min)	49.72±5.38	44.71±8.97	67.95±15.46	59.73±4.53
The content of nitrite- and nitrate-anions				
Nitrite-anions, %	100.0±6.70	95.69±6.48	181.03±25.98**	78.48±9.53##
Nitrate-anions, %	100.0±4.57	95.70±3.44	136.55±5.71***	103.16±4.16###

Note: *, **, *** – significant difference compared to the control (p ≥0.95, p ≥0.99, p ≥0.999); #, ##, ### – significant difference compared to the diabetes (p ≥0.95, p ≥0.99, p ≥0.999)

No significant changes in the studied indicator were found when the NCP extract was administered to the control animals. Normalization of the level of 3'-nitrotyrosine-modified proteins in peripheral blood leukocytes was detected after administration of the NCP extract to rats with DM (**Fig. 4**).

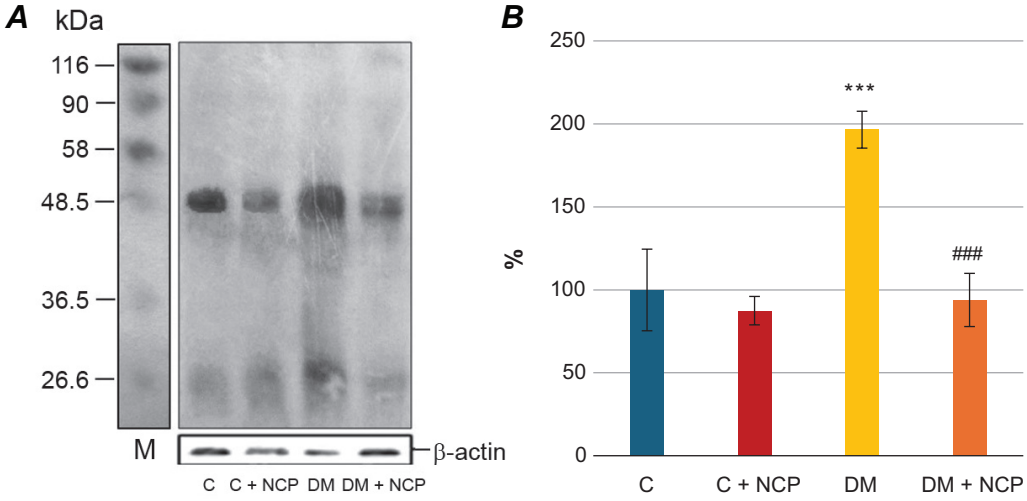

Fig. 4. Western blot analysis of 3'-nitrotyrosine-modified proteins in peripheral blood leukocytes in the norm, under experimental diabetes mellitus and after treatment with the grape pomace extract (M – molecular mass marker) (***A***). The level of 3'-nitrotyrosine-modified proteins in percentages (control taken as 100%) (***B***), obtained by densitometric analysis of Western blotting results

Note: *** – significant difference compared to the control (p ≥0.999); ### – significant difference compared to the diabetes (p ≥0.999)

An increase in the content of 3'-nitrotyrosine under DM may be due to an increased level of NO and $O_2^{\bullet-}$ (Hertsyk *et al.*, 2021). On the other hand, the decrease in nitrotyrosine levels after the NCP extract administration to animals with DM may be related to the ability of polyphenolic compounds to scavenge $ONOO^-$ (Hertsyk *et al.*, 2021), as well as reduce NOS activity.

CONCLUSION

The results of the research confirm the immunomodulatory and antioxidant properties of the grape pomace extract rich in a natural complex of polyphenols. In particular, the positive effect of the studied extract under the conditions of diabetes-induced oxidative-nitrative stress in peripheral blood leukocytes was established. The ability of the grape pomace extract to positively correct disorders of the morpho-functional state of peripheral blood cells caused by diabetes mellitus was revealed. Thus, polyphenolic compounds of the grape pomace extract should be considered as the basis for the development of medicine for concomitant complex therapy in diabetes mellitus.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Human Rights. This article does not contain any studies with human subjects performed by any of the authors.

Animal studies. All international, national and institutional guidelines for the care and use of laboratory animals were followed.

AUTHOR CONTRIBUTIONS

Conceptualization, [S.M.; S.N.]; methodology, [Ch.D.; S.M.]; validation, [Ch.D.; S.M.]; formal analysis, [Ch.D.; S.M.;].; investigation, [Ch.D.]; resources, [Ch.D.; S.M.]; data curation, [Ch.D.; S.M.]; writing original draft preparation, [Ch.D.; S.M.]; writing – review and editing, [Ch.D.; S.M.; S.N.]; visualization, [Ch.D.]; supervision, [S.M.; S.N.]; project administration, [S.M.; S.N.]; funding acquisition, [S.M.].

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REFERENCES

Altobelli, G. G., Van Noorden, S., Balato, A., & Cimini, V. (2020). Copper/zinc superoxide dismutase in human skin: current knowledge. *Frontiers in Medicine*, 7, 183. doi:10.3389/fmed.2020.00183
Crossref ● PubMed ● PMC ● Google Scholar

Bahadoran, Z., Mirmiran, P., & Azizi, F. (2013). Dietary polyphenols as potential nutraceuticals in management of diabetes: a review. *Journal of Diabetes & Metabolic Disorders*, 12(1), 43. doi:10.1186/2251-6581-12-43
Crossref ● PubMed ● PMC ● Google Scholar

Baisya, R., Katkam, S. K., KS, S., Devarasetti, P. K., Kutala, V. K., & Rajasekhar, L. (2023). Evaluation of NADPH oxidase (NOX) activity by nitro blue tetrazolium (NBT) test in SLE patients. *Mediterranean Journal of Rheumatology*, 34(2), 163–171. doi:10.31138/mjr.34.2.163
Crossref ● PubMed ● PMC ● Google Scholar

Boydens, C., Pauwels, B., Vanden Daele, L., & Van de Voorde, J. (2016). Protective effect of resveratrol and quercetin on *in vitro*-induced diabetic mouse corpus cavernosum. *Cardiovascular Diabetology*, 15(1), 46. doi:10.1186/s12933-016-0366-9
Crossref ● PubMed ● PMC ● Google Scholar

Chala, D., Sabadashka, M., Morozovych, A., Krychowiak-Maśnicka, M., Królicka, A., & Sybirna, N. (2024). Immunomodulatory and antibacterial effect of red wine concentrate rich in a natural complex of polyphenols under diabetes mellitus. *Biomedicine & Pharmacotherapy*, 170, 116023. doi:10.1016/j.biopha.2023.116023
Crossref ● PubMed ● Google Scholar

Checa, J., & Aran, J. M. (2020). Reactive oxygen species: drivers of physiological and pathological processes. *Journal of Inflammation Research*, 13, 1057–1073. doi:10.2147/jir.s275595
Crossref ● PubMed ● PMC ● Google Scholar

de Souza Ferreira, C., Araújo, T. H., Ângelo, M. L., Pennacchi, P. C., Okada, S. S., de Araújo Paula, F. B., Migliorini, S., & Rodrigues, M. R. (2012). Neutrophil dysfunction induced by hyperglycemia: modulation of myeloperoxidase activity. *Cell Biochemistry and Function*, 30(7), 604–610. doi:10.1002/cbf.2840
Crossref ● PubMed ● Google Scholar

Diabetes. (n.d.). WHO. Retrieved January 29, 2024, from https://www.who.int/health-topics/diabetes

Elejalde, E., Villarán, M. C., & Alonso, R. M. (2021). Grape polyphenols supplementation for exercise-induced oxidative stress. *Journal of the International Society of Sports Nutrition*, 18(1), 3. doi:10.1186/s12970-020-00395-0
Crossref ● PubMed ● PMC ● Google Scholar

Fountas, A., Diamantopoulos, L.-N., & Tsatsoulis, A. (2015). Tyrosine kinase inhibitors and diabetes: a novel treatment paradigm? *Trends in Endocrinology & Metabolism*, 26(11), 643–656. doi:10.1016/j.tem.2015.09.003
Crossref ● PubMed ● Google Scholar

Froy, O., Hananel, A., Chapnik, N., & Madar, Z. (2007). Differential effect of insulin treatment on decreased levels of beta-defensins and Toll-like receptors in diabetic rats. *Molecular Immunology*, 44(5), 796–802. doi:10.1016/j.molimm.2006.04.009
Crossref ● PubMed ● Google Scholar

Ganesh, R., & Meenakshi, B. (2023). Serum zinc and copper levels in children with type 1 diabetes mellitus. *Indian Journal of Pediatrics*, 90(10), 1052–1052. doi:10.1007/s12098-023-04725-3
Crossref ● PubMed ● Google Scholar

Giri, B., Dey, S., Das, T., Sarkar, M., Banerjee, J., & Dash, S. K. (2018). Chronic hyperglycemia mediated physiological alteration and metabolic distortion leads to organ dysfunction, infection, cancer progression and other pathophysiological consequences: an update on glucose toxicity. *Biomedicine & Pharmacotherapy*, 107, 306–328. doi:10.1016/j.biopha.2018.07.157
Crossref ● PubMed ● Google Scholar

Hertsyk, D. Yu., Sabadashka, M. V., Kaprelyants, L. V., & Sybirna, N. O. (2021). Corrective effect of red wine concentrate enriched with natural complex of polyphenols on activity of antioxidant defense enzymes in cardiac muscle under experimental diabetes mellitus. *Studia Biologica*, 15(1), 37–48. doi:10.30970/sbi.1501.644
Crossref ● Google Scholar

Hink, H. U., Santanam, N., Dikalov, S., McCann, L., Nguyen, A. D., Parthasarathy, S., Harrison, D. G., & Fukai, T. (2002). Peroxidase properties of extracellular superoxide dismutase: role of uric acid in modulating *in vivo* activity. *Arteriosclerosis, thrombosis, and vascular biology*, 22(9), 1402–1408. doi:10.1161/01.atv.0000027524.86752.02
Crossref ● PubMed ● Google Scholar

Insuela, D., Coutinho, D., Martins, M., Ferrero, M., Carvalho, V., Insuela, D., Coutinho, D., Martins, M., Ferrero, M., & Carvalho, V. (2019). Neutrophil function impairment is a host susceptibility factor to bacterial infection in diabetes. In: O. Fuchs & S. Shamsadin Athari (Eds.), *Cells of the immune system* (pp. 1–22). IntechOpen. doi:10.5772/intechopen.86600
Crossref ● Google Scholar

Kanikarla-Marie, P., Micinski, D., & Jain, S. K. (2019). Hyperglycemia (high-glucose) decreases L-cysteine and glutathione levels in cultured monocytes and blood of Zucker diabetic rats. *Molecular and Cellular Biochemistry*, 459(1), 151–156. doi:10.1007/s11010-019-03558-z
Crossref ● PubMed ● Google Scholar

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, 193(1), 265–275. doi:10.1016/S0021-9258(19)52451-6
Crossref ● PubMed ● Google Scholar

Magrone, T., Candore, G., Caruso, C., Jirillo, E., & Covelli, V. (2008). Polyphenols from red wine modulate immune responsiveness: biological and clinical significance. *Current Pharmaceutical Design*, 14(26), 2733–2748. doi:10.2174/138161208786264098
Crossref ● PubMed ● Google Scholar

Moradi, S., Jafarian-Kerman, S. R., Salari, & Rohani. (2012). Association between diabetes complications and leukocyte counts in Iranian patients. *Journal of Inflammation Research*, 5, 7–11. doi:10.2147/jir.s26917
Crossref ● PubMed ● PMC ● Google Scholar

Pettersson, U. S., Christoffersson, G., Massena, S., Ahl, D., Jansson, L., Henriksnäs, J., & Phillipson, M. (2011). Increased recruitment but impaired function of leukocytes during inflammation in mouse models of type 1 and type 2 diabetes. *PloS One*, 6(7), e22480. doi:10.1371/journal.pone.0022480
Crossref ● PubMed ● PMC ● Google Scholar

Promyos, N., Phienluphon, P. P., Wechjakwen, N., Lainampetch, J., Prangthip, P., & Kwanbunjan, K. (2023). Inverse correlation of superoxide dismutase and catalase with type 2 diabetes among rural thais. *Nutrients*, 15(9), 2071. doi:10.3390/nu15092071
Crossref ● PubMed ● PMC ● Google Scholar

Sabadashka, M., Hertsyk, D., Strugała-Danak, P., Dudek, A., Kanyuka, O., Kucharska, A. Z., Kaprelyants, L., & Sybirna, N. (2021). Anti-diabetic and antioxidant activities of red wine concentrate enriched with polyphenol compounds under experimental diabetes in rats. *Antioxidants*, 10(9), 1399. doi:10.3390/antiox10091399
Crossref ● PubMed ● PMC ● Google Scholar

Serreli, G., & Deiana, M. (2023). Role of dietary polyphenols in the activity and expression of nitric oxide synthases: a review. *Antioxidants*, 12(1), 147. doi:10.3390/antiox12010147
Crossref ● PubMed ● PMC ● Google Scholar

Skorobahatko, V., Sabadashka, M., Chala, D., & Sybirna, N. (2023). Diabetes-correcting and antioxidant effects of grape pomace extract rich in natural complex of polyphenols. *Studia Biologica*, 17(4), 51–62. doi:10.30970/sbi.1704.738
Crossref ● Google Scholar

Spryn, K. R., Sabadashka, M. V., & Sybirna, N. O. (2021). Effects of agmatine and red wine concentrate, enriched with polyphenolic compounds, on L-arginine/nitrogen oxide system in the brain of rats with experimental diabetes mellitus. *Studia Biologica*, 15(2), 25–34. doi:10.30970/sbi.1502.655
Crossref ● Google Scholar

Taylor-Fishwick, D. A. (2013). NOX, NOX who is there? The contribution of NADPH oxidase one to beta cell dysfunction. *Frontiers in Endocrinology*, 4, 40. doi:10.3389/fendo.2013.00040
Crossref ● PubMed ● PMC ● Google Scholar

Vermot, A., Petit-Härtlein, I., Smith, S. M. E., & Fieschi, F. (2021). NADPH oxidases (NOX): an overview from discovery, molecular mechanisms to physiology and pathology. *Antioxidants*, 10(6), 890. doi:10.3390/antiox10060890
Crossref ● PubMed ● PMC ● Google Scholar
Waterhouse, A. L. (2002). Wine phenolics. *Annals of the New York Academy of Sciences*, 957(1), 21–36. doi:10.1111/j.1749-6632.2002.tb02903.x
Crossref ● PubMed ● Google Scholar
Wood dos Santos, T., Cristina Pereira, Q., Teixeira, L., Gambero, A., A. Villena, J., & Lima Ribeiro, M. (2018). Effects of polyphenols on thermogenesis and mitochondrial biogenesis. *International Journal of Molecular Sciences*, 19(9), 2757. doi:10.3390/ijms19092757
Crossref ● PubMed ● PMC ● Google Scholar

ВПЛИВ ЕКСТРАКТУ З ВИНОГРАДНИХ ВИЧАВОК, ЗБАГАЧЕНОГО ПРИРОДНИМ КОМПЛЕКСОМ ПОЛІФЕНОЛІВ, НА МОРФО-ФУНКЦІОНАЛЬНИЙ СТАН ЛЕЙКОЦИТІВ ЗА ЕКСПЕРИМЕНТАЛЬНОГО ЦУКРОВОГО ДІАБЕТУ

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Вступ. Цукровий діабет належить до найпоширеніших захворювань у світі. За цієї патології ушкоджень зазнають усі органи та системи організму, серед яких імунна система. Важливим елементом цієї системи є лейкоцити периферичної крові, що зазнають пошкоджень у хворих на діабет, адже піддаються впливу активних форм оксигену й активних форм нітрогену, кількість яких різко зростає і призводить до розвитку оксидативно-нітративного стресу. Отже, розробка нових діабет-коригуючих препаратів, які мали би гіпоглікемічні, антиоксидантні й імуномодулюючі властивості, є однією з актуальних проблем. Такі властивості притаманні поліфенольним сполукам, велика кількість яких міститься у виноградних вичавках. Саме тому дослідження екстракту з виноградних вичавок, збагаченого природним комплексом поліфенолів, є важливим для того, щоб оцінити можливість подальшого використання цих речовин як основи препаратів для комплексної терапії цукрового діабету.

Матеріали та методи. У дослідженні використовували лейкоцити периферичної крові контрольних щурів, контрольних тварин, яким упродовж 14 днів вводили екстракт із виноградних вичавок, тварин зі стрептозотоцин-індукованим цукровим діабетом і щурів з експериментальним цукровим діабетом, яким 14 днів вводили екстракт із виноградних вичавок. Для дослідження коригуючого ефекту природного поліфенольного комплексу екстракту з виноградних вичавок на стан системи антиоксидантного захисту, систему L-аргінін/NO та функціональний стан лейкоцитів визначали активність антиоксидантних ензимів, рівень продуктів окисної модифікації білків і ліпідів, активність NO-синтази, вміст нітритів і нітратів, 3'-нітротирозин-модифікованих білків, загальну кількість лейкоцитів, лейкоцитарну формулу та фагоцитарну активність і середній цитохімічний коефіцієнт катіонних протеїнів і НСТ-тесту.

Результати. Встановлено нормалізацію загальної кількості лейкоцитів і лейкоцитарної формули, активності NADPH-оксидази, супероксиддисмутази, каталази

й глутатіонпероксидази та рівнів продуктів окисної модифікації білків і ліпідів, нітритів, нітратів і 3'-нітротирозин-модифікованих білків, а також підвищення середнього цитохімічного коефіцієнта катіонних білків та зниження активності індуцибельної NO-синтази за перорального введення упродовж 14 днів екстракту з виноградних вичавок тваринам зі стрептозотоцин-індукованим цукровим діабетом.

Висновки. Одержані результати підтверджують антиоксидантну й імуномодулюючу дію досліджуваного екстракту і обґрунтовують доцільність використання комплексу поліфенольних сполук із виноградних вичавок як основи нових препаратів для комплексної терапії цукрового діабету.

Ключові слова: виноградні вичавки, цукровий діабет, лейкоцити, оксидативний стрес, нітративний стрес, поліфеноли

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