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Study of Glycyrrhizic Acid:menthol Supramolecular Complexes on Mitochondrial Functional Activity in in-vitro Experiments

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ABSTRACT. Here we present how a supramolecular complex of Glycyrrhizic acid (GA) with Menthol (Mt) affects the blood glucose levels and glycogen synthesis in the liver of rats in in-vivo experiments with diabetes caused by alloxan. We have shown that Menthol, Glycyrrhizic acid and GA:Mt supramolecular complexes can restore functional dysfunction of the liver mitochondria in alloxan diabetes, i.e., inhibit lipid peroxidation. The hypoglycemic activity and mitochondrial membrane stabilizing properties of the supramolecular complex GA:Mt (4:1) in alloxan diabetes were more pronounced than those of menthol, GA and its GA:Mt (2:1) and GA: Mt (9:1) supramolecular complexes.

Key words: Menthol, Glycyrrhizic acid, Homeostasis, Aloxan diabetes, Glycogen, Lipid peroxidation, Supramolecular complex

INTRODUCTION

Menthol ($C_{10}H_{20}O$) is a monoterpenoid that exhibits many biological activities in experiments. Currently, their biological activity is being studied in many scientific laboratories. Menthol (Mt) monoterpenoid has been shown to exhibit gastric ulcer repair using ethyl alcohol.[1] A dose of 50 mg/kg of menthol has a gastroprotective effect and exhibits apoptosis, anti-inflammatory and antioxidant activities in cells of the gastric mucosa.[1] Menthol monoterpenoid also affects the physiological processes associated with Ca^{2+}ions present in the cell. Menthol exhibits relaxant activity by inhibiting Ca^{2+} channels in smooth muscle cells of the rat aorta and coronary blood vessels.[2] Published data suggests that the relaxant effect of menthol may lie in the blockade of potential-dependent Ca^{2+} channels. The effect of menthol on the membrane is due to its hydrophobic properties. The breakdown of menthol and other monoterpenes that are part of the biological composition affects membranes and leads to a number of changes. The effect of hydrophobic compounds on membranes is manifested by altering the physicochemical properties of integral proteins (e.g. ion channels, carriers) and the phospholipid layer and by indirectly affecting channel function.[3] In-vitro studies have shown that menthol and other hydrophobic monoterpenes affect membrane ion channels in concentrations in the range of 10 μM to 10 mM.[4]

It is known that the licorice plant (Glycyrrhiza Glabra L.) differs from other plant species in its medicinal properties. It has been used as a traditional medicine for many years. Licorice root extract has been found to contain up to 25% glycyrrhizic acid. Its aglycone, glycyrrhetinic acid, is structurally similar to the hormones of the adrenal cortex. Currently, Glycyrrhizic acid and glycyrrhetinic acid are widely used in medicine as a remedy for colds, allergies, viral and tumor diseases, in addition, in recent years, licorice drugs have been used in the treatment of viral hepatitis.[5]

Currently, antioxidant and mitoprotective properties of megaferon and GA:quercetin supramolecular complexes in rat liver and brain mitochondria have been identified, which are mainly manifested in young animals. Megaferon and Glycyrrhizinic acid:quercetin complex has been shown to have a corrective effect on the aging of animals by reducing the activity of respiratory chain enzymes in the liver and brain mitochondria, decreasing ATF (adenosine triphosphate) synthesis and protein biosynthesis.[6,7] These results may lead to formulation of drugs with geroprotective activity on the basis of supramolecular complexes of megaferon and GA:quercetin. However, the biological activity of menthol and GA-based supramolecular complexes has been studied very little at present, and their effects on the functional parameters of rat liver mitochondria have never been studied.[7] For this purpose, we have studied the effects of menthol, GA and their supramolecular complexes obtained in different proportions on rat liver mitochondria in in-vitro and in-vivo experiments.

MATERIALS AND METHODS

Preparation of the root extract of the native licorice (Glycyrrhiza glabra L.) and separation of GA (Glycyrrhizic acid) from the extract and chemical identification was carried out using standard methods (*Fig*. 1). For the production of supramolecular complexes of GA with L - (-) - menthol (*Scheme* 1), acidic solutions in dry organic solvents (ethyl alcohol, benzene, acetone) and alkaline solutions or soluble salt solutions were used in the production of L - (-) - menthol.

The feeding and keeping of the animals were carried out in vivarium conditions and the vivarium was kept at a normal level. The experiments were performed on male white

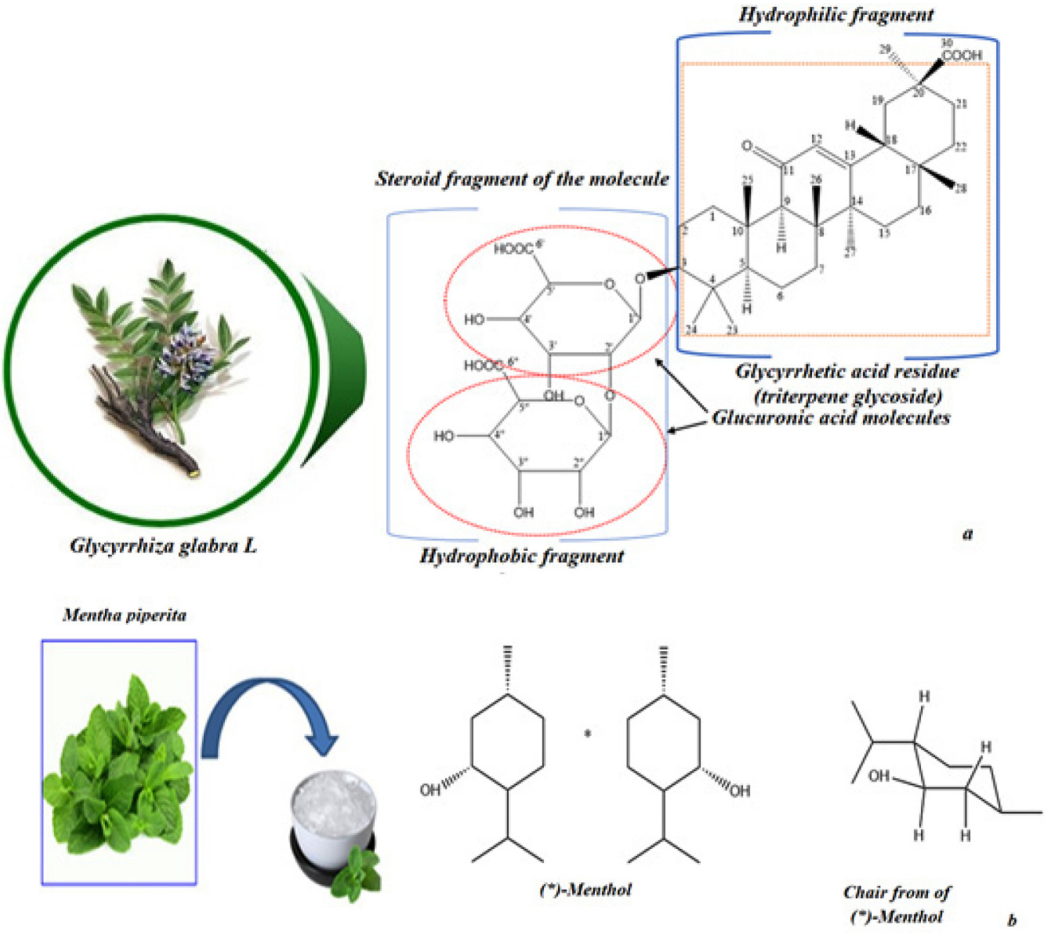
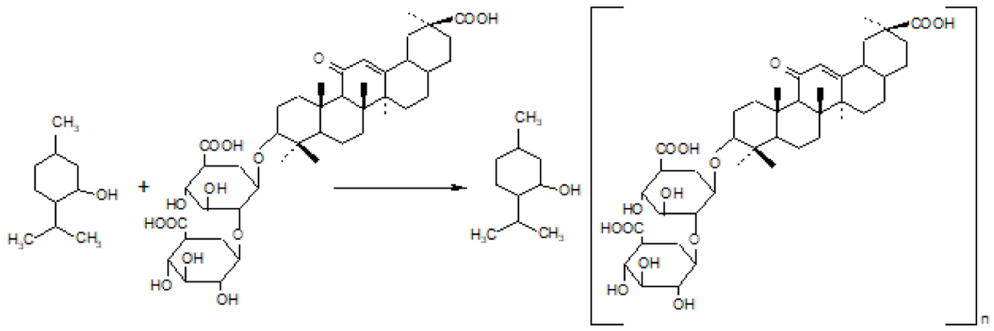


***Figure* 1.** Molecular structures of glycyrrhizic acid (GA) and Menthol. (a) Glycyrrhizic acid (Empirical formula–$C_{42}H_{62}O_{16}$; 20β-carboxy-11-oxo-30-norolean-12-en-3β-il-2-O-β-D-glucopyranuronosyl-α-D-glucopyranose-duronic acid) [2]; (b) Menthol ($C_{10}H_{20}O$).



n=2, 4, 9.

***Scheme* 1.** Formation of the supramolecular complex between GA and Menthol.

rats without off springs weighing 180-200 g.

In the first in-vitro studies on the effect of menthol, GA and supramolecular complexes obtained from menthol and GA at different ratios on the functional parameters of rat liver mitochondria, the biological activity of the supramolecular complex GA:Mt (4:1) was more pronounced than other supramolecular complexes. Therefore, in the later stages of our study, this supramolecular complex was selected to study the hypoglycemic property in IN-VIVO experiments.

Separation of Mitochondria

Mitochondria were isolated from rat liver using the Schneider[8,9] differential centrifugation method. By decapitating the animal, the liver was removed from the abdominal cavity and placed in a frozen separation medium in a beaker. Separation medium composition: 250 mM sucrose, 10 mM tris-chloride, 1 mM EDTA at pH=7.4. After the liver mass was measured, it was homogenized in a Teflon homogenizer in a 6-fold separation medium by mechanical pressing. For the first time, a 600 g rotation was centrifuged at 0-1 ℃ for 7 min at an angle-type rotor TsLR-1, and the nucleus and cell fragments were separated. The supernatant was centrifuged at 6000 g for 15 min at the same temperatures for 15 min. The precipitate was suspended in a separation medium in a 10:1 ratio (the amount of the separation medium in ml per gram of liver mass). Mitochondria were kept in an ice bath during the experiments.

Determination of Protein Content

We identified mitochondrial protein by biuret reaction.[10] To prepare the biuret reagent, 750 mg of copper sulphate ($CuSO_4 \cdot 5H_2O$) and 3 g of potassium sodium tartrate tetrahydrate ($NaKC_4H_4O_6 \cdot 4H_2O$) were dissolved in 500 ml of H_2O. To stop oxidation of the prepared solution, 150 ml of 10% NaOH and 1 g of KI were added and stored in a polyethylene container to a volume of 1 litre.

Determination of Mitochondrial Permeability Transition Pore (PTP) Permeability

Mitochondrial swelling (swelling) kinetics (0.3–0.4 mg/ml) was determined by varying the optical density of the mitochondrial suspension in an open cell (volume 3 ml) at 540 nm while constantly stirring at 26 ℃.

Determination of Lipid Peroxidation (LPO) Products in Mitochondria

Separation of LPO products was performed in the presence of thiobarbituric acid (TBA). The reaction was stopped by adding 0.220 ml of 70% trichloroacetic acid. After this step, the mitochondrial suspension was centrifuged at 15,000 rpm for 15 min. Then 2 ml of supernatant was obtained and 1 ml of 75% TBA was infused. 2 ml of N_2O and 1 ml of TBA were added to the control solution. The mixture was incubated for 30 min in a water bath. After cooling, a change in optical density at a wavelength of 540 nm was detected.

For the determination of the malondialdehyde (MDA) amount, molar coefficient extinction ($e = 1.56 \times 105\ M^{-1}\ cm^{-1}$) was used in the formula: MDA / mg protein = D/1.56×30.

Fe^{2+}/ascorbate system was also used to study the LPO process in the mitochondrial membrane. Under the influence of this system, the mitochondrial membrane lost its barrier function, resulting in an increase in organelle size and the suppression of mitochondria. This volume change was detected photometrically. IM:KCl-125 mM, tris-HCl -10 mM, pH 7.4; Concentrations: $FeSO_4$-10 μM, ascorbate - 600 μM; mitochondrial volume 0.5 mg/ml.

Alloxan Diabetes Model

Alloxan monohydrate solution was used to induce diabetes in experimental animals. In experimental animals, alloxan monohydrate 150 mg/kg (prepared using 0.9% NaCl solution) was injected once into the subcutaneous area of the abdomen after one day of starvation to induce diabetes mellitus.[10] The experimental animals were divided into groups: the control group was injected once with 0.2 ml/100 mg of saline. Alloxan diabetes 150 mg/kg once. Alloxan diabetes + menthol 50 mg/kg. Alloxan diabetes + GA 50 mg/kg. Alloxan diabetes + GA:Mt (4:1) 50 mg/kg. The animals were injected once a day by dissolving 150 mg/kg alloxan monohydrate in saline (0.2 ml/100 mg). Twelve days after alloxan injection in rats and after the blood glucose levels exceeded 11 mmol/l, the animals were given the test substance once daily for 10 days. Blood glucose levels were determined by the glucose oxidase method.

RESULTS AND DISCUSSION

It is known that many inhibitors of mitochondrial mitochondrial permeability transition pore (mPTP) have antioxidant properties, which in turn effectively affect the process of ATP synthesis. In this regard, in order to determine the antioxidant properties of menthol, GA and their supramolecular complexes in rat liver mitochondria, the effect of lipid peroxidation on mitochondria caused by Fe^{2+}/ascorbate was studied. Initially, the effect of menthol

monoterpenoid and GA triterpenoid on liver mitochondrial LPO was determined. After Fe^{2+}/ascorbate was added to the incubation medium, the induced LPO process, i.e., mitochondrial tumour rate, was assumed to be 100%. In this experiment, the products resulting from the peroxidation of lipids disrupt the barrier function of the mitochondrial membrane, resulting in an increase in its swelling rate relative to control. In experiments, the effect of menthol monoterpenoid on LPO in the mitochondrial membrane at a concentration of 10 μM was not significant. However, concentrations of menthol 20, 30, and 40 μM were reduced the peroxidation of liver mitochondria under the action of Fe^{2+}/ascorbate by 76.5 ± 4.5%, respectively, compared with the control; decrease of 89.4 ± 3.0% and 93.5 ± 1.7% was established. This result indicates that menthol concentrations of 20, 30 and 40 μM have antioxidant properties. The antioxidant properties of menthol are also consistent with the literature[11] but it was first discovered that it exhibits this activity in the hepatic mitochondria.

In our next experiment, the effect of GA concentrations in the range of 10–40 μM on the peroxidation of lipids induced by Fe^{2+}/ascorbate in rat liver mitochondria was studied (*Fig*. 2b). Results show that the concentration of 10 μM of GA did not affect the peroxidation of lipid membranes of the liver mitochondria. However, a concentration of 15 μM of GA was found to reduce LPO formed by the effect of Fe^{2+}/ascorbate on the mitochondrial membrane by 58.0 ± 5.0% relative to control. Concentrations of 20, 30, and 40 μM of GA were 87.5 ± 5.0%, respectively, relative to LPO rate control in hepatic mitochondria; Further reductions of 89.1 ± 4.0% and 92.8 ± 3.0% were found (*Fig*. 2b). Thus, a concentration of 10 μM of GA does not affect the LPO induced by the Fe^{2+}/ascorbate inducer of the mitochondrial membrane, but it has been noted that its high 15, 20, 30, and 40 μM levels may have a reliable inhibitory effect on LPO intensity. The inhibitory effect of GA and its supramolecular complexes synthesized with various flavonoids on lipid peroxidation in rat heart and brain tissue is consistent with the literature data.[12] However, we identified that GA exhibited antioxidant properties in the hepatic mitochondria under the influence of the Fe^{2+}/ascorbate inducer.

In our next experiments, the effect of GA and menthol supramolecular complexes on the LPO process induced using the Fe^{2+}/ascorbate system of the rat liver mitochondrial membrane was studied. Results show that the supramolecular compound of GA and menthol in the ratio GA:Mt (2:1) to LPO induced by Fe^{2+}/ascorbate of rat liver mitochondria in the amounts of 20 and 50 μg/ml, respectively, 46.6 ± 5.5% and 57.7 ± A 2.8% inhibitory effect was found (*Fig*. 3a).

In a subsequent experiment, the effect of GA and menthol on the rate of lipid peroxidation of the mitochondrial membrane of the rat liver supramolecular complex in the ratio GA:Mt (4:1) was studied. Results show that the rates of GA:Mt (4:1) supramolecular complex of 20 and 50 μg/ml of the lipid peroxidation rate of hepatic mitochondria caused by Fe^{2+}/ascorbate were 68.6 ± 4.4% and 94.6 ± 3, respectively. A 2% inhibitory effect was noted (*Fig*. 3b).

The GA:Mt (9:1) supramolecular complex also had an inhibitory effect on the peroxidation of lipids located in the hepatic mitochondrial membrane. Their doses of 20 and 50 μg/ml were found to inhibit mitochondrial LPO by 25.0 ± 3.4% and 58.6 ± 4.5%, respectively (*Fig*. 3c). Hence, here, the inhibitory effect of the GA:Mt (2:1) supramolecular complex on the LPO process was found to be more effective than the GA: Mt (4:1) and (9:1) compounds. GA and menthol and their derived GA:Mt (2:1), GA:Mt (4:1)

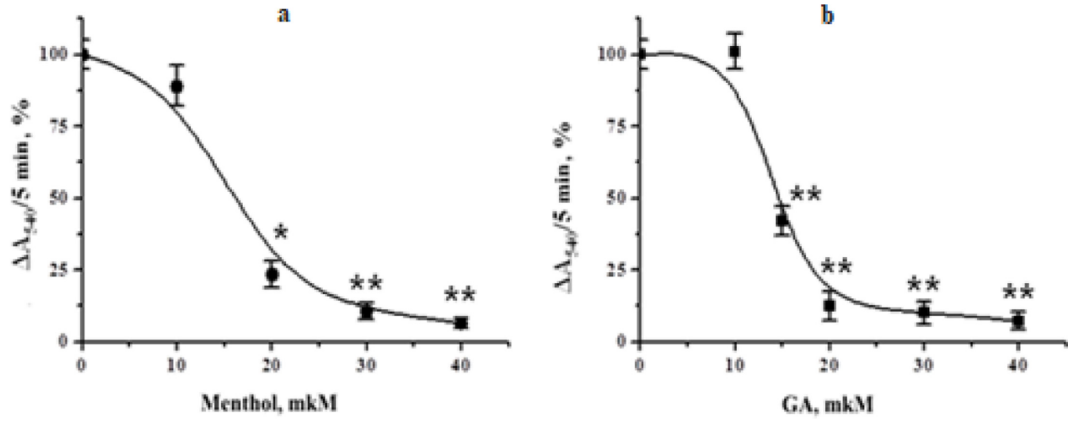


***Figure* 2.** Effect of menthol (a) and GA (glycyrrhizic acid) (b) on hepatic mitochondria to Fe^{2+}/ascorbate-induced LPO process (control reliability *R < 0.05; **R < 0.01; n = 5).

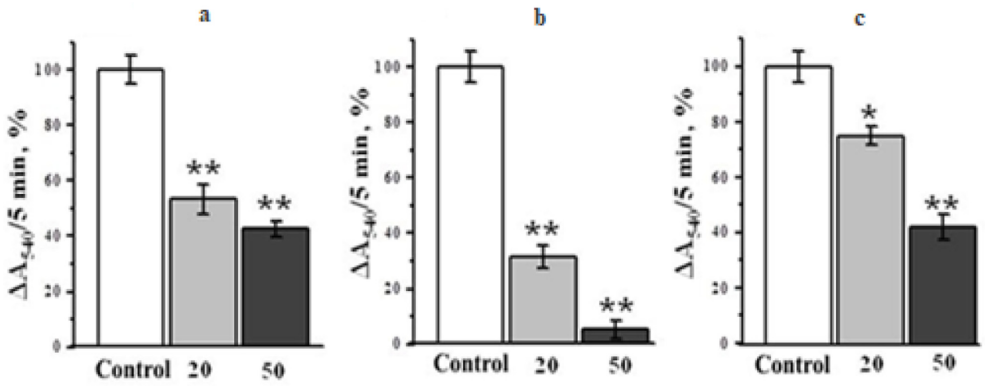


***Figure* 3.** Supramolecular compounds of GA and menthol in ratios GA:Mt (2:1) (a), GA:Mt (4:1) (b) and GA:Mt (9:1) (c) were induced by Fe^{2+}/ascorbate of rat liver mitochondria effect on LPO process (reliability with control *R < 0.05; **R < 0.01; n = 5).

and GA:Mt (9:1) supramolecular complexes to further demonstrate antioxidant activity in rat liver mitochondria the effect on MDA levels was also studied.

Glycogenesis processes in the liver play an important role in the maintenance of glucose homeostasis in blood plasma.[13] Glycogen is an important reserve for the body, a polysaccharide, which can be stored by the body up to 20% weight of the liver. In diabetes mellitus, a decrease in glycogen synthesis in the liver is observed. In determining the hypoglycemic and antidiabetic activity of many compounds, the amount of glycogen in the liver is checked along with the amount of glucose in the plasma. In our in-vivo experiments, we showed the supramolecular complex of GA and menthol partially restored blood glucose levels and glycogen synthesis in the liver of rats in diabetes conditions caused by alloxan. The effect of GA, menthol and its supramolecular complex on the amount of glucose in the blood of rats and the amount of glycogen in liver tissue in patients with diabetes caused by alloxan is presented in *Table* 1.

The results show that in alloxan diabetes, a sharp increase in blood glucose was observed compared to control, and a sharp decrease in glycogen in the liver. Impairment of glycogen synthesis in the liver in alloxan diabetes is likely to be associated with decreased glycogen synthetase activity and decreased glucose oxidation due to a deficiency in the pyruvate dehydrogenase complex. Oral administration of menthol (50 mg/kg), GA (50 mg/kg) and GA:Mt (4:1) (50 mg/kg) to animals with diabetes mellitus once daily for 10 days (16.10.21-26.10.2021) results in increased glucose levels and glycogen in the liver. the amount was found to decrease relative to control (*Table* 1). When rats were treated with GA, menthol, and supramolecular complex of the alloxan diabetes model were found to be effective at lowering blood glucose and increasing glycogen in the liver for supramolecular complex GA:Mt (4:1) (*Table* 1).

In this case, one of the mechanisms of restoration of glucose concentration in the blood of animals after taking GA:Mt (4:1) may occur as a result of restoration of carbohydrate metabolism between blood and liver due to changes in glycogenesis processes.

Effect of GA, menthol and their GA:Mt (4:1) supramolecular complex on liver mitochondria mPTP in alloxan diabetes mellitus.

One of the priorities of modern physiology and medicine is the study of the mechanisms of disruption of cell structure and function in diabetes mellitus and their correction with the help of pharmacological agents. Currently, despite the diversity of drugs used to treat diabetes, there is a growing demand for the search for new gener-

***Table* 1.** Effect of supramolecular complexes of GA, menthol and supramolecular complex GA: Mt (4: 1) on the amount of glucose in the blood of rats and glycogen in liver tissue in rats in alloxan diabetes (M ± m, n = 4)

Animal groups	The amount of glucose mmol/l	Glycogen content in mg/100 g % relative to body weight
Control	5,3	100
Alloxan diabetes	17,2	41,0
Alloxan diabetes + menthol 50 mg/kg	13,4	58,5
Alloxan diabetes + GA 50 mg/kg	11,7	65,3
Alloxan diabet + GA: Mt (4:1) 50 mg/kg	9,5	74,5

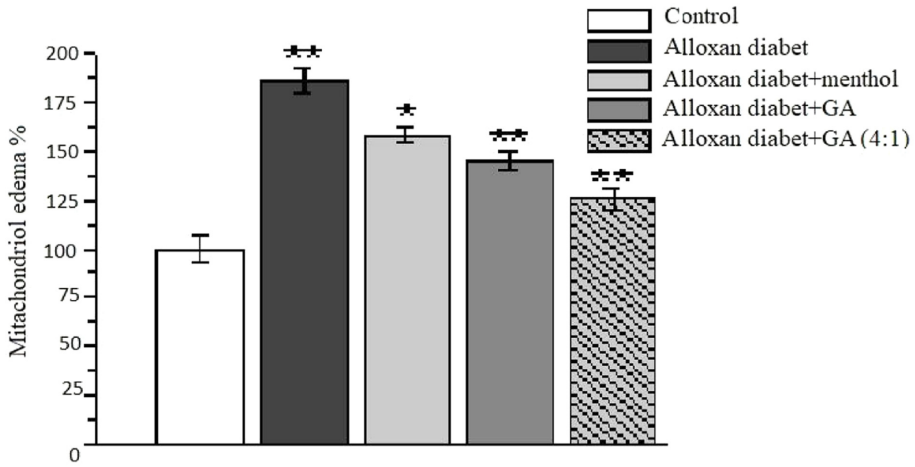


***Figure* 4.** Effect of GA, menthol compounds and its GA:Mt (4:1) supramolecular compound on mPTP of liver mitochondria under conditions of alloxan diabetes (reliability compared to control *R < 0.05; **R < 0.01; n = 5).

ations and the study of mechanisms of action of "therapeutic target" organs. One of such "targets" in the cell is the mitochondria, and in pathological conditions, their two-layered membrane and matrix structures are primarily the respiratory chain and mPTP dysfunction.[14] The role of mitochondrial high-permeability mega-channel, i.e., mPTP, in the development of various diseases, especially in diabetes mellitus, is currently being discussed separately.[15]

We have studied the effects of plant-derived menthol, GA, and its supramolecular complex GA:Mt (4:1) on alloxan in hepatic mitochondrial edema in diabetes in in-vivo experiments (*Fig.* 4).

The results showed that mitochondrial suffocation isolated from rat liver was increased by 86.5±6.0% in alloxan diabetes compared with control. As a result of administration of menthol, GA and GA:Mt (4:1) supramolecular complex (orally once daily for 10 days) to animals with alloxan diabetes, it was found that their blood glucose levels dropped to normal levels. In addition, mitochondrial suffocation in the liver of pharmacotherapeutic animals was reduced by 28.1 ± 4.3% in menthol, 41.1 ± 4.5% in GA, and 61.1 ± 5 in GA:Mt (4:1) compared with alloxan diabetes. Inhibition was found to be 5% (*Fig.* 3). Hence, the GA:Mt (4:1) complex was found to effectively inhibit the high permeability of the liver mitochondrial membrane in alloxan diabetes mellitus relative to menthol and GA. This requires further study of its pharmacological properties. We know that the open conformational state of mPTP in various pathological conditions, including diabetes mellitus, is led to the closed state by compounds with antioxidant properties.

Natural biologically active substances can exhibit antiradical properties over synthetic compounds and prevent lipid peroxidation in bio membranes. There is no literature on the effect of menthol, GA, and GA:Mt (4:1) supramolecular complex, which have been shown to be active in in-vitro experiments, on the amount of MDA (malondialdehyde), in mitochondria in diabetes mellitus. In this in-vivo experiment, we studied the effects of menthol, GA, and GA:Mt (4:1) supramolecular complex on rat liver mitochondria LPO in alloxan diabetes. Data on the amount of MDA in mitochondria isolated from the liver of animals of the diabetic group corrected with healthy, alloxan diabetes and menthol, GA and GA:MT (4:1) supramolecular complex are shown in *Fig*. 4. The results showed that the formation of MDA in mitochondria isolated from the liver of rats with alloxan diabetes mellitus increased by 73.7 ± 3.5% compared to controls (*Fig.* 5).

When the animals with alloxan diabetes mellitus were administered orally menthol, GA, and GA:Mt (4:1) supramolecular complex once daily for 10 days, a decrease in the amount of MDA in their hepatic mitochondria was noted. In this case, the reducing effect of the supramolecular complex GA:Mt (4:1) on the mitochondrial MDA in alloxan diabetes mellitus was more pronounced compared to menthol or GA. It was observed that the amount of MDA in the mitochondria isolated from the liver of alloxan diabetes animals treated with the supramolecular complex GA:Mt (4:1) decreased by 60.3 ± 3.1% compared to the diabetes group (*Fig.* 5).

The analysis of the results obtained from the experiments shows that GA, menthol and their supramolecular complexes in different ratios indicate that the antioxidant property has a stabilizing effect on the mitochondrial

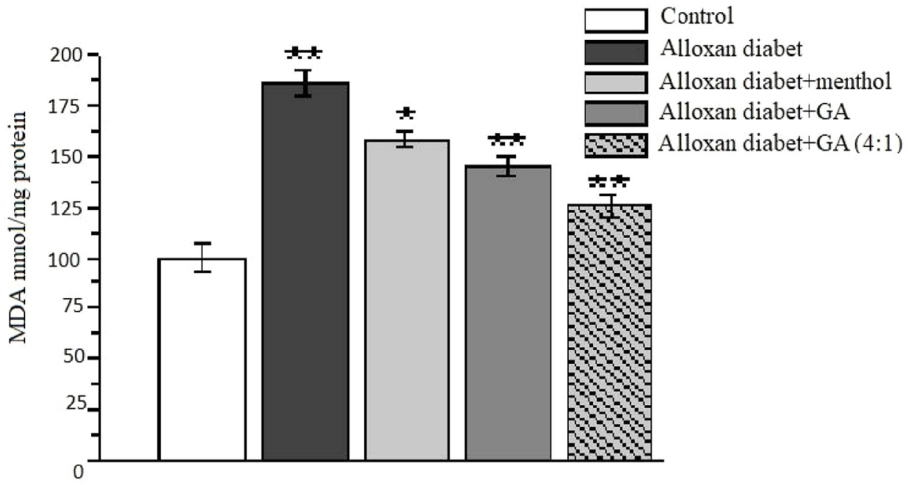


***Figure* 5.** Effect of alloxan on the amount of GA, menthol and their GA:Mt (4:1) supramolecular complex in the liver mitochondria MDA in diabetes mellitus (reliability with control *R < 0.05; **R < 0.01; n = 5).

membrane by inhibiting the mitochondrial LPO process and mPTP. It was observed that the stabilizing effect of these studied compounds on the membrane of rat liver mitochondria restores the activity of ion channels located in the membrane can also be explained by its antioxidant property.[15] GA, menthol and their supramolecular complexes can increase the synthesis of ATP by reducing the formation of active forms of oxygen from the respiratory chain of liver mitochondria and reduce the process of lipid peroxidation. This, in turn, allows the use of these compounds as corrective agents in the development of various pathologies.

CONCLUSION

It can be concluded that the membrane active properties and antioxidant activities of menthol, GA and their supramolecular complexes in different ratios may depend on the number of hydroxl groups in their structure, their mutual location. Thus, menthol, GA, and GA:Mt (4:1) supramolecular complex can restore functional dysfunction in the liver mitochondria, i.e., in diabetes and inhibit membrane lipid peroxidation. The hypoglycemic activity and mitochondrial membrane stabilizing properties of the supramolecular complex GA:Mt (4:1) in alloxan diabetes were more pronounced compared to menthol, GA and their GA:Mt (2:1) and GKA:Mt (9:1) supramolecular complexes.

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Conflict of Interest. The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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