Journal of the Korean Chemical Society
2021, *Vol. 65, No. 5*
Printed in the Republic of Korea
https://doi.org/10.5012/jkcs.2021.65.5.313

Influence of Glycyrrhizic Acid, Menthol and Their Supramolecular Compounds on the Functional Activity of Rat Mitochondria in in-vitro Experiments

L. A. Ettibaeva*, U. K. Abdurahmonova, A. D. Matchanov†, D. M. Allanazarova, and Z. T. Halmuratova‡

*Department of Chemistry, Gulistan State University, *E-mail: lola1981a@mail.ru*
†Head of the Chair Experimental Technology Laboratory, Acad. O. Sodiqov Institute of Bioorganic Chemistry
‡Department of Chemistry, Qaraqalpaq State University
(Received May 4, 2021; Accepted June 23, 2021)

ABSTRACT. Menthol (M) is a cyclic monoterpenode and is a major component of essential oils. Menthol, along with menthol, isomenton, etc., gives taste and odor of the mint plant, and when it comes to menthol in general, L- or (-) -menthol is usually used. Included in pharmaceuticals, cosmetics and pesticides. It has antimicrobial, antibacterial, antioxidant properties. It is also known that the licorice plant (Glycyrrhiza Glabra L.) differs from other types of plants by its medicinal properties. For many years it has been used in folk medicine. Extraction of licorice root revealed up to 25% glycyrrhizinic acid (GA). Its aglycone - glycyrrhizic acid is notable for its structural similarity to the adrenal cortex hormones. Currently, GA and glycyrrhizic acid are widely used in medicine as a remedy for colds, allergies, viral diseases, tumors. The biological activity of menthol and GA-based supramolecular compounds has been poorly studied, and their effect on the functional parameters of rat liver mitochondria has been studied little. For this purpose, in our experiments, the effect of menthol (M), glycyrrhizinic acid (GA) and their supramolecular complexes obtained in different proportions on in vitro and in vivo studies on rat liver mitochondria was studied.

Key words: Menthol, Glycyrrhizic acid, Supramolecular compound, Anti-inflammatory and antioxidant activity, Mitochondria

INTRODUCTION

Menthol ($C_{10}H_{20}O$) is a monoterpenoid that exhibits many biological activities in experiments. Their biological activity is currently being studied in many scientific laboratories. Studies in rats belonging to the Wistar line have shown that menthol monoterpenoid exhibited ulcer-induced wound healing properties using ethyl alcohol.[1] The amount of menthol 50 mg/kg has a gastroprotective effect, has apoptosis, anti-inflammatory and antioxidant activity in the 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 the smooth muscle cells of the rat aorta and coronary arteries.[2] 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 the smooth muscle cells of the rat aorta and coronary arteries.[2] Based on the literature data, it can be said 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 ranging from 10 μM to 10 mM.[4]

It is known that the licorice plant (Glycyrrhiza Glabra L.) differs from other types of plants by its medicinal properties. For many years it has been used in traditional medicine. When licorice root was extracted, it was found to contain up to 25% glycyrrhizinic acid (GA). Its aglycone - glycyrrhizic acid is notable for its structural similarity to the adrenal cortex hormones. Currently, GA and glycyrrhizic acid are widely used in medicine as a remedy for colds, allergies, viral diseases, tumors. In addition, in recent years, licorice drugs are used in the treatment of viral hepatitis.[5] GA has been observed to stabilize permeability changes of mitochondrial membranes under the influence of Ca^{2+} or lipid peroxidation.[6] GA has been shown to have Ca^{2+} - ionophore properties, enhance the effect of peroxidation of Ca^{2+} or lipids on the permeability of mitochondrial membranes, as well as the release of oxidative phosphor-

ylation and decrease in Ca^{2+}-capacity of mitochondria.[6] The mechanisms of action of new chemical derivatives of GA on the functional parameters of mitochondria have been studied. GA derivatives-2-(N-cytisine)-ethyl-3-0-acetyl-18βN-glycyrrhizate, 2-(N-cytisine)-isopropyl-3-0-acetyl-18βN-glycyrrhizate and N-(2-pyridyl)-3-0-acetyl-11-ketoolean-12-en-30-amides have been shown to reduce the activity of mitochondrial permeability transition pore (mRTR) on the mitochondrial membrane and have a protective effect on the mitochondrial membrane.[7] It has been shown that membrane disorders occurring in the case of toxic hepatitis can be corrected using GA derivatives.[7]

Currently, antioxidant and mitoprotective properties of megaferon and GA + quercetin supramolecular complexes have been identified in rat liver and brain mitochondria, which are mainly manifested in young animals. Aging of animals has been shown to have a corrective effect of megaferon and glycyrrhizinic acid + quercetin complex, a decrease in the activity of respiratory chain enzymes in the liver and brain mitochondria, a decrease in ATF synthesis and a decrease in protein biosynthesis.[8,9] This will allow in the future to create drugs with geroprotective activity on the basis of supramolecular complexes megaferon and GA + quercetin. However, the biological activity of menthol and GA-based supramolecular compounds is currently poorly understood and their effects on the functional parameters of rat liver mitochondria are almost unknown. For this purpose, in our experiments, the effect of menthol, GA and their supramolecular complexes obtained in different proportions on in vitro and in vivo studies was studied in rat liver mitochondria.

Research Materials and Methods

Obtaining a Supramolecular Complex. Supramolecular compounds were obtained by the following steps. Firstly, 1.68 g (0.002 M) of GA dissolved 25 ml distilled water and 25 ml ethanol. This solution widely mixed with a magnetic stirrer at 50–60 ºC. Then we added 0.156 g (0.001 M) Menthol and continued the mixing process for 5–6 hours. After then the reaction mixture was filtered. The rest of the ethanol was removed in a vacuum, and the aqueous part was dried in a lyophilic manner. Final product was a yellow powder: liquid = 205–210 ºC R_f = 0.9 (System 2) Units: 85% IR spectrum:1042 (-O-) cm^{-1}; 1655 (CO) cm^{-1} 2948 (CH_3) cm^{-1}; 3600–3200 (OH) cm^{-1}. Other supramolecular complexes of GA with M were also obtained by this synthesis method:

1. GA: MT (2: 1). Liquid = 218-220 ºC R_f = 0.8 (System 2) Productivity 95%
2. GA: MT (4: 1). Liquid = 220-225 ºC, R_f (1) Productivity: 90%.

The supramolecular complex of GA with menthol was obtained according to *Fig.* 1.

Supramolecular complexes with molar ratio 2:1; 4:1; 9:1 of glycyrrhizic acid with menthol were obtained in water:etanol system.

The experiments were performed on purebred white rats. The feeding and keeping of the animals was carried out in vivary conditions and it was kept at a normal level. The experiments were performed on male white rats weighing 180–200 g.

In the first in vitro studies of the effect of supramolecular complexes obtained on the basis of menthol, GA and their different ratios on the functional parameters of rat liver mitochondria, the biological activity of GA: M (4: 1) supramolecular complex was more pronounced than other supramolecular compounds. Therefore, in the later stages of our study, it was selected to study the hypoglycemic property in in vivo experiments.

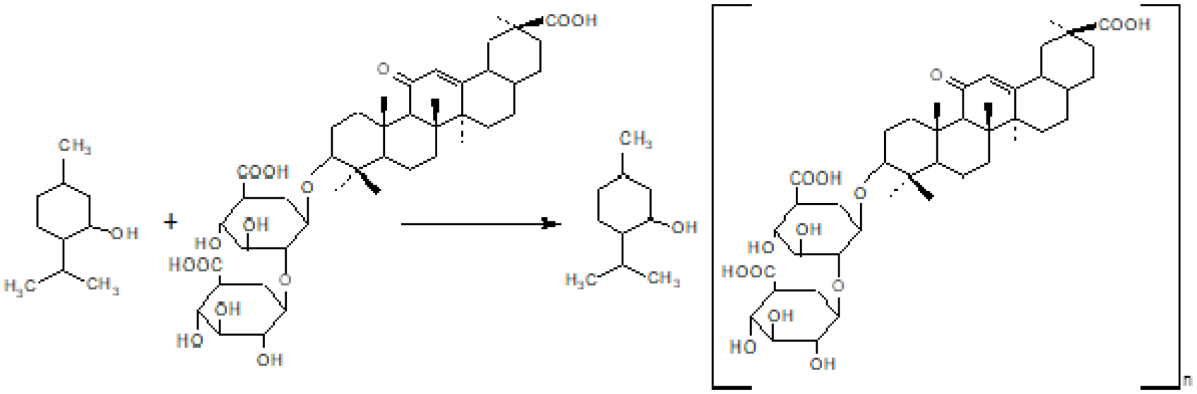

***Figure* 1.** Reaction of the supramolecular complex between GA and Menthol.

Separation of Mitochondria

Mitochondria from rat liver to Schneider[10,11] were separated using the differential centrifugation method. By decapitating the animal, the liver was extracted from the abdominal cavity and then placed in a frozen separation medium in a beaker. Separation medium composition: 250 mM sucrose, 10 mM tris-chloride, 1 mM EDTA, 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 ºC in an angular-type rotor TsLR-1 for 7 min to separate the nucleus and cell fragments. The supernatant was centrifuged at 6000 g for 15 min at high temperature for 15 min. The precipitate was suspended in a separation medium at a ratio of 10:1 (the amount of the separation medium in ml per gram of liver mass). During the experiment, mitochondria were kept in an ice bath.

Determination of Protein Content

Mitochondrial protein biuret reaction.[12] To prepare the biuret reagent, we dissolved 750 mg of copper sulphate ($CuSO_4 \times 5H_2O$) and 3 g of sodium potassium hydroxide ($NaKC_4H_4O_6 \times 4H_2O$) in 500 ml of H_2O. To stop oxidation to the prepared solution, 150 ml of 10% NaOH and 1 g of KJ were added and stored in a polyethylene container to a volume of 1l.

To determine the protein in the mitochondrial suspension, we added 0.9 ml of 2N KOH and 10 mg of deoxycholic acid to 0.1 ml of the drug suspension to break down the membranes. After complete dissolution of the protein, add 4 ml of biuret reagent and leave at room temperature for 30 min. Simultaneously, we prepared a control probe (1 ml of 2N KOH + 10 mg of deoxycholate acid + 4 ml of biuret reagent). We then colorized at 540 nm in cuvettes with a thickness of 10 nm. We calculated the protein serum albumin standards according to the calibration curve obtained by colorimetry.

Determination of Mitochondrial PTP Permeability

The swelling (swelling) kinetics of mitochondria (0.3-0.4 mg/mL) were determined by changing the optical density of the mitochondrial suspension at 26 ºC in an open cell (volume 3 mL) at 540 nm with constant stirring. The following incubation medium (IM) was used to determine the permeability of PTP in mitochondria: 200 mM sucrose, 20 μM EGTA, 5 mM succinate, 2 μM rotenone, 1 μg / ml oligomycin, 20 mM Tris, 20 mM HEPES, and 1 mM KH_2PO_4, pH 7.4.[13] We calculated the concentration of ionized calcium in the medium using a computer program BAD4 in the presence of Ca^{2+}-EGTA buffers.

Determination of Peroxidation Oxidation Products of Lipids in Mitochondria. Separation of LPO products was performed in the presence of thiobarbituric acid (TBA). The reaction stopped by adding 0.220 ml of 70% trichloroacetic acid to the IM. After this step, the mitochondrial suspension was centrifuged at 4,000 rpm for 15 min. Then 2 ml of sedimentary liquid was obtained and 1 ml of 75% TBA was infused. 2 ml of N2O 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.

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

A 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: KSI - 125 mM, tris-NSI - 10 mM, rN 7.4; Concentrations: $FeSO_4$ - 10 μM, ascorbate - 600 μM; mitochondrial volume 0.5 mg / ml; Statistical processing of the obtained results. Statistical processing of the obtained results and drawing of images was carried out using the computer program OriginPro 7.5 (Microsoft, USA). In experiments, the swelling kinetics of mitochondria were performed as a percentage of the maximum, in the form of calculating the arithmetic mean of 4–7 different experiments. The difference between the values obtained from the control, experiment, and experiment + research material was calculated on the t-test. In this case $R < 0.05$; $R < 0.01$; and values of $R < 0.001$ represent statistical reliability.

RESULTS AND DISCUSSION

Effect of Glycyrrhizic Acid, Menthol and Supramolecular Compounds GA: M (2: 1), GA: M (4: 1) and GA: M (9: 1) on Mitochondrial Functional Activity In vitro Experiments

Initially, the effect of the compounds on rat-liver mitochondria Ca^{2+} - dependent megakanal (mPTP) was studied. Ionic channels located in the inner and outer membranes of the mitochondria serve as the main pharmacological and therapeutic targets in cell protection.[14,15] One such ion channel is the mitochondrial permeability transition pore-mPTP, which is a channel that physiologically regulates

the release of Ca^{2+} ions from the matrix. Mitochondria play an important role in the homeostasis of Ca^{2+} ions between the PTP matrix and the cytosol, as well as in the Ca^{2+}-signalization. This megapora ensures the transition of mitochondrial membranes to a highly permeable state in various pathologies. In pathological conditions, permeabilization of the mitochondrial membrane takes place in the open conformational state of mPTP. Some biologically active compounds exhibit the property of inhibiting the high permeability state of PTP resulting from mitochondrial dysfunction. To determine this property, the effect of supramolecular complexes obtained on the basis of menthol, GA and their different ratios on rat liver mPTP was studied.

Initially, the effect of menthol on the PTP status of rat liver mitochondria was studied in experiments. A concentration of 10 μM of Ca^{2+} ions was used as the inductor. It is known that Ca^{2+} ions increase the permeability of mPTP and convert it to the open state, resulting in mitochondrial swelling. Studies have shown that when 10 μM of menthol is exposed to energized mitochondria induced by Ca^{2+} ions, mitochondrial swelling decreases by 24 ± 2.1% and 30 and 50 μM by 48 ± 3.8% and 82 ± 3.7%, respectively (*Fig.* 2, A). Hence, the menthol monoterpenoid had an inhibitory effect on mPTP.

The effect of GA at 10, 30, and 50 μM concentrations on rat liver mitochondrial edema was studied. In the presence of Ca^{2+} ions in the incubation medium and in the absence of GA, mitochondrial swelling was assumed to be 100%. According to the results obtained, the concentrations of GA 10, 30 and 50 μM were 18 ± 3.1% of the control of mitochondrial swelling, respectively; It was found to inhibit 55 ± 4.3% and 85 ± 2.9% (*Fig.* 2, B).

Thus, at concentrations of menthol 10–50 μM and GA 10–30 μM studied, hepatic mitochondria had an inhibitory effect on mPTP permeability and prevented mitochondrial suffocation. The identified property of these compounds can be used as a corrective agent for disorders of the mitochondrial membrane in pathological conditions.

In our next experiments, the effect of supramolecular compounds of GA and menthol in the ratios GA: M (2: 1), GA: M (4: 1) and GA: M (9: 1) on mPTP permeability of rat liver mitochondria was studied. Experimental results showed that the levels of GA: M (2: 1) at 20 and 50 μg / ml inhibited rat liver mitochondrial suffocation by 14 ± 2.3% and 66 ± 2.8%, respectively (*Fig.* 3, A).

The ratio of GA: M supramolecular complex (4: 1) was found to reduce mitochondrial swelling by 57 ± 4.1% and 87 ± 3.2% compared to control (*Fig.* 2, B). The ratio of GA: M supramolecular complex (9: 1) was found to inhibit mitochondrial permeability by 35 ± 4.4% and 71 ± 2.5% relative to control (*Fig.* 3C). Hence, supramolecular complexes of GA and menthol inhibited rat liver mitochondrial suffocation, leading to an open conformation of mPTP relative to control. Here, the effect of the supramolecular complex of GA and menthol (4:1) on mitochondrial mPTP permeability was found to be effective against its (2:1) and (9:1) ratio compounds.

These results suggest that various glyceric acid compounds.[16] Corresponds to data from the literature on inhibitory activity. Studies require experiments to further study the pharmacological activity of the supramolecular complex in the ratio of GA and menthol (4:1).

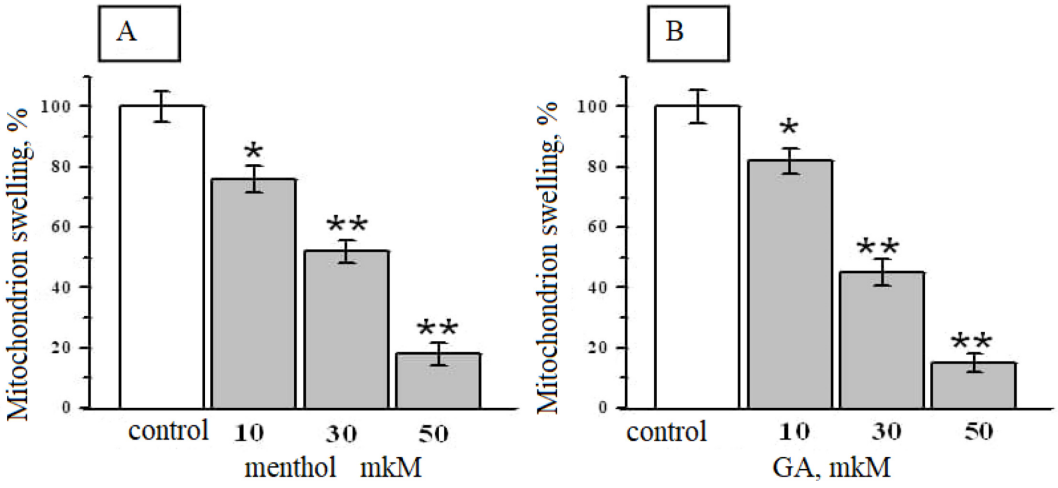

***Figure* 2.** Effect of menthol (A) and GA (B) on mitochondrial edema of rat liver (*R < 0.05; **R < 0.01; n = 5).

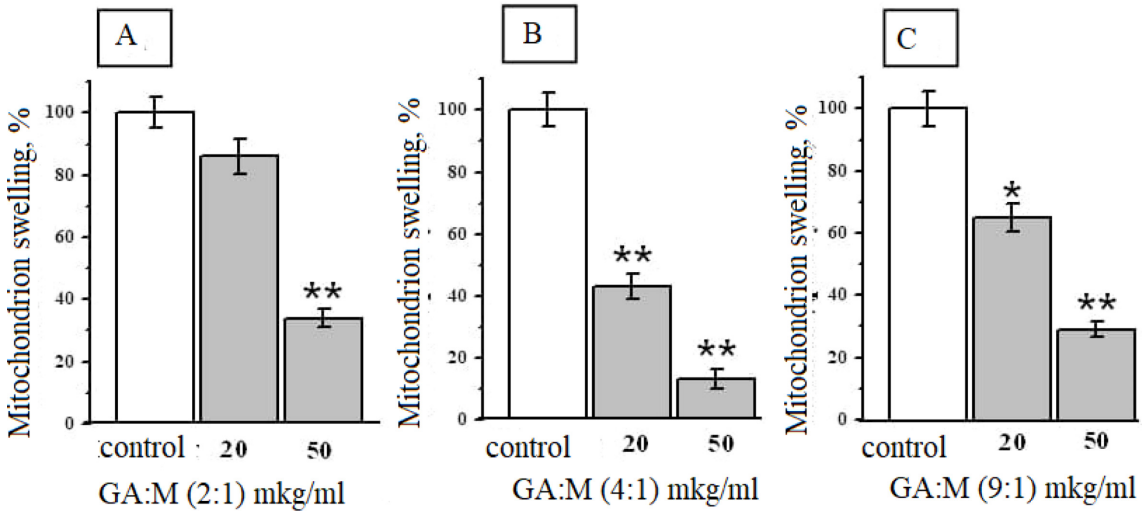

***Figure* 3.** Effect of supramolecular compounds of GA and menthol on the mitochondria of rat liver in the ratios GA: M (2:1) (A), GA: M (4:1) (B) and GA: M (9:1) (V) (* R < 0.05; ** R <0.01; n = 5).

It is known that many inhibitors of mitochondrial mPTP have antioxidant properties, which in turn effectively affect the process of ATF synthesis. In this regard, in order to determine the antioxidant properties of menthol, GA and their supramolecular complexes in rat liver mitochondria, the effect on the process of peroxidation of lipids in mitochondria caused by Fe^{2+} / ascorbate was studied. Initially, the effect of menthol monoterpenoid and GA triterpenoid on liver mitochondria LPO was determined. After Fe^{2+} / ascorbate was added to the incubation medium, the induced LPO process, i.e., mitochondrial tumor rate, was assumed to be 100%. In this experiment, the resulting products of lipid peroxidation disrupt the barrier function of the mitochondrial membrane, resulting in an increase in its swelling rate relative to control. Menthol monoterpenoid in experiments. At 10 μM, the effect on LPO in the mitochondrial membrane was negligible. However, concentrations of menthol 20, 30, and 40 μM were 76.5 ± 4.5%, respectively, relative to the control of peroxidation of hepatic mitochondria under Fe^{2+} / ascorbate; Decreases were found to be 89.4 ± 3.0% and 93.5 ± 1.7%, respectively (*Fig*. 4, A). 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.[17] But it was first discovered that it exhibited this activity in the hepatic mitochondria.

In our next experiment, we studied 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 (*Fig*. 4, B). According to the results, the concentration of GA 10 μM did not affect the lipid peroxidation of the liver mitochondrial membrane. However, a concentration of 15 μM of GA was found to reduce the LPO formed by the action of Fe^{2+} / ascorbate on the mitochondrial membrane by 58.0 ± 5.0% relative to control. GA concentrations of 20, 30, and 40 μM were 87.5 ± 5.0%, respectively, relative to LPO rate control in hepatic mitochondria; Further reductions were found to be 89.1 ± 4.0% and 92.8 ± 3.0%, respectively (*Fig*. 3, B). 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 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 compounds synthesized with various flavonoids on lipid peroxidation in rat heart and brain tissue is consistent with the literature data.[18] However, in our experiments, it was first identified that GA exhibited antioxidant properties in the hepatic mitochondria under the influence of the Fe^{2} + / ascorbate inducer.

In our next study, 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. According to the results, the supramolecular compound of GA and menthol in the ratio GA: M (2: 1) to LPO induced by Fe^{2+} / ascorbate of rat liver mitochondria at 20 and 50 μg / ml, respectively, was 46.6 ± 5.5% and 57.7 ± A 2.8% inhibitory effect was found (*Fig*. 5, A).

In a subsequent experiment, the effect of GA and menthol on the rate of lipid peroxidation of the supramolec-

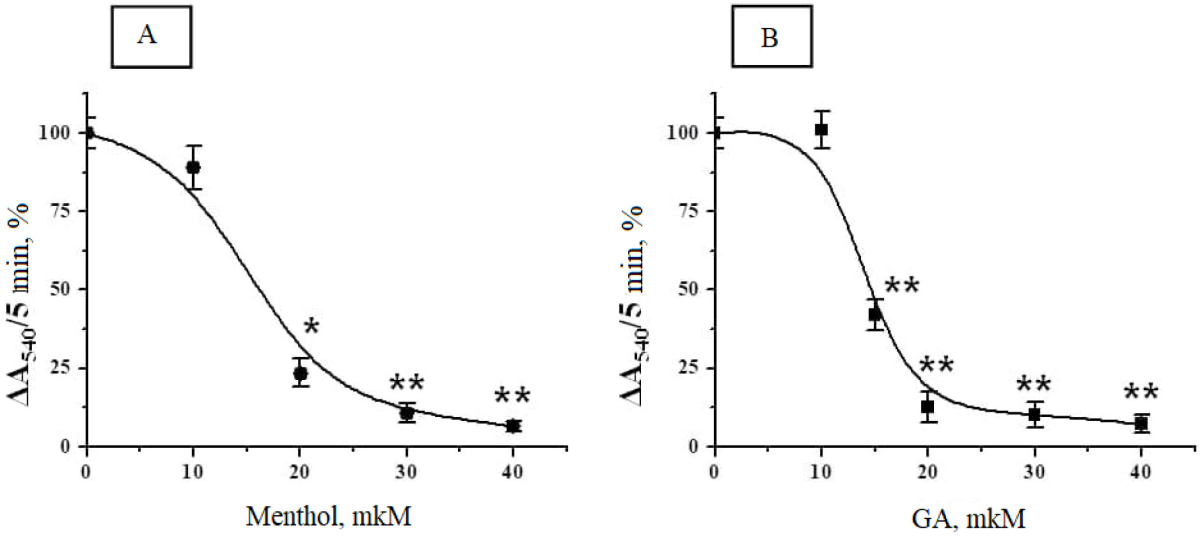

***Figure* 4.** Effect of menthol (A) and GA (B) on hepatic mitochondria in the LPO process induced by Fe^{2+}+ ascorbate (control reliability *R < 0.05; **R < 0.01; n = 5).

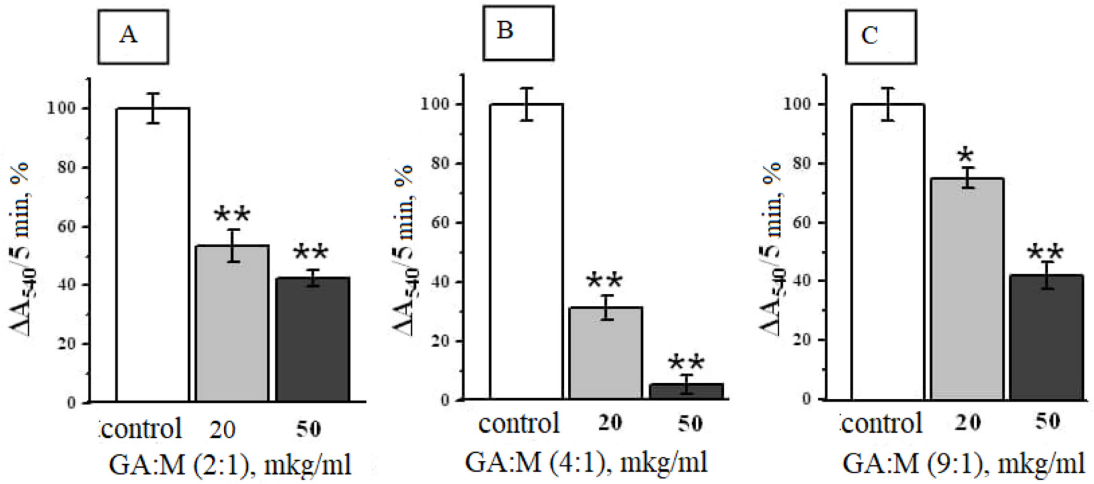

***Figure* 5.** Supramolecular compounds of GA and menthol in ratios GA: M (2:1) (A), GA: M (4:1) (B) and GA: M (9:1) (C) were induced by Fe^{2+}+ ascorbate of rat liver mitochondria Effect on LPO process (reliability with control *R < 0.05; **R < 0.01; n = 5).

ular complex of rat liver mitochondria in the ratio GA: M (4:1) was studied. According to the results of the experiment, the rates of peroxidation of lipids induced by Fe^{2+} / ascorbate in the liver mitochondria GA: M (4:1) supramolecular complex 20 and 50 μg / ml were 68.6 ± 4.4% and 94.6 ± 3, respectively, compared with controls. A 2% inhibitory effect was noted (*Fig.* 5, B).

CONCLUSION

The GA: M (9:1) supramolecular complex of GA and menthol also had an inhibitory effect on lipid peroxidation 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.* 5, C). Hence, here, the inhibitory effect of the GA: M (2:1) supramolecular complex on the LPO process was found to be effective against the GA: M (4:1) and (9:1) compounds. GA and menthol and the GA: M (2:1), GA: M (4:1) and GA: M (9:1) supramolecular compounds derived from them were further tested to ensure antioxidant activity in rat liver mitochondria. The effect on dialdehyde levels was also studied.

Acknowledgments. Publication cost of this paper was supported by the Korean Chemical Society.

REFERENCES

1. Rozza, A. L.; de Faria, F. M.; Brito, A. R. S.; Pellizzon, C. H. *Plos One* **2014**, *9*, 1.
2. Cheang, W. S.; Lam, M. Y.; Wong, W. T.; Tian, X. Y.; Lau, C. W.; Zhu, Z. *Eur. J. Pharmacol.* **2013**, *702*, 79.
3. Oz, M.; El Nebrisi, E.; Yang, K.; Howarth, F.; Al Kury, L. *Frontiers in Pharmacology* **2017**, *8*, 1.
4. Lee, A. G. *Trends. Biochem. Sci*. **2011**, *36*, 493.
5. Tolstikov, G. A.; Baltina, L. A.; Schultz, E. E.; Pokrovsky, A. G. *Bioorganic Chemistry* **1997**, *23*, 691.
6. Kamburova, V. S.; Asrarov, M. I.; Kim, N. P. *Khim. Prirod. Soed.* **2002**, Special Edition, 102.
7. Chuliev, I. N.; Kamburova, V. S.; Dzhuraev, A.; Asrarov, M. I. *DAN RUz.* **2005**, *1*, 41.
8. Dolimova, S. N.; Umarova, G. B.; Mukhamadzhonova, G. M.; Kuziev, Sh. N.; Yunusova, M. K. *European Science Review* **2018**, *3*, 43.
9. Chuliev, I. N.; Kamburova, V. S.; Dzhuraev, A.; Asrarov, M. I. *DAN RUz.* **2005**, *1*. 41.
10. Vinogradov, A. D. *Soros Educational Journal* **1999**, *9*, 11.
11. Schneider, W. *J. Biol. Chem.* **1948**, *176*, 250.
12. Gornal, A. G.; Bardawill, C. J.; David, M. *J. Biol. Chem.* **1949**, *177*, 751.
13. He, L.; Lemasters, J. J. *J. Biol. Chem*. **2003**, *278*, 16755.
14. Halestrap, A. P.; Clarke, S. J.; Javadov, S. A. *Cardiovasc Res*. **2004**, *61*, 372.
15. Javadov, S.; Karmazyn, M.; Escobales, N. *J. Pharm. Exp. Therap.* **2009**, *330*, 670.
16. Fiore, C.; Salvi, M.; Palermo, M.; Sinigaglia, G.; Armanini, D.; Toninello, A. *Biochimica et Biophysica Acta* **2004**, *1658*, 195.
17. Singh, R.; Muftah, A. M.; Belkheir, A. *Arabian Journal of Chemistry* **2015**, *8*, 322.
18. Dalimova, S. N.; Kaziev, Sh. N.; Dalimov, D. N.; Umarova, G. B.; Mukhamadzhanova, G. M.; Abdullaeva, U. A. *Nazariy va Klinik Tibbiyot Jurnali* **2017**, *3*, 14.