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STUDY OF THE BIOLOGICAL ACTIVITY OF EXTRACT MORUS NIGRA

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ABSTRACT

This article investigates the mechanism of action of the extract from the local plant *Morus nigra* (black mulberry) on the activity of Ca^{2+} channels in the smooth muscle cells of rat aorta preparations. The study determined that the extract has a significant effect on Ca^{2+} channels, specifically those associated with sarcoplasmic reticulum (SR), including IP3R and RyR receptors, as well as endothelial-dependent mechanisms. These results, along with further details, are discussed in the article.

KEYWORDS: smooth muscle, Ca^{2+} channels, blocker, IP3R, RyR receptors, endothelium, *Morus nigra*, extract.

INTRODUCTION

The sarcoplasmic reticulum (SR) plays a crucial role in the physiological and pathological conditions of vascular smooth muscles. The main function of the SR is to regulate calcium ion levels within cells, which is vital for maintaining vascular tone. Components such as IP3R, RyR, and SERCA in the SR are of utmost importance. IP3R and RyR receptors control the release of calcium ions from the SR into the cytosol, supporting various cellular activities. The SERCA pump is responsible for returning calcium to the SR, ensuring the cell's return to its baseline state.

The SR's structure includes key components such as choline (~65%), ethanolamine (~15%), and inositol phospholipids (~7%), which form the membrane and provide the optimal conditions for its functions. Choline and ethanolamine phospholipids ensure membrane elasticity and stability, while inositol phospholipids participate in intracellular signaling. Disruptions in this system can affect vascular smooth muscle tone and lead to various diseases.

It is evident that the structure and functions of the SR are complex and vital for supporting cellular activity under different conditions. Thorough research of these processes is essential for developing new methods in physiology, biophysics, and pharmacology.

Disruptions in Ca^{2+} transport play a critical role in the development of many diseases. Although Ca^{2+} ions are present in very low amounts in the cytosol, their higher concentrations in extracellular environments and intracellular stores (mainly the SR, Golgi apparatus, lysosomes, nucleus, and mitochondria) regulate various cellular functions. An imbalance in Ca^{2+} concentration affects several physiological, biophysical, and pharmacological processes, potentially leading to a range of pathologies, including cardiovascular, neurological, and metabolic diseases.[1]

The movement of calcium ions along concentration gradients is essential for biological processes, including muscle contraction and relaxation, transmission of nerve impulses, cellular signaling pathways, and material exchange across cell membranes. Proper regulation of calcium ions is crucial for generating strong, coordinated muscle movements and restoring muscle function post-contraction. Mismanagement of calcium distribution can lead to imbalanced muscle contractions or relaxation difficulties, which may result in various diseases and pathologies.[2]

MATERIALS AND METHODS

Chemicals

Morus nigra, a medicinal plant commonly used in Uzbekistan, was utilized in this research. The *Morus nigra* extract employed in the experiments was supplied by "Bioton" LTD, based in Tashkent, Uzbekistan. The chemical compounds phenylephrine, phentolamine, and verapamil were sourced from Sigma-Aldrich Chemie, a division of Sigma-Aldrich, located in St. Louis, MO, USA.

Determination of Antiradical Activity

The antiradical properties of *Morus nigra* were tested against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical using the method described by.[3] An ethanolic solution of the *Morus nigra* extract was added to a cuvette containing 100 μM of DPPH. The mixture was vigorously stirred, and the absorbance change at 517 nm was continuously monitored over a 30-minute period.

Tissue Preparation

All experimental procedures and preoperative care were approved by our institution's animal use committee. The animals were housed under standard vivarium conditions (humidity: 55%–65%, temperature: 22°C ± 2°C), with access to water and laboratory food. All procedures followed the European Directive 2010/63/EU for the protection of animals used for scientific purposes. The protocol was approved by the Animal Ethical Committee of the Institute of Bioorganic Chemistry, AS RUz (Protocol Number: 133/1a/h, dated August 4, 2014). The experiments were conducted on the aortic tissues of white male rats (weighing 200-250 g). The rats were euthanized by cervical dislocation, the chest cavity was opened, and the aorta was carefully isolated and placed in an organ bath (5 ml) containing Krebs-Henseleit physiological solution (mM): NaCl 120.4; KCl 5; NaHCO3 15.5; NaH2PO4 1.2; MgCl2 1.2; CaCl2 2.5; C6H12O6 11.5; HEPES pH 7.4. For some experiments, Krebs solutions without Ca^{2+} were prepared by adding EGTA (1 mM). The physiological solutions were oxygenated with carbogen (95% O2, 5% CO2) and maintained at +37°C using a DAIHAN WATER BATH ultrathermostat. After removing the surrounding connective tissue and fat, the aorta was cut into 3-4 mm rings.

Aortic Ring Contraction Studies

The aortic rings were attached to a Radnoti isometric transducer (USA) using platinum wire hooks and left for 60 minutes to equilibrate. An initial tension of 1 g (10 mN) was applied to each preparation. The contraction force

generated by the aortic rings was transferred from the transducer to a signal amplifier and recorded using a Go-link automated digital converter. The data were analyzed statistically using OriginLab OriginPro v.8.5 SR1 (Northampton, MA, USA). The isometric contraction force (mN) of the rat aortic rings under in vitro conditions was expressed as a percentage (%) during statistical analysis.[4] (Fig.1.)

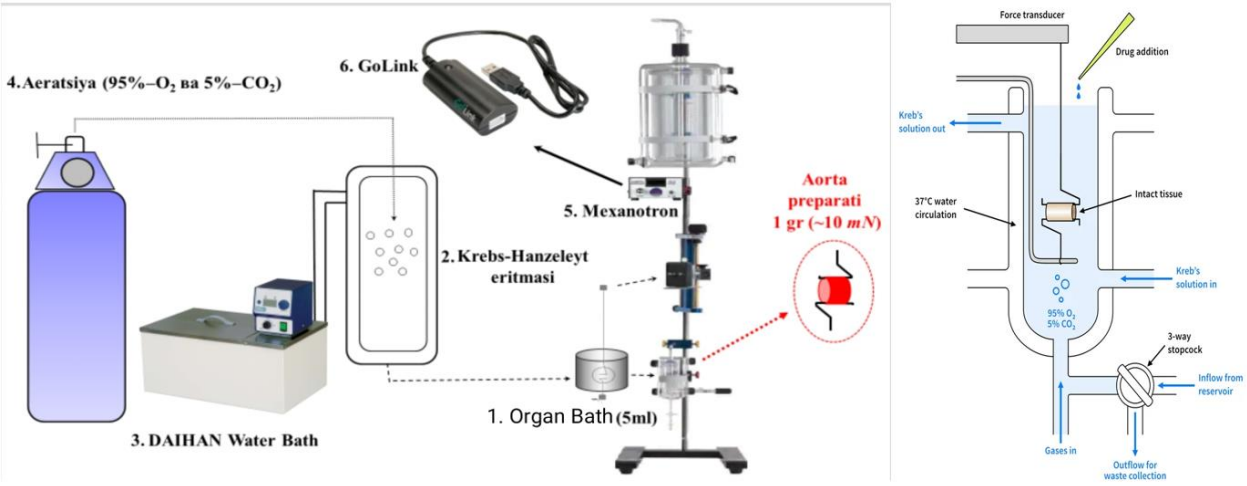

Fig. 1: General manufacturing view of the apparatus for regulating isometric contraction of a rat aortic vascular muscle preparation [Vandier et al., 2002]. 1. Organ bath (5 ml) is circulated through a special reservoir 2. Krebs-Henseleit physiological condition and 3. Thermostat support is provided with constant physiological temperature 4. Aerated with production of gases with 5%–CO2 95%–O2. The contractile activity of the aortic vascular prepart fixed to the experimental cell 5. ISOMETRIC TRANSDUCER mechanotron (Grass Instrument, USA) and the signal amplifier device 6 - GoLink devices support support system.

RESULTS AND DISCUSSION

In this study, the effects of *Morus nigra* extract on rat aortic vascular smooth muscle cells and its antiradical activity were investigated in vitro. Oxidative stress can lead to vascular dysfunction, resulting in conditions such as hypertension and atherosclerosis. *Morus nigra* extract, known for its antioxidant properties, demonstrated strong antiradical activity against the DPPH radical, indicating its ability to neutralize free radicals and protect vascular cells from oxidative stress. This aligns with recent scientific studies, which highlight *Morus nigra's* phenolic compounds, such as antioxidants like morusin, in reducing vascular permeability and oxidative stress.[5]

To assess the antioxidant radical scavenging activity (ARA) of the *Morus nigra* extract, a concentration of 50 µL of the extract from an ethanol solution (1 mg/mL) was used. The results showed a decrease in the optical density of the DPPH-containing ethanol solution, demonstrating the extract's antiradical effectiveness. Based on the experimental data, it can be concluded that the *Morus nigra* extract has a significant capacity to neutralize free radicals.

To quantify the antiradical potential, parameters such as t50 (the time required for the substances to reduce the initial radical concentration by 50%), the chemical reaction constant (k), and the half-maximal inhibitory concentration (IC50) were used. The IC50 value for *Morus nigra* extract was calculated to be approximately 38.48 µL, meaning that at this concentration, the extract achieves 50% inhibition of free radicals.

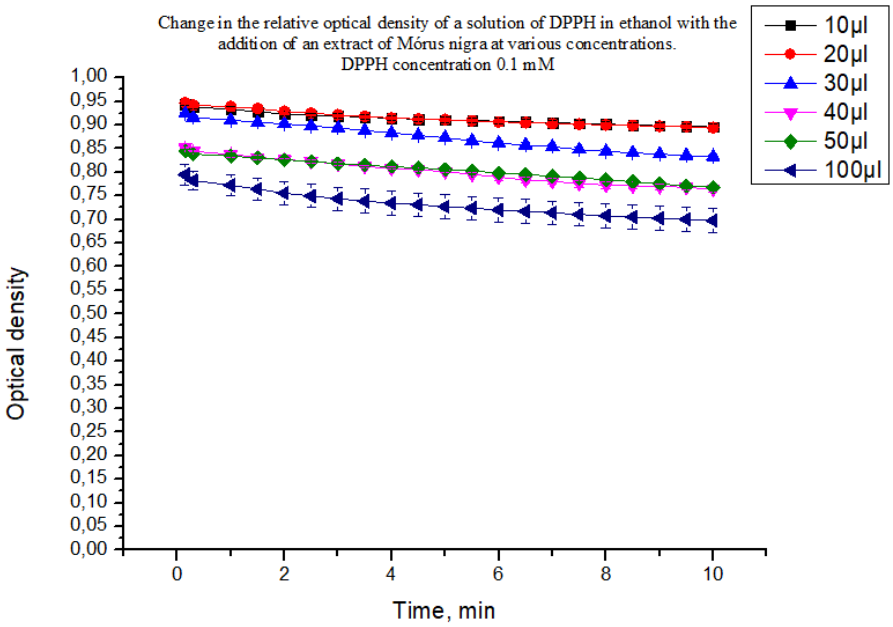

Fig. 2: Change in the relative optical density of a DPPH solution in ethanol with the addition of Mórus nígra extract in various concentrations. DPPH concentration 0.1 mM.

The graph demonstrates the dynamics of changes in the optical density of the DPPH solution in ethanol upon addition of Mórus nígra extract in various concentrations (10-100 µl) over time. It is observed that with an increase in the concentration of the extract, a proportional decrease in optical density is observed, indicating an increased reduction of DPPH radicals. The most pronounced decrease in optical density is recorded at concentrations of 50 and 100 µl, confirming the high antiradical activity of Mórus nígra extract in these doses. At the same time, at lower concentrations (10 and 20 µl), there are practically no changes in optical density, indicating a low antioxidant activity in these doses. These data confirm the dose-dependent effect of Mórus nígra extract in relation to DPPH radicals, emphasizing its effectiveness in high concentrations.

In our subsequent studies, it was found that *Morus nigra* extract effectively influences the activity of voltage-dependent Ca^{2+} channels located in the smooth muscle cells of rat aortic preparations.[6] The results of this study confirmed the additional relaxant properties of this extract. The research examined the effect of *Morus nigra* extract on the contraction activity of rat aortic preparations induced by phenylephrine (1 µM). According to the obtained data, the extract demonstrated a strong relaxant effect. For example, at a concentration of 10 µg/mL, *Morus nigra* extract reduced the contraction activity of the aortic preparation by 95.5±2.7%, while at a concentration of 80 µg/mL, this figure was 22.2±2.8%. This indicates that *Morus nigra* extract exhibits a dose-dependent muscle relaxation property. Thus, it was determined that *Morus nigra* extract has a potent vasorelaxant effect on the smooth muscle cells of rat aortic blood vessels (Figure 2).

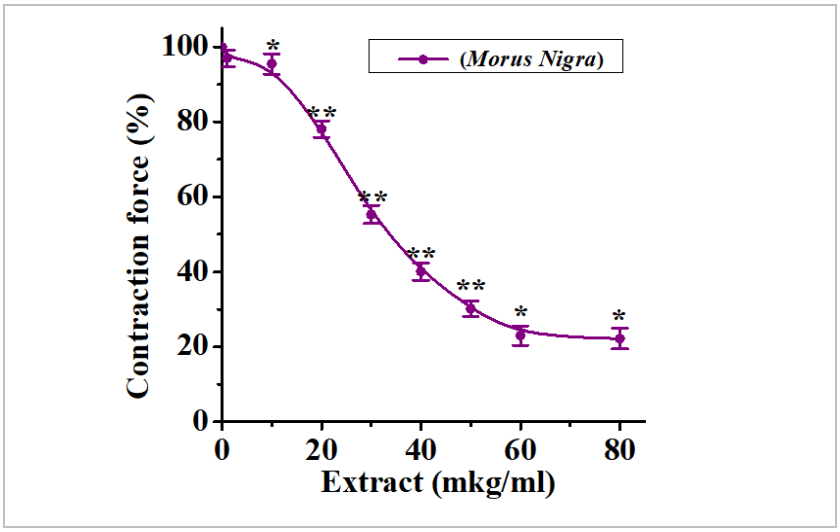

Figure 3: The effect of 80 µg/ml mulberry extract on phenylephrine-induced contraction of rat aortic preparations. On the ordinate axis – the contraction strength of the aorta induced by 1 µM phenylephrine (PE) is taken as 100%. On the abscissa axis – mulberry extract concentration. (In all cases, significance indicator: *p<0.05, **p<0.01; n=5-7).

Based on the obtained results, it can be hypothesized that the relaxant effect of the studied extract is mediated through the blockade of receptor-operated Ca^{2+} channels. To clarify this hypothesis further, in subsequent experiments, the effects of flavonoids were compared with the α-adrenoreceptor blocker phentolamine (PA).

The results of the experiments showed that in the absence of phentolamine, *Morus nigra* extract at a concentration of 80 µg/mL reduced the contraction force of the aortic preparation induced by 1 µM phenylephrine, as previously mentioned. When checking the contraction force of the aortic preparation with phentolamine (10 µM), it was found to be 80.7±2.9%. In the presence of phentolamine, the effect of *Morus nigra* extract was 33.1±2.4% (Figure 3).

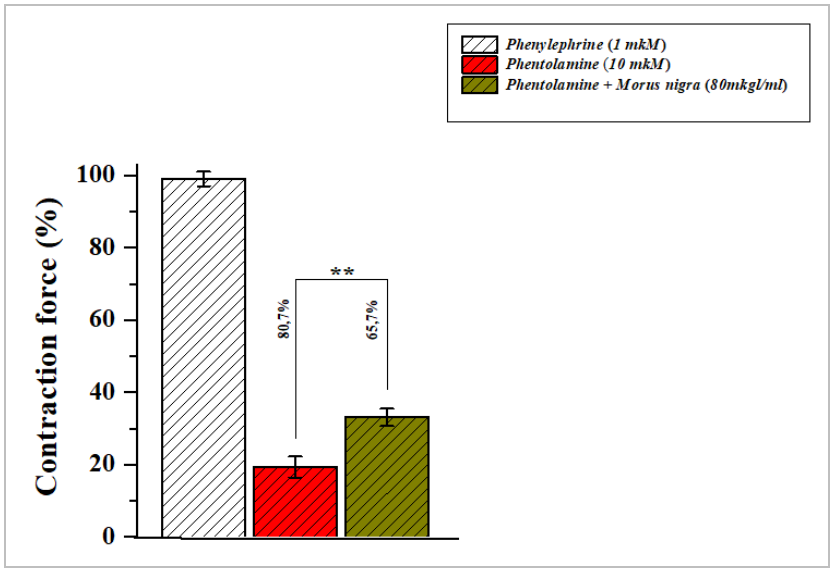

Figure 4: The relaxant effect of mulberry extract in the presence of phentolamine (10 µM) and verapamil (0.1 µM). The contraction strength of the aorta preparation induced by 1 µM phenylephrine (PE) is taken as 100%, and in all cases, the significance indicator is *p<0.05, **p<0.01; n=5.

The conducted experiments confirm that the relaxant activity of *Morus nigra* extract is achieved through the blockade of receptor-operated Ca^{2+} channels. Additionally, experiments conducted with the α-adrenoreceptor blocker, phentolamine, further support this conclusion.

In the following experiments, the effect of *Morus nigra* extract on the release of Ca^{2+} ions from the sarcoplasmic reticulum (SR) through IP3 receptors (IP3R) was studied. In an incubation environment without Ca^{2+} ions, the contraction force induced by phenylephrine (1 µM) was found to be primarily driven by the release of Ca^{2+} ions from the SR via IP3R. Literature suggests that this mechanism plays an important role in the contraction process of vascular smooth muscles.[7] In the current studies, the contraction force induced by phenylephrine (1 µM) in normal Krebs solution was 69±3.1%, which was considered 100%. Under these conditions, *Morus nigra* extract at a concentration of 80 µg/mL reduced the contraction force by 31.1±2.3% compared to the control (Figure 4).

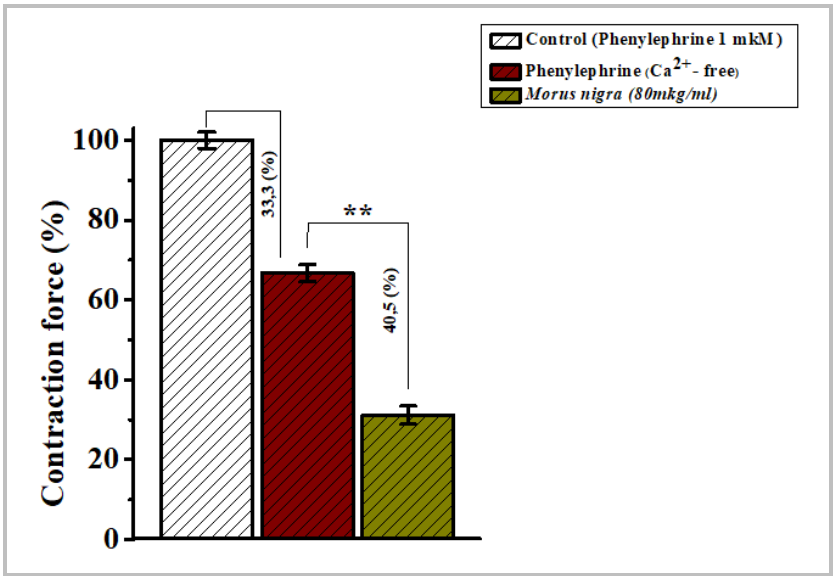

Figure 5: The dose-dependent relaxant effect of mulberry extract on phenylephrine-induced contraction of rat aortic preparations in Ca2+-free Krebs solution. The contraction of the aorta induced by 1 µM phenylephrine (PE) in a Ca2+-free Krebs solution is taken as 100% in the control (In all cases, significance indicator: *p<0.05, **p<0.01; n=4-6).

Based on the obtained results, it was determined that *Morus nigra* extract significantly reduced the contraction force induced by phenylephrine (1 µM) in the absence of Ca^{2+} ions. This indicates that the extract influences intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) by reducing the release of Ca^{2+} ions from the SR. Additionally, the relaxant effect of *Morus nigra* extract in the aortic preparation in the absence of Ca^{2+} ions in Krebs solution is likely associated with the inhibition of Ca^{2+} release from the SR through IP3R. To clarify this hypothesis, additional experiments were conducted using caffeine. According to scientific literature, caffeine activates ryanodine receptors (RyR) in the SR, leading to the release of Ca^{2+} ions into the cytosol. The contraction force induced by caffeine is an important indicator in evaluating the amount of Ca^{2+} ions in the SR. In the conducted experiments, under normal Krebs solution conditions with Ca^{2+} ions (2.5 mM), caffeine (10 µM) induced a contraction force of 66.7±2.2% compared to phenylephrine (1 µM). In this condition, the incubation of *Morus nigra* extract reduced the caffeine-induced contraction by 30.2±2.4% compared to the control (Figure 5).

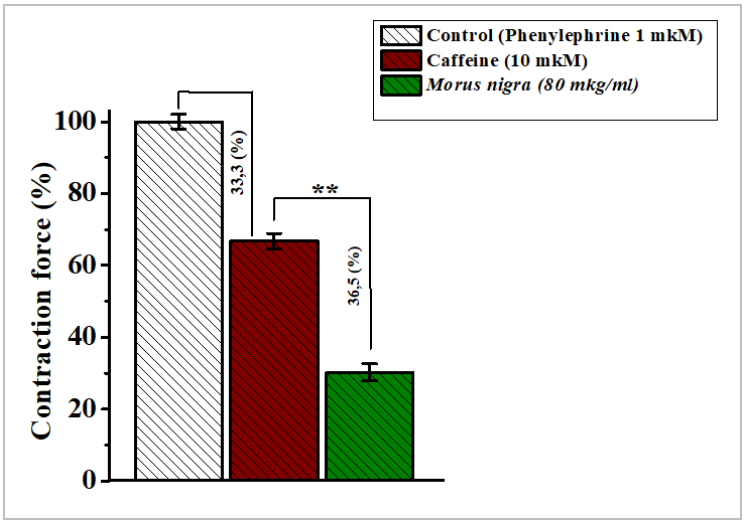

Figure 6: The relaxant effect of mulberry extract on caffeine-induced contraction of rat aortic preparations in normal Krebs solution. The contraction of the aorta induced by 1 µM phenylephrine (PE) is taken as 100% in the control (In all cases, significance indicator: *p<0.05, **p<0.01; n=5).

The obtained results indicate that the relaxing effect of the mulberry extract on caffeine-induced contraction strength is related to a decrease in the amount of Ca^{2+} released from the sarcoplasmic reticulum (SR). However, in this process, the contraction of smooth muscle cells may be influenced not only by Ca^{2+} ions released from the SR but also by Ca^{2+} ions entering from the external environment through the plasmalemma.[8] Therefore, additional experiments were conducted in conditions where Ca^{2+} ions were absent in the incubation medium to fully clarify the mechanism of action of the extracts on caffeine-induced contraction. Under these conditions, the contraction strength induced by caffeine was recorded as 35.0±2.3% compared to the condition where Ca2+ ions were present. Experiments were then conducted with various concentrations of mulberry extract under these conditions. The results showed that the mulberry extract reduced caffeine-induced contraction by 16.6±1.8% compared to the control (Figure 6).

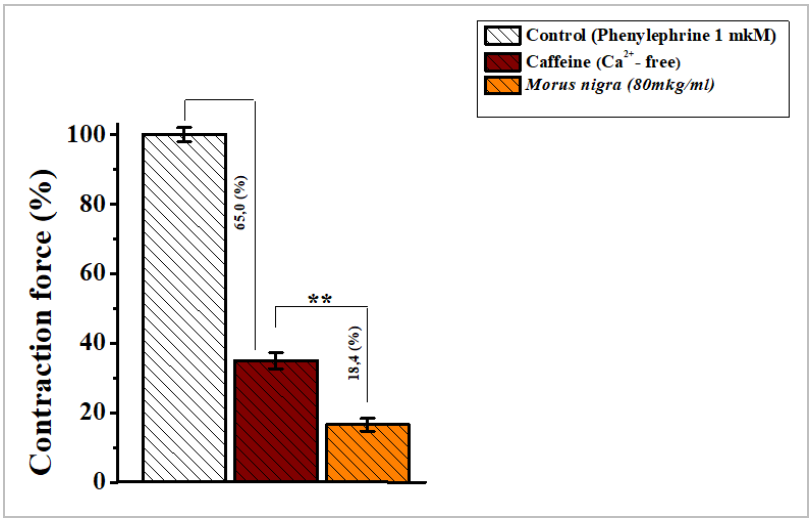

Figure 7: The dose-dependent relaxant effect of mulberry extract on caffeine-induced contraction of rat aortic preparations in a Ca^{2+}-free Krebs solution. The contraction of the aorta induced by 1 µM phenylephrine (PE) in a Ca^{2+}-free Krebs solution is taken as 100% in the control (In all cases, significance indicator: *p<0.05; **p<0.01; n=4-5).

From the obtained results, it is clear that the studied extracts effectively reduce the aortic contraction induced by phenylephrine (PE) in Ca^{2+}-free Krebs solution. This process mainly occurs due to the release of Ca^{2+} from the SR through the IP3 receptor (IP3R). These findings confirm that inhibiting the release of Ca^{2+} from the SR via IP3R may play a significant role in the relaxing effect of mulberry extract. Investigating the role of the endothelium in the relaxing effect of mulberry extract According to the literature, the endothelial layer plays an important role in maintaining vascular tone and the functional activity of blood vessels. Endothelial cells, located in the inner layer of the vascular wall, participate in regulating blood flow in organs by synthesizing locally acting mediators. One of the main active substances of endothelial cells is nitric oxide (NO), which is considered an endothelial vasodilator.[9] Endothelial dysfunction is important in the pathogenesis and clinical evaluation of diseases such as arterial hypertension and atherosclerosis. In many cardiovascular diseases, the initial damage to endothelial cells is observed, leading to a cascade of pathological changes and large-scale dysfunction.[10]

Endothelial dysfunction is now described as an imbalance in endothelial-dependent processes. Vascular changes associated with cardiovascular diseases are often accompanied by the inability of blood vessels to respond to external stimuli and impaired production of endothelial vasoactive factors.[11] Many factors contribute to endothelial dysfunction, including physical inactivity, smoking, excessive salt consumption, intoxications, estrogen deficiency, and disturbances in carbohydrate and lipid metabolism.[12]

Under normal conditions, eNOS is bound to caveolin, which reduces its activity. As a result of receptor stimulation (by acetylcholine, bradykinin, serotonin, etc.), eNOS detaches from caveolin and is transported to the plasma membrane. Statins increase NO synthesis by reducing excessive levels of caveolin-1, which inhibits eNOS activation, helping to eliminate endothelial dysfunction.[13]

Thus, in endothelial cells, NO-synthase (eNOS) activates the guanylate cyclase enzyme, which increases the concentration of cGMP and provides relaxation via protein kinase G.[14]

Endothelial cells play a key role in regulating smooth muscle contraction activity and Ca^{2+} homeostasis. NO acts as the primary mediator in this process, activating the NO/sGC/cGMP/PKG signaling pathway in smooth muscle cells, reducing Ca^{2+} levels, and leading to relaxation.[15] The endothelium-dependent effect of the extracts has also been noted in our previous studies.[16] Therefore, in our current research, experiments were conducted on aorta preparations with the endothelium removed to study the relaxing effect of mulberry extract. The functional activity of the endothelium in response to the extracts was evaluated using standard methods.

Aortic smooth muscle preparations with and without an intact endothelial layer were contracted with 1 μM phenylephrine. Under the influence of phenylephrine, the contraction amplitude of the aorta preparation reached 10 mN. To test the presence or absence of the endothelial layer, 1 μM acetylcholine was used. In isometric conditions, the contraction amplitude of rat aortic vascular preparations induced by the α1-adrenoreceptor agonist – 1 μM phenylephrine – was reduced by 57.8±4.3% under the influence of 1 μM acetylcholine. In the aorta preparation where the endothelial layer was mechanically removed, the effect of acetylcholine on phenylephrine-induced contraction was almost negligible.

According to the results, in isometric conditions of rat aortic vascular preparation, the relaxing effect of the mulberry extract significantly changed when the endothelial layer was removed. The mulberry extract, at various concentrations (µg/ml), reduced relaxation by 68.2±2.8%, which was significantly higher compared to the 30.0±2.4% reduction observed with an intact endothelium (Figure 7).

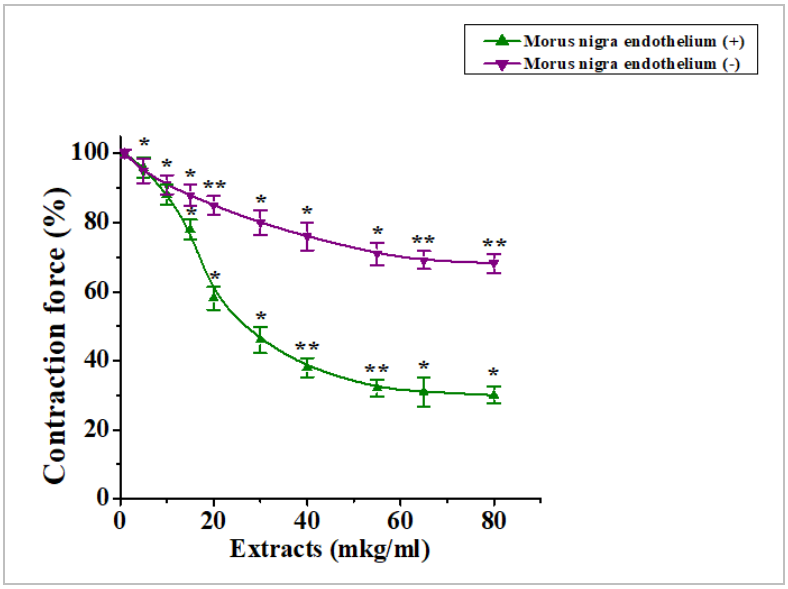

Figure 8: The concentration-dependent relaxant effect of mulberry extract on the contraction of rat aortic vessel preparations with (+) and without (-) an endothelial layer induced by 1 µM phenylephrine (PE). The contraction strength induced by 1 µM PE is taken as 100% in the control. (In all cases, significance indicator: *p<0.05, **p<0.01; n=5).

The experimental results show that the effect of the examined extract on aorta preparations significantly changed after the removal of the endothelial layer. These findings may assist in determining the endothelial-dependent effects of the examined substances. Additionally, to further clarify this hypothesis, subsequent experiments were conducted using the eNOS inhibitor – L-NAME (100 µM).

The results showed that in aorta preparations incubated with L-NAME, the relaxing effect of the mulberry extract significantly diminished. Under conditions of 100 µM L-NAME, the mulberry extract reduced the phenylephrine-induced contraction strength of aorta preparations by 63.3±2.2%, compared to a 33.1±2.1% reduction in preparations with an intact endothelial layer (Figure 8).

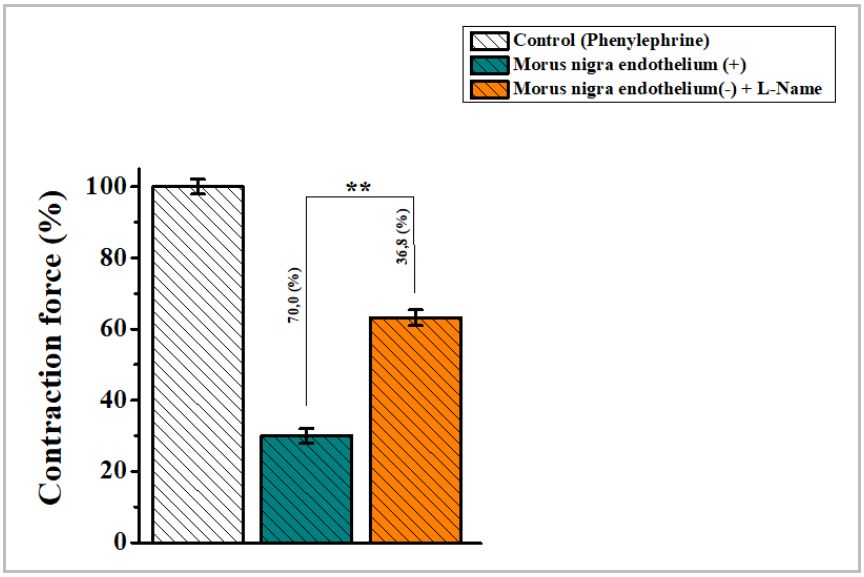

Figure 9: The concentration-dependent relaxant effect of mulberry extract on the contraction of rat aortic vessel preparations incubated with the eNOS inhibitor – L-NAME (100 µM). The contraction strength induced by 1 µM phenylephrine (PE) is taken as 100% in the control. (In all cases, significance indicator: *p<0.05, **p<0.01; n=6).

The experimental results clearly indicate that the main cause of the observed relaxing effect of the studied extracts is endothelial-dependent processes. The relaxing effect of the extracts significantly diminished in conditions where the endothelium was removed or in the presence of L-NAME, highlighting the importance of NO-synthase. By activating the NO-synthase and sGC/cGMP/PKG signaling pathway, these molecules reduce the entry of Ca^{2+} ions through Ca^{2+}L and Ca^{2+}R channels in the plasmalemma, as well as inhibit their release from the SR. As a result, the [Ca^{2+}]i level decreases in smooth muscle cells, leading to muscle relaxation.

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