

The Combined Inotropic and Vasorelaxant Effect of DHQ-11, a Conjugate of Flavonoid Dihydroquercetin with Isoquinoline Alkaloid 1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline

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This study investigated the positive inotropic and vasorelaxant activity of DHQ-11, a conjugate of flavonoid dihydroquercetin with isoquinoline alkaloid 1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline. A study was performed using anterior papillary muscle removed from the left ventricle and thoracic aorta dissected from rats. DHQ-11 produced a concentration-dependent positive inotropic effect which was more potent than their parent compounds alone. The positive inotropic effect of conjugate DHQ-11 was significantly attenuated by the α -adrenoreceptor inhibitor propranolol and L-type Ca^{2+} channel blocker nifedipine. Also, conjugate DHQ-11 markedly potentiated first post-rest responses indicating that it can modulate Ca^{2+} loading/release processes in the sarcoplasmic reticulum. These results suggest that positive inotropic effect produced by conjugate DHQ-11 may be mediated through activation of the α -AR/AC/cAMP/PKA pathway that leads to increased Ca^{2+} influx and rises in Ca^{2+} loading/release in the SR, resulting in increased $[Ca^{2+}]_i$ and enhanced contraction force. DHQ-11 significantly relaxed both high KCl- and phenylephrine-induced contractions of rat aortic rings which were significantly inhibited by lowering extracellular Ca^{2+} concentration and in the presence of verapamil. DHQ-11 significantly inhibited phenylephrine-induced contractions in a Ca^{2+} -free medium, in the presence of verapamil. The vasorelaxant effect of the DHQ-11 was significantly reduced by the removal of endothelium and in the presence of L-NAME and methylene blue as well as glibenclamide and TEA. These results suggest that the vasorelaxation produced by conjugate DHQ-11 may be mediated by an endothelium-independent mechanism involving activation of K_{ATP} and BK_{Ca} channels and inhibition of L-type VDCCs and Ca^{2+} release from the sarcoplasmic reticulum and endothelium-dependent mechanism through activation of the NO/sGC/cGMP/PKG signaling pathway resulting in a decrease of intracellular Ca^{2+} levels. These observations reveal that the conjugate DHQ-11 due to its high positive inotropic and vasorelaxant activity could be a promising compound for the design and development of new drugs for the treatment of heart failure.

Keywords: Positive inotropic effect, vasorelaxation, papillary muscle, aorta, endothelium, alkaloids, flavonoids, nitric oxide, calcium channel.

Heart failure (HF) is a major health problem worldwide which is the leading cause of death in many developed countries^{1]} HF is a

common condition in which the heart cannot pump enough blood to meet the body's needs due to loss in cardiac contractility and ejection fraction².

The major pathophysiologic mechanisms leading to HF include increased hemodynamic overload, ischemia-related dysfunction, increased oxidative stress, reduced energy utilization, disrupted Ca^{2+} homeostasis, and reduced contractility of cardiac muscle³. Current pharmacological approaches for treating HF are still based on using renin-angiotensin-aldosterone system inhibitors (ACEIs), beta-blockers, positive inotropes, and vasodilators which by modulation of hemodynamics, cardiac output, and ventricular filling pressures, improve contractile performance of hearts⁴. ACEIs act by prevention the conversion of angiotensin I to angiotensin II, and inhibition of angiotensin II receptor which leads to lower blood pressure, reduced afterload, and improved cardiac output⁵. The effect of beta-blockers is related to inhibition of the AMP/PKA-dependent pathway which leads to lower blood pressure, slowed heart rate, and reduced force of heart contraction⁶. The most commonly used positive inotropes are $\hat{\alpha}_1$ - and $\hat{\alpha}_2$ -adrenergic receptor agonists which by activation of L-type Ca^{2+} channels and Ca^{2+} release from the sarcoplasmic reticulum (SR) restore Ca^{2+} homeostasis thus improve the function of failing cardiomyocytes and cardiac contractility⁷. Other positive inotropes that extensively used in HF are cardiac glycosides which by inhibition of Na/K-ATPase and increasing intracellular Ca^{2+} concentration in cardiomyocytes enhance cardiac contractility and improve cardiac output⁸. Vasodilators by dilation of arterial vessels and reducing arterial pressure decrease the left ventricular afterload and thus enhances stroke volume and increase cardiac output, while venous dilators which reduce venous pressure, decrease preload and cardiac output⁹.

However, despite the benefits of the drugs commonly used for the treatment of HF almost all of them are far from ideal because they cannot completely correct underlying abnormalities implicated in its pathogenesis and have adverse effects.

The most common side effect of these drugs is; reduced kidney function, hyperkalemia, reduced blood pressure, increased metabolic demand, slowing the heart rate and rhythm disturbances, that limit their usefulness^{10,11,12}.

Therefore, current strategies for the treatment of HF focuses on the development of a

new generation of effective drugs that would avoid undesirable side effects and act through novel mechanisms involving potential therapeutic targets contributing to the progression of the disease.

Recent advances in understanding the pathophysiology of HF have provided insights into novel pathways and molecular sites as promising therapeutic targets to improve the treatment efficacy of HF. According to numerous experimental evidence, the most effective therapeutic strategy in HF is to improve blood supply by dilation of the coronary artery through the restoration of altered endothelial function and bioavailability of nitric oxide (NO), and enhancing contractility of cardiac muscle by correction of impaired Ca^{2+} signaling and Ca^{2+} homeostasis in cardiomyocytes^{13,14}. In the last decade, natural compounds like plant flavonoids and alkaloids due to their high bioavailability and low cytotoxicity are recognized as the most efficient candidates for the development of novel approaches to treatment HF. There is growing evidence, that plant flavonoids and alkaloids displays antioxidant, antihypertensive, antiarrhythmic effects and protect the heart from ischemia-reperfusion injury mediated through several different mechanisms including free-radical scavenging, vasorelaxant and inotropic activities^{15,16}.

Nowadays, one of the promising approaches for the rational design of novel drugs for the treatment of HF is molecular hybridization based on the combination of compounds with distinct pharmacologic activities in one molecule to produce a new hybrid compound with improved affinity and efficacy¹⁷. Recently, using a hybridization technique and *Mannich reactions*, a conjugate of flavonoid dihydroquercetin with isoquinoline alkaloid 1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline DHQ-11, both exhibiting potent vasodilatory and positive inotropic activity, respectively, was synthesized^{18,19}.

In the present study, we aim to evaluate how the hybridization of flavonoid dihydroquercetin with isoquinoline alkaloid 1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (F18) affects their vasorelaxant and inotropic activity. This study would provide evidence for the use of DHQ-11 in the treatment of HF.

MATERIALS AND METHODS

All experimental protocols and conditions for preoperative care were approved by the animal use committee of our institution. Adult male Wistar rats weighing 200 – 250 g were anesthetized with sodium pentobarbital and their hearts were removed rapidly and placed in oxygenated Krebs's solution. The left ventricle was opened and an anterior papillary muscle was removed. The papillary muscles about 0,5-0,8 mm in diameter and 1-3 mm in length were mounted in an organ bath (STEIRT, HSE, Germany) between a fixed hook and an isometric tension transducer (Type F30, HSE). The muscle was superfused with oxygenated (gas mixture 95% oxygen, 5% carbon dioxide; pH 7.4) Krebs solution contained (in mmol/l); NaCl, 118; KCl, 4.7; MgSO₄, 1.2; KH₂PO₄, 1.2; glucose 5.8; NaHCO₃, 24; CaCl₂, 2.54) maintained at 37^o C by a thermostat controlled water bath. The muscle was stretched to a length at which maximum developed force was evoked and allowed to equilibrate for at least 1 h before the commencement of the experiments. The muscle was stimulated at a rate of 0,1-5 Hz through the platinum field electrodes with rectangular pulses of 5 ms duration at twice the threshold voltage. The amplitudes of elicited maximal isometric contraction were used as the control (100%) and changes in the contraction after drug action was expressed as a percentage of the maximal response. Contractions were recorded on a chart recorder (TZ 4620, Chech Rep.) and after digitalization stored on an online computer. To characterize the mechanism of inotropic action of conjugate DHQ-11 its effect on contractile responses of papillary muscles at the various experimental conditions were investigated. To examine the effects of conjugate DHQ-11 on calcium homeostasis its effect on the cumulative dose-response curve of Ca²⁺ was studied. In these experiments, the papillary muscle was washed three times in a Ca²⁺ free Krebs solution containing 2.5 mM EGTA. To further clarify the possible involvement of the Ca²⁺ channels and β-adrenoreceptor in the inotropic action of the conjugate DHQ-11 its effect in the presence of their blockers nifedipine and propranolol, respectively, were studied. To test the effect of the conjugate DHQ-11 on loading and release functions of sarcoplasmic reticulum (SR) its effect on post-

rest potentiation was examined. The post-rest potentiation of contraction was studied at an [Ca²⁺]_o of 0.5 mM, after a rest period of 30 s and at a stimulation frequency of 1Hz.

The vasorelaxant effect of conjugate DHQ-11 was evaluated using the thoracic aorta, dissected from the rat. The isolated aorta was immediately placed in Krebs solution contained (in mmol/l); 118 mM NaCl, 5 mM KCl, 25 mM NaHCO₃, 1.2 mM MgSO₄, 2 mM CaCl₂, 1.2 mM KH₂PO₄, 11 mM glucose. The aorta was cleaned of adipose and connective tissue and cut into rings (2–3 mm long). The rings were mounted using two stainless hooks with one fixed to the bottom of the organ bath and the other connected to a force transducer. The organ bath was superfused with Krebs solution, bubbled with a 95% O₂-5% CO₂ gas mixture, maintained at 37^oC. The aortic rings were equilibrated in Krebs solution under the tension of 1 g, for 60 min during which period the Krebs solution was replaced at least twice. Aortic ring contraction was recorded, isometrically using a force transducer (FT-03; Grass Instrument Company, USA) connected to a chart recorder Endim 621-02 (Germany). The rings were contracted with 1 μM of phenylephrine (PE) or 50 mM KCl and allowed to plateau before the addition of tested drugs. After the contraction had reached a stable plateau, conjugate DHQ-11 was added cumulatively. The vasorelaxant effect of conjugate DHQ-11 was expressed as a percentage relaxation of the pre-contraction induced by PE or KCl (50mM). To assess the effect of conjugate DHQ-11 on extracellular Ca²⁺ influx, concentration–response curves to CaCl₂ were tested using Ca²⁺-free Krebs solution with 100 mM EGTA and high-K⁺ (50 mM), prepared by replacing an equimolar concentration of NaCl with KCl. To investigate the role of Ca²⁺ released from intracellular stores in the vasorelaxant action of conjugate DHQ-11, its effects on aortic rings contraction induced by 1 μM PE in Ca²⁺-free Krebs solution contained 50 mM EGTA, were studied. To examine the participation of the K⁺ channels in the vasorelaxant action of conjugate DHQ-11, its effects in presence of the 4-aminopyridine (4-AP), a specific blocker of voltage-dependent K_v channels, tetraethylammonium (TEA), a nonspecific blocker of the calcium-activated large conductance BK_{Ca} channels, BaCl₂, a specific blocker of the

inward rectifying K_{IR} channels, and glibenclamide, a specific inhibitor of ATP-sensitive K_{ATP} channels were studied. To determine the involvement of endothelium in vasorelaxant action of conjugate DHQ-11 the endothelium was removed from ring specimens by rubbing the intimal surface with a cotton ball and the absence of ACh-induced relaxation was taken as an indicator of successful denudation. To further clarify the role of the endothelium in the vasorelaxant action of conjugate DHQ-11 its effects on PE-induced contraction of endothelium-intact aortic rings preincubated with L-NAME (nitro-L-arginine methyl ester, a NO synthase inhibitor) and methylene blue (a guanylyl cyclase inhibitor) were studied.

Drugs and reagents

All chemicals were of analytical grade commercially available. Nifedipine, verapamil, phenylephrine, propranolol, L-NAME, and methylene blue were obtained from Sigma Ltd Co., (St. Louis, MO, USA). Conjugate DHQ-11 was synthesized at the Institute of Chemistry of Plant Compounds of Uzbek Academy of Sciences and was kindly provided by V.I. Vinogradova.

Data and statistical analysis

Throughout this article, all data are

represented as the mean \pm standard error of the mean (s.e.m.) of n observations. Statistical analysis was performed using an unpaired Student's t -test. The EC_{50} and IC_{50} values, the concentration of drugs causing a 50% contraction or relaxation of the maximal response (E_{Max}), were obtained from the concentration-response curve and calculated using the sigmoidal curve fitting routine in Origin 6.0 (Microcal, Northampton, MA, U.S.A.). The differences between control and experimental values were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

The positive inotropic effect of the conjugate DHQ-11. In the isometric tension recordings in isolated rat papillary muscle, it was found that the conjugate DHQ-11 exerted a pronounced positive inotropic effect (PIE) in a concentration-dependent manner. In these studies, the application of 35 μ M of conjugate DHQ-11 caused a maximal increase in the contractile force of rat papillary muscle to $77.4 \pm 4.2\%$ from the baseline value set as 100% (Fig.1). Under the same experimental conditions, the flavonoid dihydroquercetin and alkaloid 1-aryl-

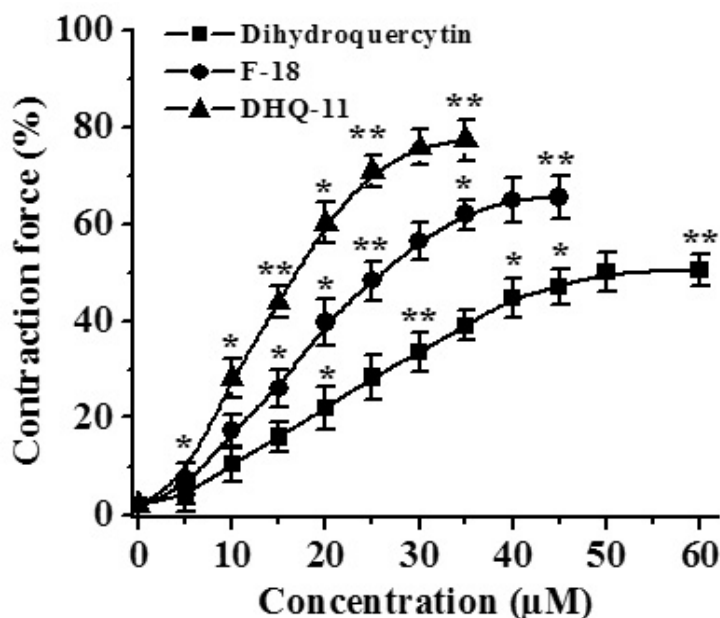


Fig. 1. Concentration-dependent positive inotropic effects of DHQ-11, dihydroquercetin and 1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline in rat papillary muscles. The force of muscle contraction obtained in control at 0.5 Hz is expressed as 100%. Data are presented as mean \pm SEM ($n = 5-6$). * $p < 0.05$, ** $p < 0.01$

6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline maximally increased the force of contraction by $51.4 \pm 3.9\%$ and $65.6 \pm 4.4\%$ from the baseline value. EC_{50} values (the concentration of compounds causing 50% of the maximum effect) obtained from these results were 21.2 ± 4.1 μ M, 14.6 ± 3.5 μ M, and 9.7 ± 4.3 μ M for dihydroquercetin, alkaloid 1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline and conjugate DHQ-11, respectively. These data indicate that the conjugate DHQ-11 has a more strong PIE compared to flavonoid dihydroquercetin and alkaloid 1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline. The most commonly used positive inotropes enhanced cardiac contractility through the activation of different mechanism resulted mainly in increased intracellular Ca^{2+} level ($[\text{Ca}^{2+}]_i$) in cardiomyocytes¹⁹. The increase in $[\text{Ca}^{2+}]_i$ mainly may be mediated by Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels (VDCCs) and Ca^{2+} release from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyR2) which are activated by stimulation of α -adrenergic receptors²⁰.

The stimulation of α -adrenergic receptors leads to activation of adenylyl cyclase (AC) and increased cAMP which by activation of protein kinase A (PKA), leads to the phosphorylation of the

L-type VDCCs and RyR2 resulting in the elevation in $[\text{Ca}^{2+}]_i$ thereby allowing forceful contraction²¹.

To determine the possible involvement of the α -AR/AC/cAMP/PKA signaling pathway in the PIE of conjugate DHQ-11 its effect in the presence of propranolol, a α -adrenoreceptor blocker was examined. In these experiments, it was found that after pretreatment of papillary muscle with 10 μ M propranolol the PIE of conjugate DHQ-11 (35 μ M) reduced from $77.4 \pm 4.2\%$ to $43.6 \pm 3.8\%$ (Fig. 2, A). These results indicate that the PIE of conjugate DHQ-11 possible is due to the activation of the α -AR/AC/cAMP/PKA signaling pathway, which may be accompanied by an increase in the influx of Ca^{2+} ions into cardiomyocytes through VDCCs. To confirm this further the experiments with nifedipine, a specific blocker of Ca^{2+} -channels were performed. As can be seen from Fig. 2, B application of conjugate DHQ-11 (35 μ M) on the background of 0.01 μ M nifedipine, the concentration corresponding to its IC_{50} value, increased the force of contraction by $26.8 \pm 3.4\%$ compared to a $77.4 \pm 4.2\%$ observed in the absence of nifedipine. These data suggest that PIE of conjugate DHQ-11 possible is mediated through activation of the α -AR/AC/cAMP/PKA signaling pathway and subsequent enhancing Ca^{2+} influx into

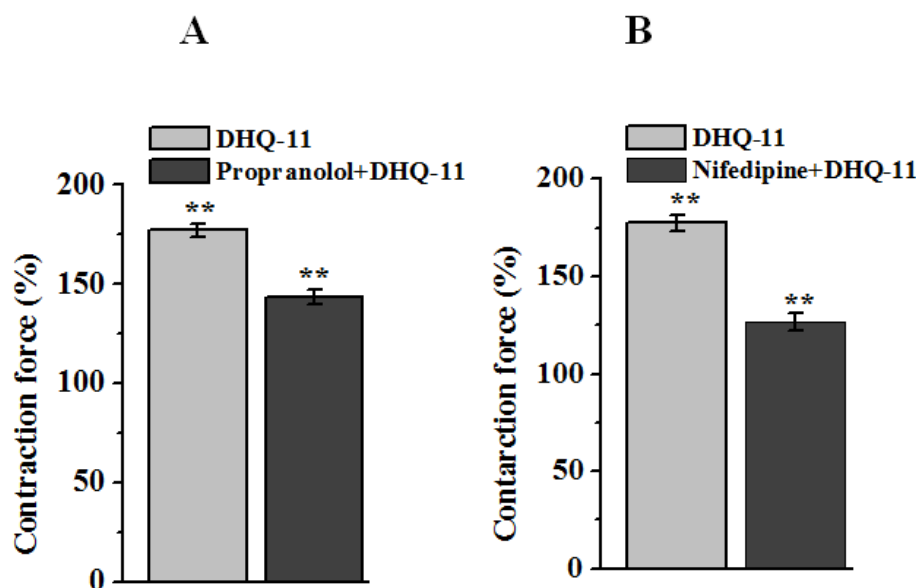


Fig. 2. Effects of propranolol (A) and nifedipine (B) on the positive inotropic effect of DHQ-11. The force of muscle contraction obtained in control at 0.5 Hz is expressed as 100%. Each column represents the mean \pm SEM (n=6). ** $p < 0.01$, as compared with the control.

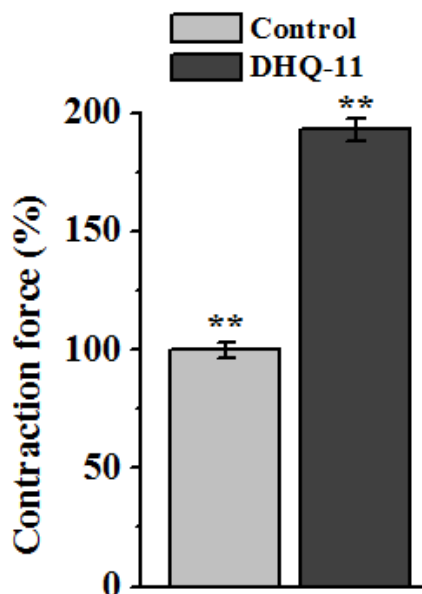


Fig. 3. Effect of DHQ-11 on post-rest potentiation of contraction in rat papillary muscle. Post-rest potentiation of contractions following regular stimulation at 0.5 Hz was tested after a 30 s rest period before and after the addition of DHQ-11 (35 μ M). Values obtained in control were expressed as 100%. Each column represents the mean \pm SEM (n=5). ** $p < 0.01$, as compared with the control.

cardiomyocytes through VDCCs. However, our observation that PIE of conjugate DHQ-11 was partially preserved in the presence of nifedipine suggested that its PIE may be mediated not only by enhancing Ca^{2+} influx via L-type VDCCs but other mechanisms may also be involved.

The key determinant of cardiac muscle contraction force is the Ca^{2+} released from SR via RyR2 mediated by calcium-induced release of calcium mechanism activated by Ca^{2+} influx through L-type VDCCs in the sarcolemma²². Therefore, to determine the possible role of Ca^{2+} released from SR in PIE produced by conjugate DHQ-11 its effect on post-rest potentiation of the force of contraction which reflects the amount of Ca^{2+} accumulated within and released from the SR was studied²³. The results obtained in these experiments showed that in the presence of conjugate DHQ-11 (35 μ M), the first contraction after rest intervals (30 s) increased from the control level taken as 100% by 93.1 \pm 3.8% (Fig. 3).

These results indicate that treatment of papillary muscle with conjugate DHQ-11 caused an additional accumulation of Ca^{2+} in the SR that lead to an increased Ca^{2+} release from the SR resulted in enhanced contraction force. These findings suggest that conjugate DHQ-11 can activate not only Ca^{2+} influx via VDCCs but also modulate Ca^{2+}

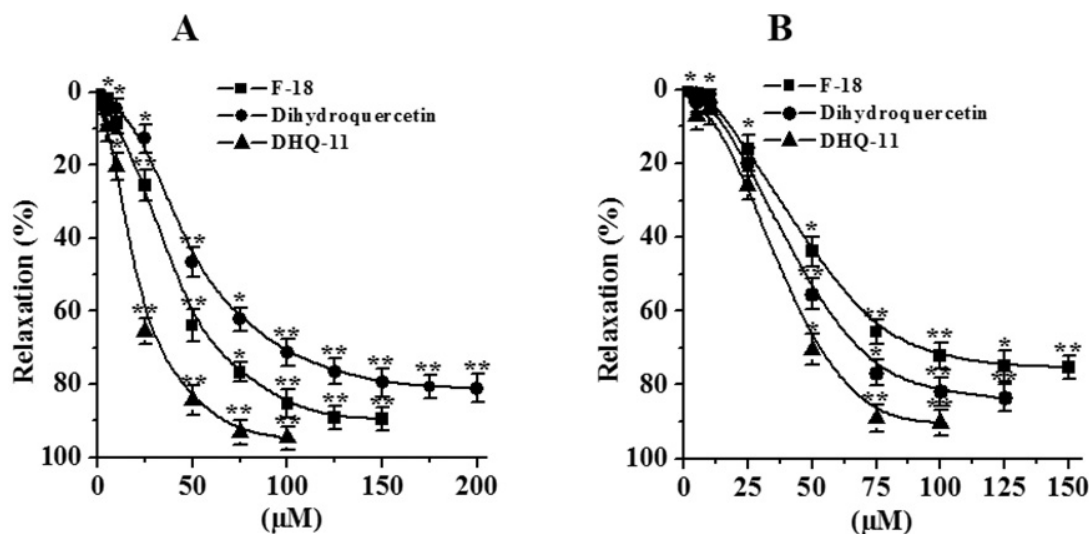


Fig. 4. Concentration-dependent vasorelaxant effects of DHQ-11, dihydroquercetin and 1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline on endothelium-intact rat aortic rings precontracted with KCl (A) and phenylephrine (B). Vasorelaxant effects of drugs were expressed as the percentage inhibition of contraction induced by KCl (50mM) and phenylephrine (1 μ M). Data are presented as mean \pm SEM (n=5–8). * $p < 0.05$, ** $p < 0.01$, as compared with the control

loading/release processes in SR. This suggestion was supported by fact that nifedipine inhibits PIE produced by conjugate DHQ-11 (Fig.2), presumably through suppression of Ca^{2+} influx via L-type VDCCs which play important role in refilling Ca^{2+} store [20]. The data obtained in these study suggest that PIA produced by conjugate

DHQ-11 may be mediated through activation of $\hat{\alpha}$ -AR/AC/cAMP/PKA signaling pathway that leads to increased Ca^{2+} influx and rises in Ca^{2+} loading/release in the SR, which results in increased $[\text{Ca}^{2+}]_i$ and enhanced contraction force.

The vasorelaxant effect of the conjugate DHQ-11. In rat aortic rings precontracted with

Table 1. The participation of K-channels in vasorelaxation induced by DHQ-11

K-channel blockers	Vasorelaxation %	
	E_{\max}	IC_{50} (μM)
Control	97.7 \pm 1.3%**	11.7
Glibenclamide (50 μM)	38.7 \pm 3.5%*	19.1
TEA (1 mM)	59.3 \pm 3.5%*	17.2
4- $\hat{\text{A}}$ P (1 mM)	77.6 \pm 3.9%**	14.4
BaCl ₂ (0,1 mM)	90.9 \pm 3.4%**	12.3

The results are expressed as the percentage vasorelaxation of rat aortic rings contraction induced by 20 mM KCl. Data are presented as mean \pm SEM (* p <0,05, ** p <0,01; n =5-6).

Table 2. The involvement of endothelium in vasorelaxation induced by DHQ-11

K-channel blockers	Vasorelaxation %	
	E_{\max}	IC_{50} (μM)
Endothelium (+)	90.3 \pm 3.4**	23.7
Endothelium (-)	60.5 \pm 3.9**	28.7
L-NAME (100 μM)	65.8 \pm 3.2*	26.9
Methylene blue (10 μM)	69.8 \pm 3.3**	26.5
Indomethacin (10 μM)	86.4 \pm 3.3*	24.9

The results are expressed as the percentage vasorelaxation of rat aortic rings contraction induced by 1 μM phenylephrine. Data are presented as mean \pm SEM (* p <0,05, ** p <0,01; n =5-6).

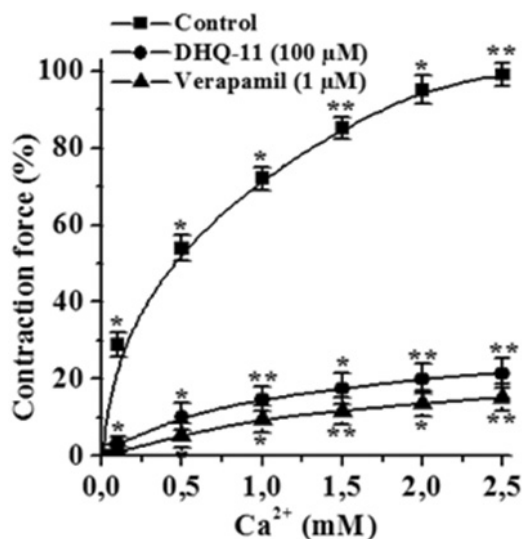


Fig. 5. Effect of DHQ-11 on Ca^{2+} - induced contraction in rat endothelium-intact aortic rings. The effect of DHQ-11 on extracellular Ca^{2+} influx was studied in aortic rings preincubated in a Ca^{2+} -free Krebs solution containing 50 mM KCl and followed by cumulative addition of CaCl_2 . The maximal contraction induced by 2.5 mM CaCl_2 was expressed as 100%. Data are presented as mean \pm SEM (n = 4-6). * p <0.05, ** p <0.01, as compared with the control

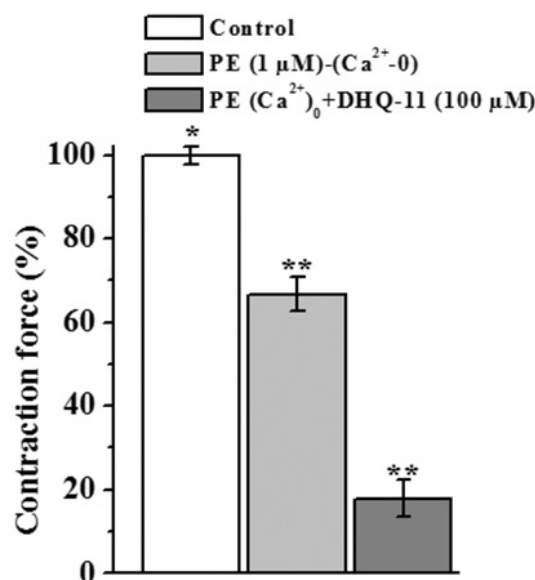


Fig. 6. Effects of DHQ-11 on rat aortic rings contraction induced by phenylephrine in Ca^{2+} - free Krebs solution. The contraction induced by 1 μM PHE in Ca^{2+} -free Krebs solutions contained 100 mM EGTA and 1 μM verapamil expressed as 100%. Data are presented as mean \pm SEM (n = 4-6). * p <0.05, ** p <0.01, as compared with the control.

high KCl (50 mM) and phenylephrine (PE), the conjugate DHQ-11 produced a significant vasorelaxant effect in a concentration-dependent manner. In these experiments, the conjugate DHQ-11 maximally reduced the KCl-induced contraction by $94.7 \pm 3.2\%$, of control, at a concentration of 100 μM (Fig 4, A). At the same concentration (100 μM) the conjugate DHQ-11 caused the maximal relaxant effect up to $90.3 \pm 3.4\%$ in rat aortic rings precontracted with PHE (1 μM) (Fig 4, B). Under similar experimental conditions, the flavonoid dihydroquercetin and alkaloid 1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline maximally reduced the KCl-induced contraction by $81.1 \pm 3.8\%$ and $89.5 \pm 3.1\%$, as well as, PE-induced contraction by $83.6 \pm 3.7\%$ and $75.2 \pm 3.2\%$ respectively (Fig.4, A, B).

The IC_{50} values (the concentration to produce a 50% maximal relaxant effect) for DHQ-11, dihydroquercetin, and alkaloid 1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline were 23.7 μM , 30.9 μM and, 41.6 μM , respectively. These data indicated that tested compounds produced a significant vasorelaxant effect and that the vasorelaxant potency of the conjugate DHQ-11 was markedly greater than that of flavonoid dihydroquercetin and alkaloid 1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline.

The contractility of smooth muscle cells (SMC) is dependent on $[\text{Ca}^{2+}]_i$, which is mainly regulated by Ca^{2+} influx from the extracellular space through L-type VDCCs and by Ca^{2+} release from the SR ²⁴ KCl-induced contraction of SMC is mainly related to the extracellular Ca^{2+} influx through L-type VDCCs activated by the SMC membrane depolarization²⁵ Therefore, to evaluate the role of the L-type VDCCs in the vasorelaxant action of conjugate DHQ-11 its effects on aortic rings contractions induced by the cumulative addition of Ca^{2+} in Ca^{2+} -free Krebs solution containing 50 mM KCl were studied. Fig.5 shows that preincubation of the aortic ring with conjugate DHQ-11 (100 μM), reduced the contractile response to 2.5 mM CaCl_2 by $78.5 \pm 32\%$ of the control obtained in a normal Krebs solution containing Ca^{2+} .

These results suggest that the vasorelaxant effect of conjugate DHQ-11 possible is related to inhibition of the Ca^{2+} influx via the L-type VDCCs. This is confirmed in experiments with

L-type VDCCs blocker verapamil that have shown that despite the great difference between the used concentrations, the percentage of inhibition of Ca^{2+} -induced contractions produced by verapamil was almost similar to that produced by conjugate DHQ-11 (data not shown). These results support the notion that the vasorelaxant effect of conjugate DHQ-11 is associated with the blockage of Ca^{2+} influx through L-type VDCCs.

In contrast to KCl, phenylephrine (PE), an α -adrenergic agonist, induced SMC contraction mainly by releasing intracellular Ca^{2+} from the SR via activation of inositol-1,4,5-trisphosphate (IP_3) receptors, To investigate the involvement of Ca^{2+} released from SR in the vasorelaxant action of conjugate DHQ-11 its effect on the PE-induced contractions of aortic rings in Ca^{2+} -free Krebs solutions containing 100 mM EGTA (100 μM) and verapamil (1 μM) was examined. As shown in Fig.6 in the presence of conjugate DHQ-11 (100 μM) the PE-induced (1 μM) contraction of aortic rings in Ca^{2+} -free Krebs solution reduced from control level $66.7 \pm 4.2\%$ to $17.9 \pm 4.3\%$. These results indicate that this effect of conjugate DHQ-11 is due to inhibition of Ca^{2+} release from SR through IP_3R , suggesting that this mechanism could be involved in its vasorelaxant action.

A critical role in the regulation of smooth muscle contractility plays a variety of the K⁺-channels, which as the dominant ionic conductance in the regulation of the membrane potential contribute to the modulation of L-type VDCCs activity and regulation of $[\text{Ca}^{2+}]_i$, as well as the Ca^{2+} release/loading in SR [26]. To evaluate the participation of K⁺ channels in the vasorelaxant action of conjugate DHQ-11, its effect in the presence of the K⁺ channels blockers 4-AP, TEA, BaCl_2 , and glibenclamide were studied. In these experiments, the endothelium-intact aortic rings were precontracted with KCl 20

mM to depolarize the SMC membrane and enhance K-channel activity. The results present in Table 1 show that the vasorelaxant effect of conjugate DHQ-11 was significantly attenuated by glibenclamide and TEA and partially by 4-AP. The greater potency of glibenclamide and TEA to inhibit the effect of conjugate DHQ-11 suggest that K_{ATP} and BK_{Ca} channels involved in its vasorelaxant action indicating that this effect of

conjugate DHQ-11 may be related to the activation of these channels.

A key role in the control of vascular tone plays endothelial cells through the production of a variety of relaxing and constricting factors that modulate the smooth muscle cells reactivity [27]. The nitric oxide (NO) produced from its precursor L-arginine by endothelial NO synthase (eNOS), is one of the major relaxing factors responsible for the endothelium-dependent dilatation in various vasculature [28].

To assess the role of the endothelium in the vasorelaxation produced by conjugate DHQ-11, its effects on aortic preparations with the removed endothelial layer were studied. In this study, was found that the removal of the endothelium significantly blunted the vasorelaxant effect of conjugate DHQ-11. As illustrated in Table 2 the vasorelaxant effect of conjugate DHQ-11 (100 μ M) in aortic rings precontracted with PE (1 μ M) significantly decreased from $90.3 \pm 3.4\%$ to $60.5 \pm 3.1\%$ after removal of the endothelium. The IC_{50} values for conjugate DHQ-11 obtained in aortic rings with and without endothelium were 23.7 μ M and 38.2 μ M, respectively. These results showed that there was a significant difference in the vasorelaxant potency of conjugate DHQ-11 in the aortic rings with and without endothelium indicating that the vasorelaxant effect of conjugate DHQ-11 is endothelium-dependent and may involve the NO/sGC/cGMP/PKG pathway.

To further examine the role of NO/sGC/cGMP/PKG pathway in the vasorelaxant action of conjugate DHQ-11 its effect on PE-induced contraction of endothelium-intact aortic rings preincubated with NOS inhibitor L-NAME and guanylate cyclase inhibitor methylene blue, as well as with indomethacin, a cyclooxygenase inhibitor were studied. The results presented in Table 2 showed that pretreatment of the intact aortic ring with L-NAME (100 μ M), significantly reduced the vasorelaxant effect of conjugate DHQ-11 from $90.3 \pm 3.4\%$ to $60.5 \pm 3.9\%$. Similarly, the pretreatment of the intact aortic ring with methylene blue (10 μ M) reduced the vasorelaxant effect of conjugate DHQ-11 to $69.8 \pm 3.3\%$ of control (Table 2).

From these data is evident that treatment of the aortic rings with L-NAME and methylene blue attenuated the vasorelaxant activity of

conjugate DHQ-11 to a comparable extent as did by the mechanical removal of the endothelium. In contrast, pretreatment of the aortic rings with indomethacin did not affect the vasorelaxant activity of conjugate DHQ-11. These results indicate that the activation of NO/sGC/cGMP/PKG pathway but not the prostaglandin signaling pathway is involved in vasorelaxation induced by conjugate DHQ-11. These results revealed that conjugate DHQ-11 may exert a marked vasorelaxant effect mediated by endothelium-dependent and -independent mechanisms. Taken together obtained results suggested that the endothelium-dependent mechanism is likely involved NO/sGC/cGMP/PKG pathway, while blockage of the L-type VDCCs and inhibiting intracellular Ca^{2+} release as well as activating K_{ATP} and BK_{Ca} channels, might contribute to the endothelium-independent mechanism.

CONCLUSION

We have been demonstrated that DHQ-11, a conjugate of flavonoid dihydroquercetin with isoquinoline alkaloid 1-aryl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline, exert pronounced positive inotropic and vasorelaxant effects mediated by multiple mechanisms. In the rat papillary muscle, the conjugate DHQ-11 increased the force of contraction in a concentration-dependent manner. The blockage of α -adrenoreceptor and L-type VDCCs with propranolol and nifedipine, respectively, significantly attenuated the conjugate DHQ-11-induced positive inotropic effect suggesting that it may activate the α -AR/AC/cAMP/PKA pathway and thus enhance the Ca^{2+} influx in cardiomyocytes through L-type VDCCs. Also, the conjugate DHQ-11 significantly increased the first contraction after rest intervals indicating that it enhanced the post-rest potentiation of force contraction. These findings suggest that the conjugate DHQ-11 may cause an additional accumulation of Ca^{2+} in the SR that leads to an increased Ca^{2+} release from the SR resulted in enhanced contraction force. This suggestion was supported by fact that nifedipine inhibits the positive inotropic effect produced by conjugate DHQ-11, presumably through suppression of Ca^{2+} influx via L-type VDCCs which play important role in refilling Ca^{2+} store. These results demonstrate

that positive inotropic effect produced by conjugate DHQ-11 mediated through activation of α -AR/AC/cAMP/PKA signaling pathway that leads to increased Ca^{2+} influx and rises in Ca^{2+} loading/release in the SR, resulting in increased $[\text{Ca}^{2+}]_i$ and enhanced contraction force.

The conjugate DHQ-11 significantly relaxed both high KCl- and phenylephrine-induced contractions of rat aortic rings in a concentration-dependent manner. The vasorelaxant effect of the conjugate DHQ-11 was significantly reduced by lowering extracellular Ca^{2+} concentration in both high KCl- and phenylephrine and the presence of verapamil. Also, the conjugate DHQ-11 significantly inhibited phenylephrine-induced contractions in a Ca^{2+} -free medium, indicating inhibition of Ca^{2+} release from the sarcoplasmic reticulum (SR). At the same time, the vasorelaxant effect of conjugate DHQ-11 was more potent in aortic rings precontracted with 20 mM KCl- than 50 mM KCl and significantly attenuated by glibenclamide and TEA. Furthermore, the vasorelaxant effect of the conjugate DHQ-11 was significantly reduced by the removal of endothelium and in the presence of L-NAME and methylene blue, an NO synthase and guanylate cyclase inhibitors.. indicating that it is endothelium-dependent and related to the stimulation of the NO/sGC/cGMP/PKG signaling pathway. These results suggest that the vasorelaxation produced by conjugate DHQ-11 may be mediated by an endothelium-independent mechanism involving activation of K_{ATP} and BK_{Ca} channels and inhibition of L-type VDCCs and Ca^{2+} release from the sarcoplasmic reticulum and endothelium-dependent mechanism through activation of the NO/sGC/cGMP/PKG signaling pathway resulting in a decrease of intracellular Ca^{2+} levels.

These observations reveal that the conjugate DHQ-11 due to its high positive inotropic and vasorelaxant activity could be a promising compound for the design and development of new drugs for the treatment of heart failure.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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