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VASCULAR REACTIVITY TO VASOACTIVE AGENTS FOLLOWING EXPOSURE TO KOLAVIRON IN ISOLATED AORTIC RINGS

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ABSTRACT

Effect of kolaviron (KV), a biflavanoid-complex of *Garcinia* kola fraction was studied on [histamine (HM), phenylephrine (PE)]-induced and/or (8 x 10^{-2} or 4 x 10^{-2})M K^+ contractile responses in ring preparations of rabbit aortae. Ring segments 2-5 mm were suspended in 20 ml organ bath containing normal physiological salt solution (PSS) and bubbled with 95% O_2, 5% CO_2 gas mixture for recording of isometric contractions at 37^0C and pH 7.4. Following precontractions with EC_{70} (10^{-5} HM; 10^{-7} PE)M and/or (8; 4 x 10^{-2})M K^+, KV(0.3-20.0µg/mL) was added cumulatively and responses evaluated in intact (+E) and endothelium denuded (-E) aortic rings. To elucidate further mode of action of KV, relaxant effects were also examined in rings exposed to methylene blue (MB) or ouabain (OB) as well as on extracellular Calcium-dependent contractions. Kolaviron caused concentration-depended relaxation of contractile responses in both +E and -E rings. Comparatively, KV-induced relaxant effect was significantly ($p<0.05$) greater in -E than in +E rings in both receptor- and non-mediated responses. Relaxant effect was significantly ($p<0.05$) more potent in HM-induced contraction than in PE- and/ or high K^+ depolarized rings. Also KV inhibited and decreased maximal extracellular Calcium-dependent contractions; whereas KV-relaxant effect was completely abolished in MB and OB incubated precontracted rings. From these results, kolaviron causes smooth muscle relaxation in arterial blood vessel of rabbit aorta in a concentration-dependent manner in non-specific mode of action. The mechanism of action may not be unconnected with guanylate cyclase enzyme-cGMP pathway, calcium supply blockade and Na+-K+ ATPase cellular activity.

Key words: kolaviron, vasorelaxation, histamine, phenylephrine, aortic rings.

INTRODUCTION

The Pharmacological properties and therapeutic effects of phytochemical components of *Garcinia* kola Heckel seed (GKola) have been characterized and outlined by several workers.[1,2,3,4,5] Kolaviron (KV)(fig.1), a biflavanoid-complex and major component of GKola-fraction has been widely reported to have a broad spectrum of medicinal values and therapeutic properties including high antioxidant and safety profile[6], cardioprotective, hepatoprotective[2,7], gastroprotetive[8,9], hypolipidemic, hypoglycaemic[5] anti-asthmatic[10] and anti-malaria[11] effects on experimental animal models.[7,9,12] The importance of flavonoids has been attributed to their actions as antioxidants, free radical scavengers, quencher of singlet and triplet oxygen and inhibitors of peroxidation reactions.[13] Also, flavonoids have been identified as one of the important physiological compounds that regulate and modulate the gene and activity expression of eNOS (one of the three distinct isoforms of NO synthase (NOS).[14] Regulation of vascular tone is essentially dependent upon the release of NO from eNOS. and NO dilates all types of blood vessels.[14] Additionally, structural and functional changes in the vasculature by host of physiological and non-physiological stimuli contribute to variations in vasoconstrictor and dilator responses which may alter regional blood flow. Recently, administration of kolaviron and sulfasalazine ameliorated Dextran-Sulphate-Sodium-induced colitis in rats by increasing antioxidant status, decreased hydrogen peroxide and lipid peroxidation levels.[15] However, there is paucity of information on the effects of Kolaviron on vascular reactivity and the possible mechanisms of action on vascular smooth muscle cells have not been fully characterised. The present study was designed to evaluate the effects of kolaviron on vascular reactivity of vascular smooth muscle of isolated arterial blood vessel of rabbit aorta following histamine (HM), Phenylephrine (PE)- and/or high-K^+ -induced contractions

MATERIALS AND METHODS

Tissue Preparation: Matured New Zealand male and female rabbits were used throughout the experiment. All experimental procedures complied with the standard protocols for the use of laboratory animals (National Institute of Health USA, 2002). The study was approved by the Institutional ethical committee on the use of animals for experiments. The rabbits were sacrificed by cervical dislocation. The thoracic aorta was carefully dissected out and quickly removed, placed in Petri dish containing physiological salt solution and freed of connective tissues, cut into 2-5 mm ring segments and suspended between two L-shaped fine stainless steel holders in a 20 ml jacketed organ baths containing physiological salt solution (PSS) of the following composition (mM/L): NaCl 119.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, $CaCl_2$ 1.6, and glucose 11.5. The medium was bubbled continuously with 95% O_2 and 5% CO_2 gas mixture and maintained thermostatically at 37°C and pH7.4. Isometric contractions were recorded with force displacement transducers FT.03 connected to Grass model 7D polygraph (Grass instrument Co, Quincy, MA, USA) under an initial load of 1g. An equilibration period of 90 minutes was allowed; following this, aortic rings were stimulated twice with 8 x 10^{-2}M K^+ PSS, at 20-minute interval. The average of these contractions represented the maximum (100%) agonist/KCl which subsequent contractions to phenylephrine or histamine were evaluated.

Protocols.

Concentration-response tests to agonists: Cumulative concentration-response tests (1x10^{-9} to 2.5 x10^{-4}) to each of the 2 agonists (PE and HIST) were examined in normal PSS (control) (n = 8). Concentration-response curves were constructed from which EC_{70}M PE and HM were graphically determined.

Vascular effect of kolaviron. Aortic rings were each precontracted with 10^{-5}M HM; 10^{-7}M PE and/or high-K^+ (8 ; 4 x 10^{-2}M). When the contractions were stable, KV (0.3, 0.6, 1.3, 2.5, 5.0, 10.0, 20.0)µg/mL was added cumulatively and the effects examined. The resultant relaxation responses were determined and expressed as percentage (%) of the initial tension.

Role of endothelium: The role of vascular endothelium in vascular reactivity by KV was studied in intact (+E) and endothelium denuded (-E) HM; PE or high-K^+ precontracted arterial rings to elucidate the mode of action. Endothelium removal was effected mechanically[16] by gently rubbing the inner lining surface of the rings with a pair of forceps.[17] The effectiveness of de-endothelisation was confirmed by lack of relaxation response to 10^{-5}M Acetylcholine (Ach) and more than 70% relation in phenylephrine - precontracted endothelium-denuded and intact arterial rings respectively.[17,18]

Effects of exposure to methylene blue and ouabain

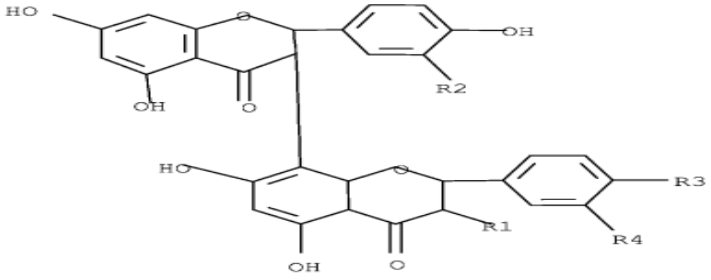
To further elucidate the possible mechanism of the relaxant effects of kolaviron, the authors took advantage of previous reports that haemoglobin and methylene blue were inhibitors of guanylate cyclase activation by nitric oxide.[19] Following 20 minutes exposure to 10^{-6}M methylene blue or 10^{-5}M ouabain, +E and -E rings were precontracted with (EC_{70})M (10^{-5}M HM; 10^{-7}M PE) and at stable contractions, KV was cumulatively added to bath and vasorelaxant effects examined.

Effect of KV on calcium (Ca^{2+}). In the experiment to study the effect of KV on extracellular calcium-dependent contractions, arterial rings were first depleted of Ca^{2+} for 30 minutes (15 min nominal Ca^{2+}-free and 15 min period of exposure to a Ca^{2+}-free) 40 mM K^+ depolarizing solution.[20] Ca^{2+} as ($CaCl_2$) was then added cumulatively and concentration -response curve was then constructed.[18,20] In test arterial ring KV was applied 10 minutes before the end of Ca^{2+} -free exposure and maintained throughout the duration of the protocol.

Drugs and solution: Ca^{2+}-free PSS was prepared by omitting $CaCl_2$ from the normal PSS. In some experiments, Ca^{2+}-free PSS contained 1.0mmol/L EGTA. Potassium free as well as high-K^+ PSS were prepared by equimolar replacement of KCl by NaCl, or NaCl by KCl. Drugs used were: Acetylcholine Hydrochloride (Sigma, UK), histamine hydrochloride (HIST) (Sigma USA), L.phenylephrine hydrochloride (PE) (Sigma USA), ethyleneglycol bis(β-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA)(Sigma Chemical Co; Saint Louis, MO, USA) tween-80, ouabain and methylene blue. Chemicals used for PSS are: calcium chloride, glucose, magnesium sulphate, potassium dihydrogen phosphate, sodium bicarbonate and sodium chloride (Sigma Chemical Co; Saint Louis, MO, USA). The solutions were prepared fresh on the day of experiments.

Statistical analysis: Data are presented as means ± SEM. Graphs and statistical analyses were by means of OriginPro 8.0 software and Students t-test. P-values less than 0.05 ($p<0.05$) was considered significant; while n-values denote number of animals from which blood vessels were obtained. EC_{70} (concentration producing 70% of maximal contraction) and IC_{50} (concentration producing 50% relaxation) values were derived graphically.

RESULTS AND DISCUSSION



	R1	R2	R3	R4
GB1	OH	H	OH	H
GB2	OH	H	OH	OH
Kolaflavanone	OH	H	OMe	OH

Fig.1 Structure of kolaviron

Table1. Maximal contraction (E_{max})

Agent	Maximal contraction (g)	Maximal relaxation (%)	IC_{50} (M)
10^{-7}M PE	+E 1.73 ± 0.15 -E 1.82 ± 0.36	21.00 ± 2.26 30.07 ± 5.97	0.28 ± 0.04* 0.24 ± 0.08*
10^{-5}M HM	+E 0.83 ± 0.69 -E 0.85 ± 0.45	48.23 ± 2.47 44.89 ± 3.57	0.19 ± 0.03* 0.31 ± 0.07*
40 mM K	-E 1.07 ± 0.33 +E 1,23 ± 0.68	46.80 ± 2.4 35.17 ± 1.99	0.31 ± 0.06* 0.18 ± 0.08*
80 mMK+	+E 1.83 ± 0.33 -E 1.92 ± 0. 14	21.15 ± 2.01 32.40 ± 5.15	0.27 ± 0.09* 0.25 ± 0.09*

values for EC_{70}% 10^{-7}M (PE; HM) and high-K^+ and maximal relaxation response (%), as well as IC_{50} M (concentration producing 50% relaxant effect values for KV-induced relaxation.

The maximal relaxation and IC_{50} values (Table 1) for kolaviron-induced relaxation following PE or high-K^+ (80 mM) contractions in intact (+E) was not significantly different, whereas in endothelium-denuded (-E) arterial rings, KV-induced relaxation was significantly ($p<0.05$) different. HM E_{max} was significantly ($p<0.05$) different from PE and high K^+-induced contractions.

Relaxation responses induced by kolaviron following receptor and non-receptor-mediated precontractions in +E and /or -E aortic rings.

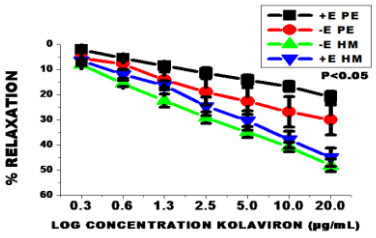


Fig.2

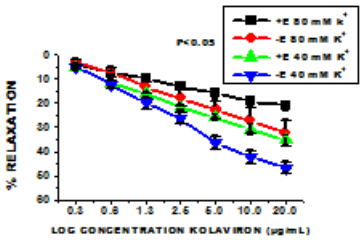


Fig.3.

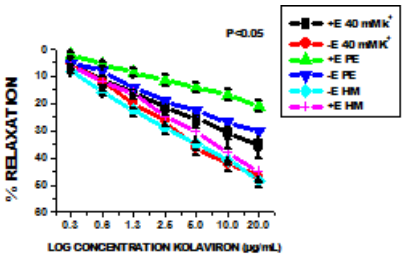


Fig.4

In Figures 2,3 and 4, kolaviron caused relaxation of contractile responses to receptor-mediated (phenylephrine, histamine) and non-receptor-mediated (high-K^+)-induced contractions in concentration-dependent manner in both intact (+E) and endothelium denuded (-E) aortic rings. The magnitude of KV-induced relaxation in precontracted rings was in the order: HM > 40 mM K^+ > PE>80 mMK^+. N = 6; means, ± SEM. Comparatively, KV-induced vasorelaxant effect was greater in -E than in +E rings; suggesting that KV-induced relaxant effect is less sensitive in +E than in -E rabbit aortic rings. The respective IC_{50} values in the absence and presence of endothelium were significantly different ($p<0.05$): PE (0.24 ± 0.08*, 0.28 ± 0.04*), HM (0.19 ± 0.03* , 0.31 ± 0.07* and high-K^+ (0.27 ± 0.09*, 0.25 ± 0.09*). Whereas KV-induced relaxation is comparable in both receptor- and non-receptor-mediated contractions (Table-1).

Role of endothelium.

In other experiments to elucidate further mechanisms of KV-induced inhibition of contractile responses in isolated aortic arteries, the influence of vascular endothelium on KV-induced relaxation was studied

following 20 minutes exposure to methylene blue and ouabain solutions.

Responses to KV in precontracted +E or -E rings incubated in methylene blue (MB) or ouabain (OB).

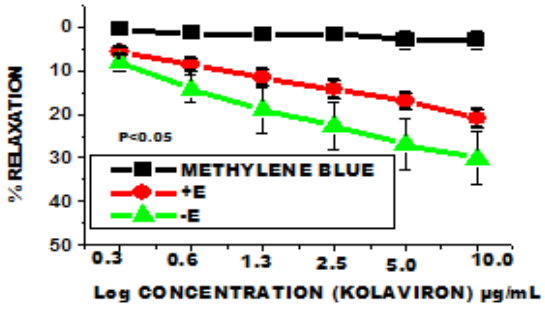


Fig.5 (PE)

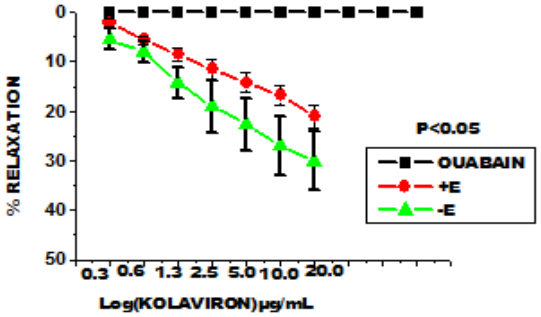


Fig.6

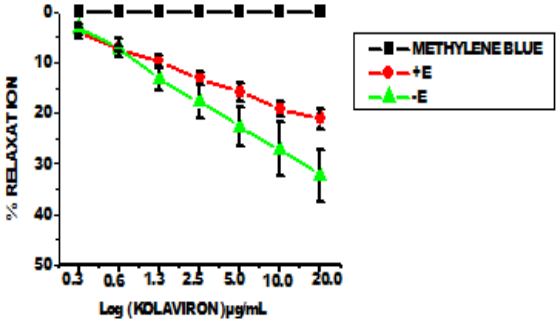


Fig.7.(High K^+)

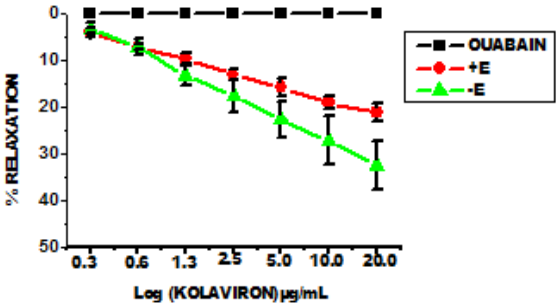


Fig.8

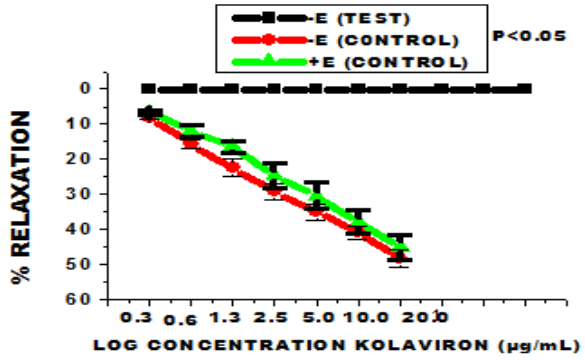


Fig.9. HM

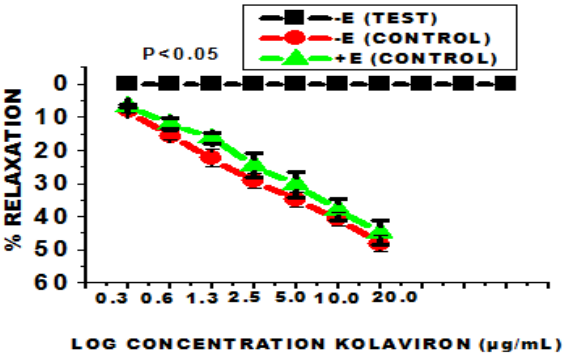


Fig.10

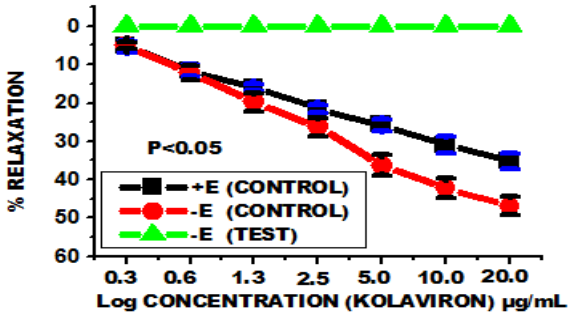


Fig.11.(HM)

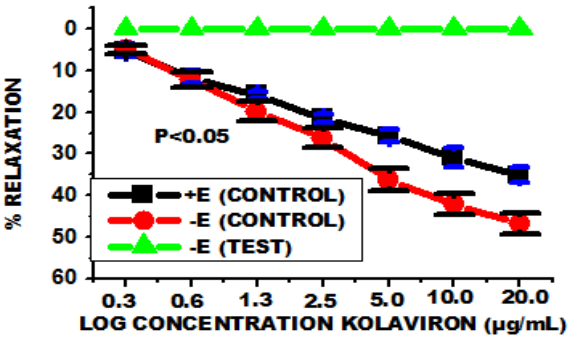


Fig.12.

Figures 5-12. KV-induced relaxation to PE, high-K^+ or HM- precontracted +E or –E aortic rings, following 20 minutes exposure to 10^{-6} M methylene blue or 10^{-5}M ouabain; (n = 6); means ± SEM. KV-induced relaxation was completely abolished in rings exposed to both methylene blue or ouabain solutions suggesting KV interference with soluble guanylate cyclase and Na-K^+ ATPase pump activities.

Effect of Calcium on kolaviron (KV) Reactivity

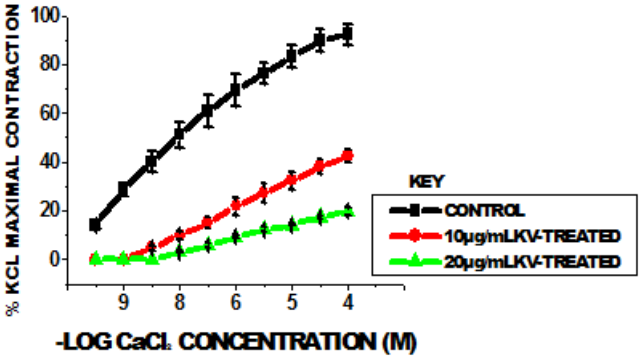


Fig. 13.

Figure 13 illustrates typical observations on the effect of kolaviron on extracellular Calcium-dependent contractions. Kolaviron (● 10 µg/mL and ▲20 µg/mL) produced a parallel right ward shift of the concentration-curves and inhibited and decreased maximal extracellular Calcium-dependent contractions of 40 mM K^+ depolarized aortic rings; suggesting calcium blockade-interference by kolaviron in relaxation of blood vessel. N=6; means ± SEM.

DICUSSION

The effects of kolaviron, a biflavanoid complex of ***Garcinia*** kola-fraction on vascular reactivity have been studied in vitro on arterial blood vessel of rabbit thoracic aorta. The protocols employed involved three contractile agents: phenylephrine, histamine (an α_1-adrenergic- and H_1-receptor agonists respectively) and high-K^+; and evaluation of endothelial function as well as effect of kolaviron on extracellular Calcium-dependent contractions. From our data, we clearly identified and demonstrated that kolaviron caused relaxation of contractile responses to receptor-mediated (phenylephrine, histamine) and non-receptor-mediated (high-K^+)-induced contractions in concentration-dependent manner in both intact (+E) and endothelium denuded (-E) aortic rings. KV-induced relaxation was however significantly greater in -E than in +E arterial rings and also greater in 4 x10^{-2} M K^+ compared to 8 x 10^{-2} M high-K^+, Table 1 and figures 2,3 & 4). The observation that KV caused vasorelaxation in both +E and -E aortic arterial rings suggest that the mechanism of KV-induced relaxation is non-endothelium-dependent specific. Whereas the greater relaxant effect observed in -E than in +E arterial rings although tensions developed by arterial rings were relatively higher in denuded than intact endothelial rings suggest an increased sensitivity of Kolaviron mode of action to de-endothelized vascular tissues or increased responsiveness of Kolaviron mode of action to soluble guanylate cyclase and /or Na/K+ ATPase related cellular activity. Vascular endothelium has been reported to modulate smooth muscle cells reactivity.[16,21,22] The vascular endothelium synthesizes and releases a broad spectrum of vasoactive substances and plays a fundamental role in the regulation and maintenance of cardiovascular homeostasis.[14] Among the major endothelial-derived factors that result in relaxation of arterial smooth muscle are NO, PGI_2 and the EDH mechanisms, which are associated with Ca^{2+}-activated K^+ channels activation. However, greater percentage of the endothelium-dependent relaxation is reported to occur through the NO/cGMP pathway.[14] In general, the magnitude of relaxation of vascular smooth muscle in response to a vasodilator agent is related to the force of contraction prior to addition of the vasodilator.[23,24] In the present study, there was no significant different in phenylephrine and high-K^+ *Emax* consequently, the differential effects of kolaviron is unrelated to the level of precontractions except in histamine-induced precontractions. Therefore, the greater relaxation effect of kolaviron to histamine-induced contraction compared to PE and high-K^+-mediated contractions could be related to the differential Emax (figures 2.3.& 4).

This study also reports that kolaviron-induced relaxation of VSM of rabbit aorta in both receptor-mediated and non-receptor-mediated contractions. Contractile agents employed in this study elicit VSM contraction via increased intracellular Ca^{2+}-concentration by mobilizing intracellular calcium pools as well as stimulating Ca^{2+} influx through receptor operated Ca^{2+} channels (ROCCs) and voltage-operated Ca^{2+} channels (VOCCs).[25,26] Therefore, the observation that KV caused relaxation of phenylephrine, histamine-evoked contractions and high-K^+ depolarization suggest a non-specific interference with receptor-and non-receptor-mediated responses.

In view of our observation of the non-specific endothelial mode of action in kolaviron relaxant effects on rabbit aortic rings, and in an attempt to characterised the mode of kolaviron effect on vascular reactivity, we took advantage of previous reports that haemoglobin and methylene blue were inhibitors of guanylate cyclase activation by nitric oxide[19,27], that suppresses the EDNO-induced relaxation by decreasing the production of cellular cGMP[28] and ouabain (inhibitor of sodium-potassium ATPase activity).[29] +E and -E arterial rings incubated in methylene blue and ouabain in separate bath

solutions, completely abolished kolaviron-induced relaxation of phenylephrine, histamine and high-K^+ precontractions (Figures 5-12); suggesting a guanylate cyclase cGMP-pathway and Na/K+-ATPase cellular activity involvement in kolaviron-induced relaxation. Methylene blue by definition permeates cell membranes and not only inhibits vascular smooth muscle relaxation elicited by all the nitric oxide-forming or nitric oxide-releasing vasodilator drugs but also their stimulatory effects on vascular cyclic GMP accumulation[27] without influencing relaxation elicited by other drugs such as certain prostaglandins, and calcium antagonists.[19] The Na-K ATPase enzyme controls the activity of vascular membranal Na^+-K^+ pump, which plays a major role in the regulation of peripheral vascular resistance.[30] And a functional indicator of Na-K ATPase activity.[31]

Figure 13 permits the assessment of extracellular Ca-dependent contraction.[32] The parallel right ward shift of $CaCl_2$-concentration curves and the significant decrease in magnitudes of maximal calcium-induced contractions by kolaviron clearly demonstrated calcium blockade-related kolaviron mode of action in relaxation of VSMs of rabbit aorta.

In conclusion, the present study showed that kolaviron elicits relaxation of rabbit arterial blood vessel of thoracic aorta concentration-dependently. The mechanism of kolaviron-induced vasorelaxant effect involves non-specific interference with receptor-and non-receptor-mediated responses and endothelium non-specific. But may be associated with Ca^{2+}-supply interference as well as activation of soluble guanylate cyclase cGMP-pathway and/or Na/K+ ATPase cellular activities. Interestingly, the action of kolaviron was more effective in relaxing endothelium denuded arterial rings compared to intact endothelial rings.

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