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ORIGINAL ARTICLE

In vitro Investigation Effects of 4-Hydroxyacetophenone on Rat Thoracic Aorta's Vasomotor Activity

Volkan Gelen, Fikret Çelebi[1]

Department of Physiology, Faculty of Veterinary, Kafkas University, Kars, [1]Department of Physiology, Faculty of Veterinary, Atatürk University, Erzurum, Turkey

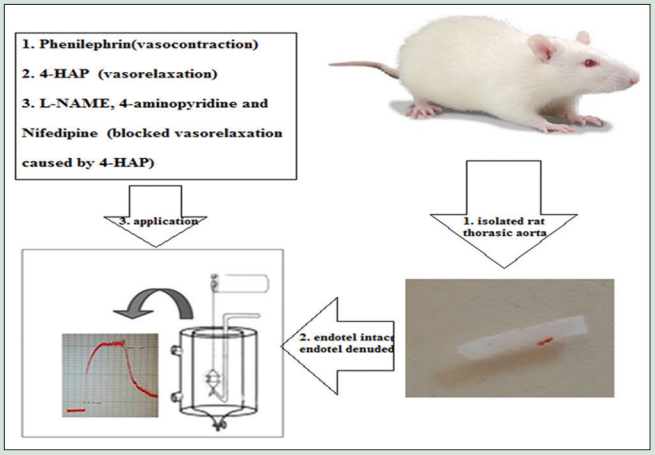
ABSTRACT

Background: *Z. clinopodioides Lam.* is also known as a "Field Mint" Seven compounds which have vasodilator activity have been isolated from Z. clinopodioides Lam. and two of them are phenolics compounds and these are acetovanillone, 4-HAP, four of them are flavonoids and these are acacetin, apigenin, chrysin, thymonin, one derivat of cinnamic acid and ethyl 4-coumarate. **Objective:** In this study, it was aimed that was defined vasodilator activiy mechanisms of fenolic compound 4-HAP on isolated rat thorasic aorta. **Material and Method:** In this study 40 male adult Sprague Dawley rats were used. Prepared rings were laid out into the 20 ml organ bath with Krebs solution. Rings were stretched by 1g and they were subjected to 1 hour incubation period. In the end of the incubation period, PE, KCl, nifedipine, L-NAME, 4-HAP, SQ22.536, ODQ, ACh, SKF96365, Propranolol, Atropin, TEA, Gibenclamide, 4-aminopyridine and U73122 were implemented to bath with a protocol. **Results:** Mechanisms of relaxed effect the of 4-HAP were assigned by using antagonists. It was observed that vasorelaksan effect of 4-HAP on endothelilal aorta smooth muscle contractions which had been inductioned by PE under the existance of L-NAME was considerably inhibited. **Conclusion:** It was stated that 4-HAP relaxed PE and KCl contractions and owing to this activity endothel intact tissues on L-NAME existance notably decreased because of NO pathyway. It is firmly believed that as relaxed effect of 4-HAP declines remarkably under the existance of 4-aminopyridine and nifedipine on endothel denuded aorta rings, activity could be on K^+ channel and L-type Ca^{+2} channel.

Key words: 4-hydroxyacetophenone, aorta, PE, potassium chloride, rat

SUMMARY

- This is the first study that reveals significant vasorelaxation effect induced by 4-HAP. Vasorelaxation maybe one of the possible mechanisms for its ability to reduce blood pressure.



Abbreviations Used: 4-HAP: 4-hydroxyacetophenone, SQ22536: 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine, 9-THF-Ade, L-NAME: N (omega)-nitro-L-arginine methyl ester, DMSO: Dimethyl sulfoxide, 4-AP: 4-aminopyridine, TEA: Tetraethylammonium, ODQ: 1H-(1,2,4) oxadiazolo(4,3-a)quinoxalin-1-one, SKF96365: 1-[β-(3-(4-Methoxyphenyl) propoxy)-4-methoxyphenethyl] 1H-imidazolehydrochloride, 1-[2-(4-Methoxyphenyl)-2-[3-propoxy]ethyl]imidazole, U73122: 1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione, ACh: Acetylcholine, KCl: Potassium chloride, IP3: Inositol triphosphate, DAG: Diacylglycerol.

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Correspondence:
Dr. Volkan Gelen,
Department of Physiology, Faculty of Veterinary,
Kafkas University, Kars, Turkey.
E-mail: gelen_volkan@hotmail.com
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INTRODUCTİON

Improved methods in the treatment of diseases are extremely important for human health, related for instance to cardiovascular diseases, cancer, and diabetes. Thus, ways to utilize the compounds of medical plants to find new treatment methods are currently being extensively investigated. One plant investigated for these purposes is *Ziziphora clinopodioides Lam.* a member of the laminae family. *Z. clinopodioides Lam.* is a green-or-gray colored 10–15 cm long perennial aromatic plant species. This plant, which also grows in Iran and Azerbaijan, is widely found in Turkey, especially in the Mediterranean region and Western, Central, and Eastern Anatolia.[1] It is widely used by Iranian and Turkish people as a traditional treatment of colds, gastrointestinal disorders, and inflammation.[2] When agar is added to the ethanol extract obtained from the leaves of this plant, it has been determined that antibacterial activities result on many Gram-positive and Gram-negative bacteria.[3-5] It has been reported that the plant's antioxidant activity is due to an oxidant-scavenging effect. One study also reported that it revealed antifungal activity on fungi such as *Aspergillus niger, Trichophyton rubrum, Trichoderma reesei,* and *Microsporum gypseum*, and another revealed anti-inflammatory activity in an acetic acid-induced colitis model in mice.[6,7] Traditional cigarettes are used by the Uyghur and Kazakh people in the treatment of diseases such as hypertension, fever,

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edema, heart diseases, insomnia, tracheitis, pulmonary insufficiency, and hemorrhoids. Studies have determined that a number of compounds such as pulegone, cis-isopulegone, cineol, thymol, α-pinene and β-pinene, piperitenone, apigenin, acacetin, 4-hydroxyacetophenone (4-HAP), ethyl-4 coumarate, thymonin, cinnamic acid, and chrysin. In another study, these compounds were identified as flavonoids such as acacetin, apigenin, chrysin, and thymonin and as phenolic compounds such as acetovanillone and 4-HAP and reported vasodilator activities on the isolated rat thoracic aortic smooth muscle by ethyl 4-coumarate and a cinnamic acid derivative.[8,9] However, the study did not examine how and by which molecular mechanisms their effects were produced or through which physiological functions. The aim of this study was to determine the effects of 4-HAP, one of the phenolic compounds found in *Z. clinopodioides Lam*. on the rat thoracic aorta *in vitro*, and to determine the pathways of this mechanism of action.

MATERIALS AND METODS

Chemicals and drugs

4-HAP, glibenclamide, SQ22536, propranolol, N (omega)-nitro-L-arginine methyl ester (L-NAME), dimethyl sulfoxide, 4-aminopyridine (4-AP), tetraethylammonium (TEA), 1H-(1,2,4) oxadiazolo(4,3-a)quinoxalin-1-one (ODQ), Atropine, SKF96365, U73122, Phenylephrine, Indomethacine, acetylcholine (ACh), potassium chloride (KCl), Nifedipine, NaCl, KCl, KH_2PO_4, $NaHCO_3$, $MgSO_4$, $CaCl_2$, and glucose were purchased from Sigma.

Animals

Male Sprague–Dawley rats (200–250 g) were obtained from the Laboratory Animal Unit, Atatürk University, Erzurum, Turkey. All the animals were housed under a 12:12-h light–dark cycle condition with a steady temperature maintained (24°C ± 1°C). The animals were allowed free access to rodent diet and tap water. The experimental protocol was approved by the Animal Ethics Committee in accordance with the guidelines for the care and the use of laboratory animals, as prepared by Atatürk University.

Preparation of isolated rat thoracic aortic rings

Rats were anesthetized, after they attained complete unconsciousness, the rats were sacrificed, and the thoracic aorta was immediately removed to be cleaned of the connective tissue and fat. The vessels were cut into rings approximately 3–4 mm in length. For the endothelium-denuded rings, the endothelial layer was removed by gently rubbing the internal surface of the vascular lumen. The aortic rings were immersed in a 10-ml chamber bath which contained Krebs solution (composition, mM: MM: NaCl 119, KCl 4.75, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 1.5, $CaCl_2$ 2,5, glucose11, and pH 7.3), maintained at a 37°C temperature, mounted on steel, and continuously bubbled with O_2 (95%) and CO_2 (5%). A resting tension of 1 g was applied to each tissue and equilibrated at least 1 h. During the equilibrium period, the Krebs solution was changed every 15 min. After equilibration, the endothelial integrity was verified with a submaximal contraction of pheniephrine (PE) (10^{-7} M). After the tension was stabilized, ACh (1 μM) was directly added into the chamber bath to detect and evaluate the presence or absence of the endothelial cell layer, more than 80% relaxation of the rings was considered to be an endothelium-intact ring, and whereas <10% relaxation was considered to be endothelium-denuded ring. Relaxation was calculated as a percentage of the maximal contraction induced by PE. Before each experimental protocol, the presence or absence of the endothelial cell layer was tested and washed out with the Krebs solution for at least 30 min. Changes in tension were detected using isometric force transducers (ELJ-S045C-EMKA-R04003 ve R04004). At the end of the incubation period, agonists, antagonists, and 4-HAP treatments were evaluated as active substances. Maximal contraction responses of the aortic smooth muscle rings to the agonists in grams were accepted as 100% (reference values). In the presence and absence of antagonist in the bath using 4-HAP relaxant responses were calculated according to the reference value %.

Experimental protocol

Effects of 4-hydroxyacetophenone on PE-induced and potassium chloride-induced contractions in endothelium-intact and endothelium-denuded rat thoracic aortic rings

The vasorelaxant effects of 4-HAP were investigated in both endothelium-intact and endothelium-denuded aortic rings. After the rings were equilibrated, they were contracted with PE (10^{-7} M) and KCI (40 mM/ml) until a stable tension was established. This was followed by cumulative exposure to 4-HAP (10^{-6}–10^{-2} M) [Figure 1].

Effect of β-adrenergic and muscarinic receptor in 4-hydroxyacetophenone-induced relaxation

The roles of the β-adrenergic and muscarinic receptors were obtained by the vasorelaxation response on preincubating the endothelium-intact aortic rings with one of the following specific antagonists of the β-adrenergic receptor-propranolol (1 μM) and the antagonist of muscarinic receptor atropine (1 μM)-for 20 min before PE (10^{-7} M) contraction. Then, 4-HAP (10^{-6}–10^{-2}M) was added cumulatively.

Effect of N (omega)-nitro-L-arginine methyl ester, 1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-one, SQ 22536, and indomethacin in 4-hydroxyacetophenone-induced relaxation

The roles of L-NAME, ODQ, SQ 22536, and indomethacin were elucidated through the vasorelaxation response on preincubating the endothelium-intact aortic rings with one of the following-L-NAME (10 μM) (inhibitor of nitric oxide synthase [NOS]), ODQ (1 μM) (inhibitor of guanylyl cyclase), SQ 22536 (100 μM) (inhibitor of adenylyl cyclase), and indomethacin (10 μM) (inhibitor of prostacyclin enzyme)-for 20 min before PE (10^{-7} M) precontraction. Then, 4-HAP (10^{-6}-10^{-2}M) was added cumulatively to the bath.

Effect of K^+ channels in 4-hydroxyacetophenone-induced relaxation

The role of the K^+ channels was elucidated through the vasorelaxation response upon preincubating the endothelium-denuded aortic

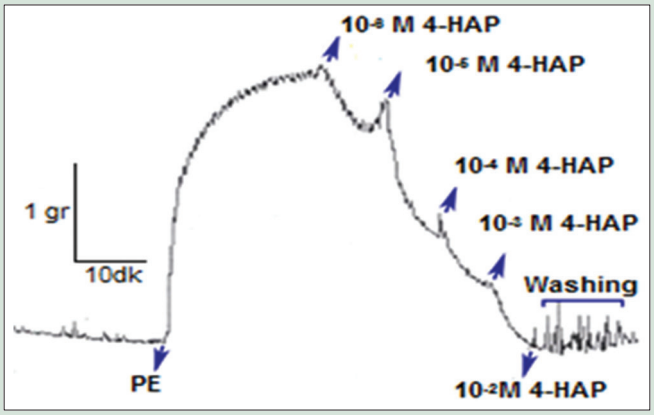

Figure 1: Vasodilatator effects of 4-hydroxyacetophenone on endothelium-intact rat thoracic aorta-induced contractions with PE

rings with one of the following specific K^+ channel blockers-TEA (1 mM), 4-AP (1 mM), and glibenclamide (10 μM)-for 20 min before PE (10^{-7} M) precontraction. Then 4-HAP (10^{-6}–10^{-2} M) was added cumulatively.

Effect of Ca^{+2} channels in 4-hydroxyacetophenone-induced relaxation

The role of the Ca^{2+} channels was elucidated through the vasorelaxation response on preincubating the endothelium-denuded aortic rings with one of the following specific Ca^{2+} channel blockers-nifedipine (10^{-4} M) (antagonist of the L-type Ca^{+2} channel), SKF96365 (10 μM) (inhibitor of the store-mediated Ca^{+2} channel), and U73122 (10 μM) (inhibitor of phospholipase C)-for 20 min before PE (10^{-7} M) contraction. Then, 4-HAP (10^{-6}–10^{-2}M) was added cumulatively.

Statistical analysis

All values are expressed as means ± standard errors. Data were analyzed using a one-way analysis of variance. Tests were performed using SPSS 20.00 system (SPSS_COMMUTE_MAX_LIFE), the value of $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Effect of 4-hydroxyacetophenone on PE-induced tonic contractions in endothelium-intact and endothelium-denuded rat thoracic aortic rings

To relax PE-induced endothelial rat thoracic aorta smooth muscle contractions, certain doses of 4-HAP were used as follows: 10^{-6} M (31.48 ± 14.88%), 10^{-5} M (47.37 ± 28.60%), 10^{-4} M (68.86 ± 25.44%), 10^{-3} M (90.45 ± 16.36%), and 10^{-2} M (112.61 ± 7.93%) [Figure 2a] ($P < 0.05$, $n = 6$). To relax PE-induced endothelium-denuded rat thoracic aorta smooth muscle contractions, the following doses of 4-HAP were used: 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} M doses of 4-HAP were relaxed 10^{-7} M, respectively, 9.71 ± 3.18%, 14.70 ± 5.29%, 32.64 ± 10.81%, 95.35 ± 3.52%, and 106.20 ± 3.27% [Figure 2b] ($P < 0.05$, $n = 6$).

Effect of β-adrenergic and muscarinic-receptor in 4-hydroxyacetophenone-induced relaxation

It was determined that all doses of 4-HAP in the PE-induced endothelium-intact *in vitro* rat thoracic aortic smooth muscle were not inhibited by atropine (1 μM) or propranolol (1 μM) on vasorelaxation [Figure 3b and c] ($P > 0.05$).

Effect of N (omega)-nitro-L-arginine methyl ester, 1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-one, SQ 22.536, and indomethacin in 4-hydroxyacetophenone-induced relaxation

Doses of 10^{-6} M, 10^{-5} M, and 10^{-4} M of 4-HAP in PE-induced contractions in endothelium-intact aortic smooth muscle were significantly inhibited in the presence of the NOS inhibitor L-NAME (10 μM) on vasorelaxation activity [Figure 3a] ($P < 0.05$). However, it was determined that the vasodilator activity of 4-HAP did not change in the presence of indomethacin (10 μM), the cyclooxygenase inhibitor, adenylyl cyclase enzyme inhibitor, SQ 22.536 (100 μM), or the guanylyl cyclase enzyme inhibitor ODQ (1 μM) [Figure 3 d-f].

Effect of K^+ channels in 4-hydroxyacetophenone-induced relaxation

In a further group of endothelium-denuded aorta rings, various K^+ channel blockers were used to test the involvement of K^+ channels. Pretreatment was done with the nonselective K^+ channel inhibitor TEA (1 mM), the voltage-activated K^+ channel inhibitor 4-AP (1 mM), or the ATP-sensitive K^+ channel inhibitor glibenclamide (10 μM). When compared to the control vasodilator activity, the 10^{-6} M, 10^{-5} M, and 10^{-3} M doses of 4-HAP decreased significantly in the presence of 4-AP [Figure 4 a-c] ($P < 0.05$).

Effect of Ca^{+2} channels in 4-hydroxyacetophenone-induced relaxation

In the endothelium-denuded aorta rings, various Ca^{+2} channel blockers were used to test the involvement of Ca^{+2} channels. As shown in Figure 4d, pretreatment was done with the L-type Ca^{+2} channel inhibitor nifedipine (1 mM), U73122 (10 μM) (phospholipase C enzyme inhibitor), and SKF96365 (10 μM) (store Ca^{+2} channel antagonist) [Figure 4e and f]. When compared to the control vasodilator activity, the 10^{-6} M, 10^{-5} M, and 10^{-3} M doses of 4-HAP decreased significantly in the presence of nifedipine ($P < 0.05$).

DISCUSSION

Z. clinopodioides Lam. is a type of plant that is widely used in the treatment of ailments such as fever, edema, heart disease, insomnia, tracheitis, lung abscess, and hemorrhoids. Some studies have determined that this plant, from the *Ziziphora* family, contains components such as essential oils,

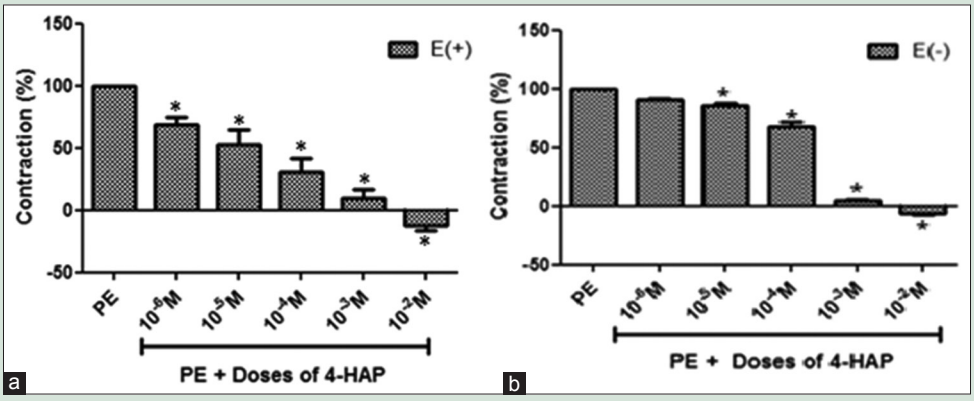

Figure 2: Relaxing effect of different doses of 4-hydroxyacetophenone on 10^{-7} M PE-induced *in vitro* rat endothelium-intact (a) and endothelium-denuded (b) aortic smooth muscle contractility (* = $P < 0.05$, $n = 6$)

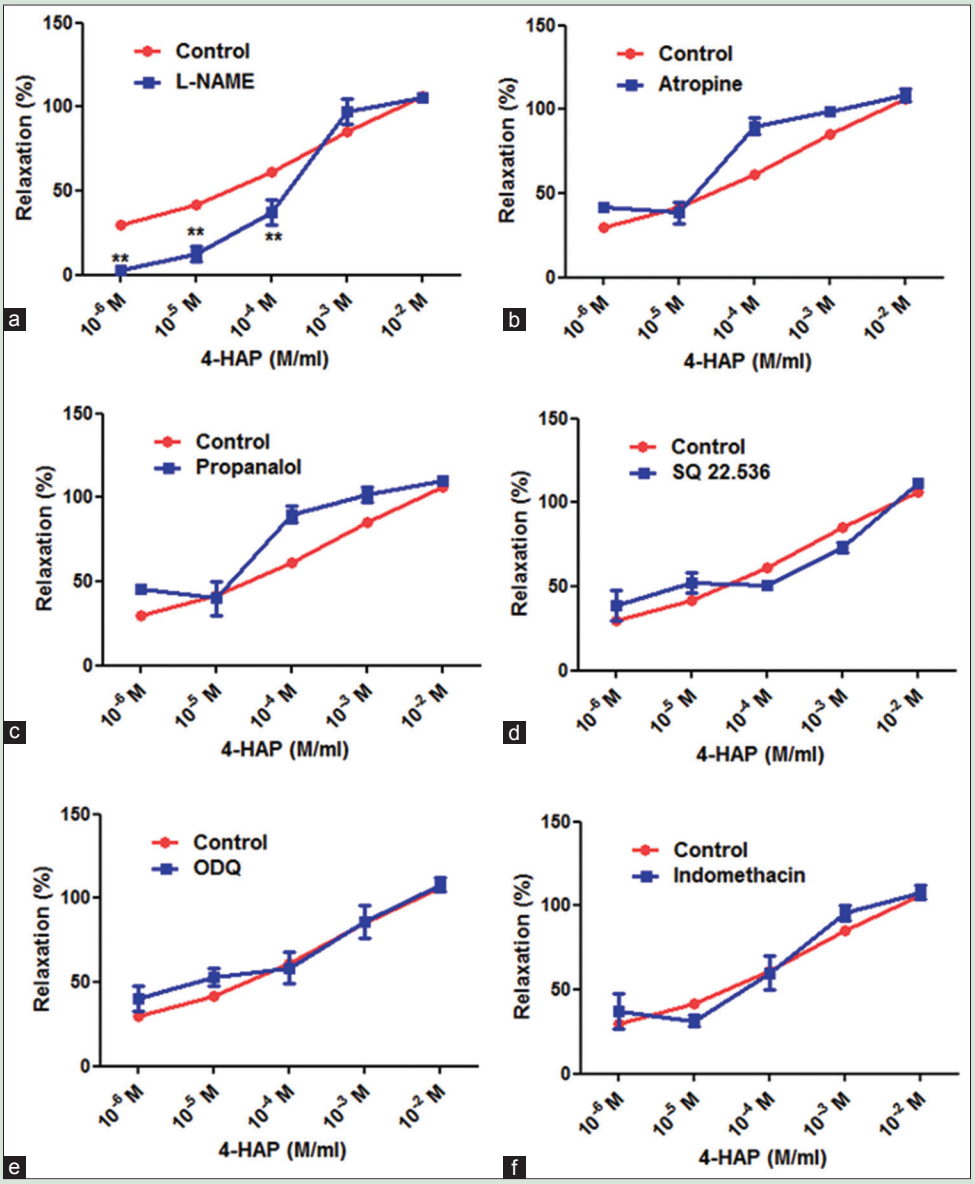

Figure 3: The effects of N (omega)-nitro-L-arginine methyl ester (a), Atropin (b), Propranalol (c), SQ22.536 (d), 1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-one, (e) and indomethacin (f) on 4-hydroxyacetophenone (10^{-6}-10^{-2}M) induced relaxation in endothelium-intact aortic rings precontracted with PE (10^{-7}M). ** = $P < 0.01$, * = $P < 0.05$, $n = 6$ rats

flavonoids, fatty acids, caffeoyl derivatives, and sterols. Several studies have reported that the essential oils present in *Z. clinopodioides Lam.* have antimicrobial, antifungal, antioxidant, and sedative properties.[5,6,10-12] In a study by Girard-Thernier *et al.*, seven compounds were isolated from *Z. clinopodioides Lam.* including flavonoids such as apigenin, chrysin, acacetin, and thymonin and phenolic compounds such as acetovanillone, 4-HAP, and ethyl 4-coumarate, a cinnamic acid derivative.[9]

When smooth muscle contractions are induced by PE, the phospholipase C enzyme becomes active. The activated enzyme stimulates the formation of inositol triphosphate (IP3) and diacylglycerol (DAG). The resulting IP3 binds to the receptor on the sarcoplasmic reticulum and allowing the release of Ca^{+2} ions to the cytoplasm. DAG inhibits the activity of myosin light-chain phosphatase through PKC, thus stimulating contraction. The PE also provides a Ca^{+2} flow into the cell from the extracellular area with receptor-mediated Ca^{+2} channels located on the cell surface. Thus, the vascular smooth muscle contracts.[13,14] In this study, PE-induced endothelial aortic smooth muscle contractions were found to be significantly relaxed with all doses of 4-HAP (10^{-6} M, 10^{-5} M, 10^{-4} M, 10^{3} M, and 10^{-2} M).

L-NAME is an inhibitor of the enzyme NOS and is used to prevent nitric oxide (NO) synthesis. NO is a substance released from endothelial cells, and it plays a very important role in the regulation of vascular tone. NO is the endothelial NOS induction endothelial cell produced. NO induces guanylyl cyclase in the vascular smooth muscle, resulting in the formation of cyclic guanosine monophosphate (cGMP). The resulting cGMP stimulates relaxation in the vascular smooth muscle cell in three ways. The first way involves the stimulation of the protein kinase G, opening the K^+ gates in the endothelial smooth muscle cell wall, and causing the exit of K^+ to the extracellular space; the decrease in the number of intracellular positive ions leads to membrane hyperpolarization, thus inducing a relaxation response. The second way is the blocking of the entry of Ca^{+2} into the cell by closing the Ca^{+2} channels in the cell

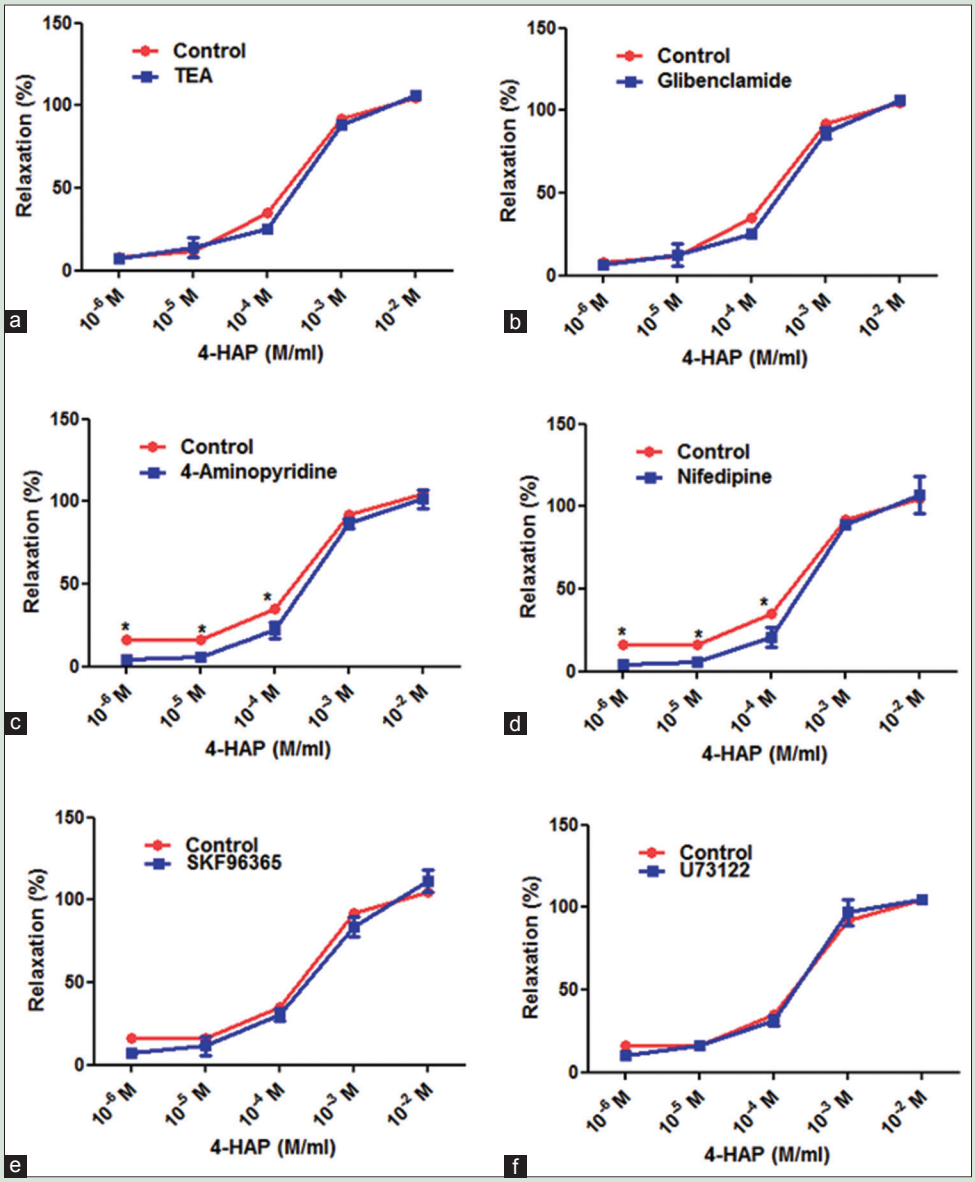

Figure 4: The effects of K^+ channel blockers as follows: tetraethylammonium (1 mM) (a), glibenclamide (10 μM) (b) and 4-aminopyridine (1 mM) (c) and L-type Ca^{+2} channel inhibitor Nifedipine (1 mM) (d), U73122 (10 μM)(e) (fosfolipaz C enzym inhibitor), and SKF96365 (10 μM) (f) (store Ca^{+2} chanalle antagonist) on 4-hydroxyacetophenone (10^{-6}-10^{-2}M)-induced relaxation in endothelium-denuded aortic rings precontracted with PE (10^{-7}M). * = $P < 0.05$, $n = 6$ rats

membrane. Moreover, the third way is to pump the Ca^{+2} ion into the intracellular storage cytoplasm.[15,16]

Jin *et al.* have reported that the Radix Paeoniae Rubra plant extract significantly inhibited vasorelaxant activity in the presence of L-NAME on PE-induced endothelial aortic smooth muscle contractions. The authors reported that the vasorelaxant activity of this plant extract occurred via NO.[17] In this study, we observed that vasorelaxant activity was significantly inhibited in PE-induced endothelial aortic smooth muscle contractions in the presence of L-NAME at 10^{-6} M, 10^{-5} M, and 10^{-4} M doses of 4-HAP. This effect of 4-HAP, a phenolic compound in the direction of these results, may be considered to be effective in the process of the NO-regulated relaxation mechanism. However, no inhibitory effect of L-NAME on the vasorelaxant effect of 10^{-2} M and 10^{-3} M doses of 4-HAP was seen. The very high concentrations of 10^{-2} M and 10^{-3} M doses of 4-HAP may have an insufficient effect on tissue-based antagonist activity.

As a voltage-sensitive K^+ channel antagonist, 4-AP was used. The activation of these channels leads to the release of K^+ ions through the opening of the K^+ channels in the cell membrane and through hyperpolarization in the cell membrane. This causes the vessel to loosen smooth muscle, which causes relaxation in the vascular smooth muscle. Pantan *et al.* determined the mechanism of vasorelaxant activity on endothelium-free aortic smooth muscle contractions of 16-O-acetyldihydroisosteviol (ADIS) and the relaxation responses obtained with 4-AP in the bath environment were not statistically significant when compared to the responses obtained without 4-AP. From these results, it was concluded that the vasorelaxant effect of ADIS was not through voltage-sensitive K^+ channels.[18] In this study, when we compared the results of 4-HAP with 4-AP in the environment of a bath of 10^{-6} M, 10^{-5} M, and 10^{-4} M in the absence of 4-AP, there was a statistically significant difference. These results suggest that this effect of 4-HAP can be achieved through voltage-sensitive K^+ channels.

Nifedipine was used as an L-type Ca^{+2} channel antagonist. When L-type Ca^{+2} channels in the cell membrane are active, the input of Ca^{+2} ions into the cell increases, and the depolarization of the cell results in contraction. The Ca^{+2} ion concentration in the cytoplasm is increased by the release of Ca^{+2} ions stored in the cell membrane and in the cell.[19,20] A study by Kang *et al.* determined the mechanism of the vasorelaxant effect of the ethanol extract of Cinnamomi ramulus (CR) on blood vessels with nifedipine compared to the values obtained without nifedipine, after vasorelaxant activity of the CR extract after induction of PE in the rat thoracic aortic smooth muscle significantly inhibited nifedipine.[21] In light of these results, it was concluded that the CR plant performed this activity through L-type Ca^{+2} channels. In this study, we determined that the effect of vasorelaxant activity of 4HAP in the presence of nifedipine in the environment of 10^{-6} M, 10^{-5} M, and 10^{-4} M baths showed a statistically significant difference between these values when compared to the results obtained without nifedipine. These results suggest that this effect of 4-HAP may occur through L-type Ca^{+2} channels.

CONCLUSION

This study showed that 4-HAP in the structure of *Z. Clinopoides Lam.* performed vasorelaxant activity with endothelium-mediated NO pathway, L-type Ca^{+2} channel inhibition, and voltage-sensitive K^{+} channel inhibition in smooth muscle.

Summary

This is the first study that reveals significant vasorelaxation effect induced by 4-HAP. Vasorelaxation may be one of the possible mechanisms for its ability to reduce the blood pressure.

Financial support and sponsorship

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Conflicts of interest

There are no conflicts of interest.

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