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The Role of K^+, Ca^{2+} channels and Endothelial Hyperpolarizing Factors in Vasorelaxation Induced by *Tribulus terrestris*

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Abstract. The present study focused on the relaxant effect of themethanolic extract (ME) of *Tribulus terristris* on rats' thoracic aortae and included the study of underlying vasorelaxation mechanisms. The methanolic extract produced concentration-dependent relaxation in rats' aorta. The methanolic extract produced concentration-dependent relaxation in the aortic rings. The use of different K^+ channel blockers ($BaCl_2$, 4-AP, GLIB, and TEA) indicated that K_v, K_{ATP}, $K_{IR,}$ and K_{Ca} and L-type Ca channels played no role in the methanolic extractinduced relaxation. However, with respect to endothelium-derived hyperpolarizing factors, PGI_2 and sGC produced a mild inhibition in the relaxation response to ME while NO produced no effect at all.
Based on the novel results of the current study, it can be concluded that *T. terrestris* methanolic extract (ME) mediated relaxation in isolated rat aortic tissues in a concentration-dependent manner. Moreover, we discovered that ME-mediated relaxation is endothelium-dependent and that potassium and calcium ion channels play no role in this relaxation with a limited role of PGI2 and sGC.

Keywords: *Tribulus terrestris*, Aorta, K^+ and Ca^{2+} channels, Methanolic extract, EDHF.

1.Introduction

Medicinal plants and herbs are widely used in folk medicine for curing and protecting from many diseases. *Tribulus terrestris* is a tropical, perennial creeping herb belong to the family *Zygophyllaceae* that is distributed all over the world, including Iraq [1-3]. *Tribulus terrestris* contains several active ingredients such as flavonoids, steroids saponins, flavonol glycosides, lignanamides, N-trans-caffeoyl tyramine, and alkaloids [4]. Thus, whole plant, fruit, and root are used in medicinal plant remedies. *Tribulus terrestris*is characterized by having diuretic, aphrodisiac [5], antiurolithic, immunomodulatory [6], antidiabetic, absorption promoting, hypolipidemic, and cardiotonic [7]. Furthermore, it also acts as a nervous system tonic, hepatoprotective, anti-inflammatory [8], antispasmodic, antibacterial, anthelmintic [9], and anticarcinogenic effects [5, 10, 11]. It also assists in curing coughs, respiratory issues, and cardiac problems [12].

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A recent study showed that *T. terrestris* reduces the production of reactive oxygen species while rising NOS levels and enhancing endothelial cell metabolism [13]. Saponin-enriched extracts of *T. terrestris* herbal medication showed to have antiglycation, antioxidant, and antiproliferative effects in human tumor cell lines. In contrast to the uniform extract, the saponin-enriched extract demonstrated higher antiglycation and antioxidant activity [14].
Due to the lack of information on the effect of *T. terrestris* leaves on blood vessels. Thus, the present study aims to investigate the vasodilation effects of methanolic extract of *T. terrestris* leaves on isolated aortic rings of therats, as well as the mechanism(s) behind these effects by using different enzymes specific inhibitors and ion channels blockers.

2. Material and methods

2.1 Plant material and preparation of the extract

The plant species used in the present study *T. terrestris* has been collected from Zakho city in July 2018. This herb was identified and authenticated by a specialized plant taxonomist. The methanolic extract of the leaves was performed in the Physiology Research Lab, Biology Dept., University of Zakho.The plant was dried in a shad and then dried in an oven at 40°C until a constant weight was obtained. Then the dried leaves were crushed in a grinder and sieved to obtain a fine powder (1000 g). The leaves powder was soaked in a suitable amount of methanol (99%) for 24 hours. The process was repeated three times for four days, with occasional shaking. The extract was filtered through Whatman No.1 filter paper, concentrated using a thin-film rotary evaporator (BŰCHI), and the obtained powder was kept in bottles stored at -4ºC until further use.

2.2 Animal model and tissue preparation

The experiments were carried out on adult male Wistar rats (300 -350 gr.), maintained in Animal House at the University of Zakho. Rats were maintained at a room temperature of about 24 ± 0.5^{o}C, mean humidity of 26.0 ± 1.2%, and exposed to a normal photoperiod cycle with free access to water and food *ad libitum*.
For the preparation of the thoracic aorta, the rats were anesthetized with ketamine (40mg/kg) and xyaline intraperitoneally. The thoracic region was dissected, opened and the thoracic aorta was removed and placed in a Petri-dish containing cold Krebs-Henseleit solution. After the removal of unwanted tissues around the thoracic aorta, it was subdivided into 4-5 pieces each of about 4 mm in length. Each aortic ring was mounted in the glass tissue chamber by a cotton thread connected from one end to a stainless hook at the lower part of the tissue holder and a force transducer from the other end. The mounted tissue was then immersed in the PanLab glass tissue bath containing warm physiological solution aerated with carbogen (95% O_2 and 5%CO_2) and maintained at 37^{o}C pH of 7.4. The aortic ring was left under initial tension of 2 gr. The aortic ring preparation was left in the glass tissue bath for one hour untiltissue stabilization. During stabilizationtime, the physiological solution in the glass chamber was changed every 15 minutes. To assess the viability of the aortic rings and the endothelium functional integrity, they were first exposed to 10µM PE and then 10µM ACh. This was achieved by periodically adjusting the bath material until a steady resting tone was recorded, after which the experiments were started. The contractile responses of the aortic rings were recorded and digitized using LabChart 7 software, which was then saved on the computer and is used for statistical analysis later.

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2.3 Protocol of the experiments

2.3.1 The role of K^+ and Ca^{2+} channels on methanolic extract of *T. terrestris* induced relaxation of the aortic rings

To studythe role of K^+ channel subtypes in methanol extract (ME) *T.terrestris* induced relaxation in isolated rats' aortic rings, the aortic rings were preincubated with the following K^+ channel blockers before the cumulative addition of ME: $BaCl_2$ (K_{IR} channel blocker, 1 mM for 10min), 4-AP (Kv channel blocker, 1 mM for10min), Glib (K_{ATP} channel blocker, 10 µM for 20min), and TEA (K_{Ca} channel blocker, 10 mM for 10min). In addition, the aortic rings were preincubated with nifedipine (the L-type Ca^{2+} channel blocker, 10µM for 20min).

2.3.2 The role of NO, prostaglandins, and sGCon methanolic extract of *T. terrestris*mediated relaxation of the aortic rings

For investigating the role of some endothelium-derived hyperpolarizing factors in ME-mediated aortic relaxation, the aortic rings were preincubated with the following EDHFs inhibitors before the addition of ME: L-NAME (nitric oxide synthase inhibitor, 300µM, 10min), indomethacin (cyclooxygenase inhibitor 30µM, 20min), and Methylene blue (MB) (soluble guanylyl cyclase inhibitor, 10µM, 10min).

2.3.3 Endothelial denudation's effects on methanolic extract of *T. terrestris*mediated relaxation of the aortic rings

The effect of the endothelium layer on ME-mediated relaxation, the endothelium layers of aortic rings were gently removed by mechanical rubbing using a wooden toothpick. This was followed by arterial rings precontraction with 10 µM PE, and the relaxation to 10 µM ACh to assure the removal of the endothelial layer. Any aortic rings that showed less than 10 % relaxation were selected as endothelium-denuded tissue, and any tissue that showed more than 50 % relaxation was selected as endothelium intact tissue. After validation, the aortic rings were precontracted with 10 µM PE, and after tissue stabilization, cumulative addition of ME at a concentration (0.1, 0.25, 0.5, 1, 5, and 10 mg/ml) was applied to 10 ml glass tissue chambers of the organ bath.

2.4 Statistical analysis

The percentage of PE-mediated contraction was used to quantify aortic reactions, which were expressed as mean±SEM after baseline adjustment. Student's t-test and one-way AVOVA with Dunnett's multiple contrast test was used for data interpretation. GraphPad Prism8 program is used to interpret the data and display it as graphs. A p-value of less than 0.05 was considered significant.

3.Results and discussion

3.1 The role of K^+ and Ca^{2+} channels on methanolic extract of *T. terrestris* induced relaxation of the aortic rings

Methanol extract of *T.terrestris*produced a dose-dependent relaxation in the rats' aortic smooth muscle in which the relaxant responses were considerably enhanced with the elevation of the concentrations used (Figure 1 and Table 1). The role of K^+ channels in ME-mediated relaxation in isolated rats' aortic rings using their respective specific K^+ channel blockers ($BaCl_2$, 4-AP, Glib, and TEA) indicated that K^+ channel subtypes K_V, K_{ATP}, $K_{IR,}$ and K_{Ca} play no direct role in ME-mediated relaxation as indicated in figure 1 and table 1. Accordingly, in the presence of various K+ channel blockers, the dose-response curves (DRC) were very close to those of the control. However, L-type Ca channels caused mild and non-significant enhancement in the

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relaxation responses in the presence of L-type Ca channel blocker nifedipine. This also indicates that the L-type Ca channel has no direct role in the ME-induced relaxation.

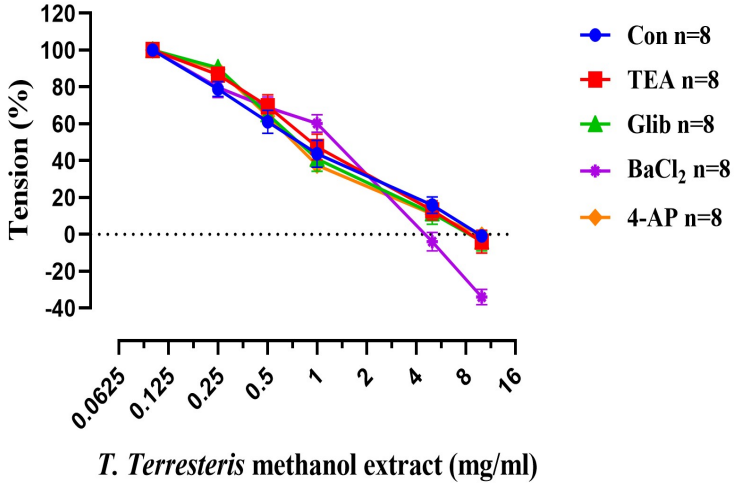

Figure 1. Dose-response curves for ME-mediate relaxation on aortic rings incubated with K^+ channel blockers.Each point reflects the mean S.E.M. of eight aortic rings taken from different rats.

Table 1. The outcomes for ME relaxation of rat aortic rings the following preincubation with specific K^+ channel blockers and precontraction with 10µM phenylephrine (PE).

Test	Mean		Mean ± SEM	P-Value
	Control	Treated		
Con vs. TEA	50.97	54.69	3.723 ± 20.32	0.8577
Con vs. Glib	72.29	39.44	-32.85 ± 19.74	0.1220
Con vs. BaCl2	58.12	61.44	3.316 ± 17.97	0.8573
Con vs. 4-AP	54.65	54.65	0.0004082± 21.13	>0.9999
Note: P-Value <0.05 is considered significant				

3.2The role of NO, prostaglandins, and sGCon methanolic extract of *T. terrestris* mediated relaxation of the aortic rings

Methylene blue caused a mild and non-significant inhibition in the relaxation responses which appeared at high ME concentrations. Indomethacin also produced a mild and non-significant inhibition in relaxation responses but at low ME concentrations and diminished at higher concentrations and return to normal. On the other hand, L-NAME did not affect the relaxation response and the DRC was very close to that of the control(Figure2 and Table 2).

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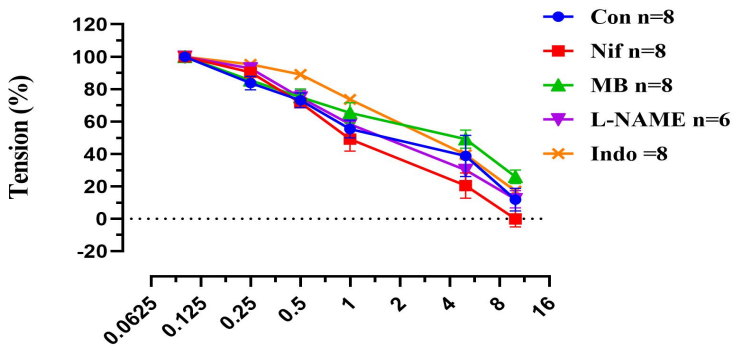

Figure 2. Role of L-type calcium channels, NO, prostaglandins, and MB in ME-mediated relaxation in isolated aortic rings. Each point represents the mean ± S.E.M. of six to eight aortic rings taken from various rats.

Table 2. Showing the data for isolated aortic rings relaxed by ME after preincubation with L-type calcium channel blocker, NO, $_{C}$GMP, prostaglandins, and contracted with 10 µM PE.

Test	Mean		Mean ± SEM	P-Value
	Control	Treated		
Con vs. Nif	63.20	55.25	-18.47 ± 19.61	0.3646
Con vs. MB	50.97	61.37	10.40 ± 16.89	0.5493
Con L-NAME	68.67	63.03	-5.643 ± 15.97	0.7299
Con vs. Indo	69.32	60.87	-8.459 ± 15.89	0.6041
Note: *P*<0.05 is considered significant				

3.3 Endothelial denudation's effects on methanolic extract of *T. terrestris*mediated relaxation of the aortic rings

Denudation of aortic rings was precontracted with 10 µM PE and incubated with different ME concentrations (0.1, 0.25, 0.5, 1, 5, and 10 mg/ml), produced a significant inhibition in the amplitude of relaxation responses at all the doses used. Thus, DRC was significantly shifted to the left-hand side as compared with that of the control (Figure 3 and Table 3). This reflects the important role of aortic endothelium and its major role in ME-induced relaxation in aortic rings.

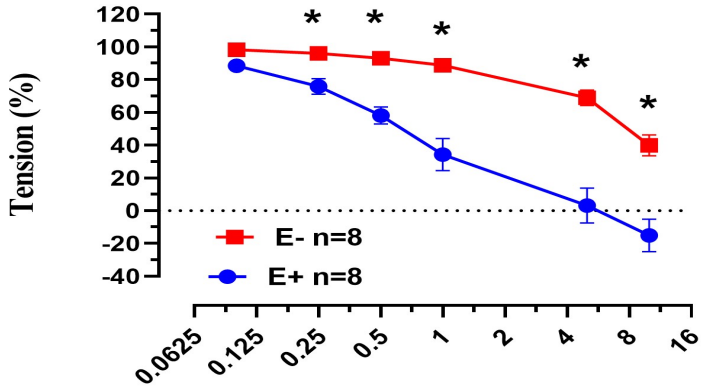

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Figure3. Endothelial denudation effects on ME-mediated relaxation in isolated rats' aortic rings. Each point represents the mean ± S.E.M. of 8 aortic rings obtained from several rats. * =P < 0.05.

Table 3.Methanolic extract-mediated relaxation results in denuded and intact aortic rings precontracted by 10µM phenylephrine (PE).

Test	Mean		Mean ± SEM	P-Value
	E-	E+		
E-vs E+	1.667	1.099	0.5680 ± 0.2510	0.0430
Note: *E*stands for endothelium denuded (E-) and intact(E+), *P-Value*< 0.05 considered significant				

4. Discussion

Due to the lack of information related to the role of ion channels and endothelium-derived hyperpolarizing factors effects of ME of *T. terrestris*mediated relaxation, it is very difficult to interpret the novel results at least now. However, the dose-dependent relaxation resulted from incubation of rats aortic ring with methanol extract of *T. terrestrisis* similar to that caused by the ethyl acetate extract of the fruits of the same plant in the rat thoracic aorta conducted by Balogun and Ashafa [15] They suggested that this vasorelaxant effect of the ethyl acetate fraction (EtOAc) of *T. Terrestris* fruits can probably be attributed to a reduction in calcium influx through the L-type calcium channel.

Usually, K^+ channel subtypes like K_{ATP}, K_{IR}, $K_{Ca,}$ and Kv channels playing a considerable role, directly or indirectly in the hyperpolarization by efflux of K^+ via smooth muscle plasma membrane and subsequent relaxation [16, 17]. Interestingly, none of these mentioned potassium channels respond to their specific blockers. This indicates that none of them played a role in the ME relaxant effect in rat aortic smooth muscle. However, [18] using TEA at 10 mM for the aortae of rats, found that it is the most common nonspecific K^+ channel blocker. Whereas, in the current study, TEA did not inhibit ME-mediated relaxation of the aortic tissues when precontracted with PE. This is consistent with the results of the studies on isolated rat aortic rings [15], they found that the K^+ channel did not play a role in EtOAc and ME-mediated relaxation in smooth muscle cells.

Phillips, Mathew [19], according to the studies on the rats' superior mesenteric artery found that aqueous and methanolic extracts of *T. terrestris*had important antihypertensive properties in spontaneously hypertensive rats and this may be due to smooth muscle relaxation via NO release. L-NAME (NOS inhibitor) and MB (sGC inhibitor) did not block the relaxant activity of ME in the isolated aortic rings of rats. Thus, ME does not relax aortic smooth muscle through the endothelium-dependent NO-cGMP pathway. On contrary, in the denuded aorta, the relaxant effect of ME is significantly inhibited, which reflects that endothelium is playing a considerable role in ME-induced relaxation.

5. Conclusion

It can be concluded from the current results that K^+ channel types (Kv, K_{ATP}, $K_{IR,}$ and K_{Ca}) had a mild effect on the relaxation induced by ME in the isolated rats' aortic rings and playing a limited direct role in the induced relaxation. In addition, also PGs and sGC play a mild role in the induced relaxation. On the other hand, neither nifedipine nor L-NAME produced any effect of the induced relaxation. While denudation experiments demonstrated that endothelium is playing a considerable role in ME-induced relaxation.

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