

INSIGHT INTO THE CARDIOVASCULAR ACTIVITIES OF *ELAEAGNUS UMBELLATA*

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Abstract

This study aimed to explore pharmacological base of the medicinal use of *Elaeagnus umbellata* in cardiovascular diseases. In normotensive and hypertensive rats under anaesthesia, crude extract of *Elaeagnus umbellata* (Eu.Cr) and selected fractions exhibited a fall in the mean arterial pressure. In isolated rat aortic rings, crude extract and selected fractions, exhibited L -NAME sensitive endothelium dependent vasodilator effect and inhibited K^+ (80 mM) pre-contractions. In isolated rabbit aortic rings pre-constricted with K^+ (80 mM) and phenylephrine (1 μ M), Eu.Cr and selected fractions induced relaxation with high potency against phenylephrine (PE). Crude extract and selected fractions had inhibitory effect on Ca^{2+} entry channels by shifting Ca^{2+} concentration - response curves (CRCs) to the right and also suppressed phenylephrine peak formation in Ca^{2+} -free medium like verapamil. When tested on basal tension, the crude extract and selected fractions were devoid of any vasoconstrictor effect. These data indicate that crude extract and selected fractions possess antihypertensive and vascular relaxing effects.

Rezumat

Acest studiu a urmărit să evalueze baza farmacologică a utilizării medicinale a *Elaeagnus umbellata* în bolile cardiovasculare. La șobolanii normotensivi și hipertensivi sub anestezie, extractul brut de *Elaeagnus umbellata* (Eu.Cr) și fracțiile selectate au produs o scădere a tensiunii arteriale medii. În inelele aortice izolate de șobolan, extractul brut și fracțiile selectate, au prezentat efect vasodilatator și au inhibat pre-contrațiile K^+ (80 mM). În inelele aortice izolate de iepure pre-contractate cu K^+ (80 mM) și fenilefrină (PE-1 μ M), Eu.Cr și fracțiunile selectate au indus un efect de relaxare cu potență mare față de PE. Extractul brut și fracțiile selectate au efect inhibitor asupra canalelor de Ca^{2+} prin deplasarea către dreapta a curbelor de răspuns la concentrația de Ca^{2+} . Asupra tensiunii bazale, extractul brut și fracțiile selectate au fost lipsite de efect vasoconstrictor. Aceste date indică faptul că extractul brut și fracțiile selectate au efecte antihipertensive și de relaxare a endotelului vascular.

Keywords: *Elaeagnus umbellata*, antihypertensive, vasorelaxant, Ca^{2+} concentration - response curves

Introduction

Elaeagnus umbeletta Thunb is a wide shrub, belongs to the *Elaeagnaceae* family [1]. Locally it is known as “kankoli”. It is a common shrub growing wild at a height of 4500-6000 feet above sea level in Himalayan region of Pakistan [2]. The fruits seeds and flowers of *Elaeagnus umbeletta* are locally used as remedy in cardiac disorders like hypertension and also as stimulants in cough and gut disorders [1, 3]. Medicinal uses have been reported for various species of *Elaeagnaceae* [4, 5]. The plant contains different phytochemicals, such as vitamins (A, C and E), flavonoids, essential fatty acids, 7 lycopene, β -carotene, lutein, phytofluene and phytoene [6]. *Elaeagnus umbellata* has not been explored pharmacologically in the past. However, few studies are available on its fruit extract such as antibacterial activity against various Gram positive and Gram negative bacteria

including *Staphylococci* and *Escherichia coli* [7], and free radical scavenging capacity and antiproliferative activity [8]. In the tradition system of medicine *Elaeagnus umbellata* is reported in cardiac disorders [1, 3]. We assumed that *E. umbellata* might have potential effects on cardiovascular system. Therefore, the present investigation was carried out to study the potential antihypertensive and vasorelaxant effects of *E. umbellata* with the intention to provide a pharmacological base to its medicinal use in hypertension.

Materials and Methods

Plant material, preparation of crude extract and fractions

Fresh fruits of *Elaeagnus umbellata* were collected from Nowsherwan (Abbottabad) in the month of July in 2012 and identified by taxonomist, Dr.

Ghulam Mujtaba, Assistant Professor Govt Degree College, Abbottabad. The sample voucher (Eu. Fr. 07/12) was submitted to the Department of Pharmacy, COMSATS Institute of Information Technology, Abbottabad. Extraction and fractionation were carried out as described previously in literature [9]. About 5 kg of fresh fruits were soaked in methanol at room temperature (23 - 25°C) for 15 days with occasional shaking. It was filtered through a muslin cloth and then through a Whatman qualitative grade 1 filter paper. This procedure was repeated twice and the combined filtrates were evaporated on a rotary evaporator under reduced pressure to the crude extract (Eu.Cr), yielding approximately 16.81%. Fractionation was carried out, using solvents of increased polarity. Eu.Cr was mixed in 10% tween 80 and distilled water, n-hexane was added and shaken vigorously in a separating funnel. The upper n-hexane layer was collected and evaporated on a rotary evaporator in order to obtain the n-hexane fraction (Eu.nHex) (yielding about 30%). The lower layer was taken in a separating funnel and chloroform was added. The lower chloroform layer was collected and evaporated on a rotary evaporator to obtain the chloroform fraction (Eu.Chl) (yielding about 6%). The other upper layer was again taken into a separating funnel, ethyl acetate was added, separated and was also evaporated in a rotary evaporator in order to obtain the ethyl acetate fraction (Eu.EtAc) (yielding about 13%). The remaining lower layer was collected and evaporated to yield the aqueous fraction (Eu.Aq) (yielding about 36%) [9].

Animals

Animals used in this study were housed in the conditions complied with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (NRC 1996) [10], and approved by the Ethical Committee of COMSATS Institute of Information Technology, Abbottabad, in its meeting held on 17-06-2013 vide notification EC/PHM/07-2013/CIIT/ATD.

In-vivo blood pressure measurement in normotensive and high salt-induced hypertensive rats anesthetized rats

These experiments were performed on male Sprague-Dawley rats (200 - 220g) as described [11], and the protocol of Lawler *et al* [12] and Vasdev *et al* [13] was followed for high salt induced hypertensive rats with some modifications. Hypertension was induced in rats by providing a high-salt diet (8% NaCl) and water *ad libitum* for 6-weeks [12]. One day prior to the experiment, the rats were given normal diet and water. Animals were anesthetized with an intraperitoneal injection of sodium pentothal, 80 - 100 mg/kg bw. After cannulation 20 to 30 minutes, in order to stabilize

hypotensive and hypertensive responses, respectively, the animal received acetylcholine (0.1 mL) and norepinephrine (0.1 mL) were used to check the stability of the animals toward. Drugs, extract and their fractions were then infused intravenously and followed by a flush with 0.1 mL saline. Changes in mean arterial pressure (MAP) were recognized as the difference between the steady-state values before and the lowest readings after injection. The MAP was calculated as the diastolic blood pressure (BP) plus one third pulse width (systolic BP – diastolic BP).

Isolated tissue preparations

Rat thoracic aorta

The procedure of Taqvi *et al* [14] was performed with some modifications. Thoracic aorta was isolated from Sprague-Dawley rats carefully to avoid any damage to the endothelium. The aorta was then transferred into the Krebs's solution aerated with carbogen. It was cautiously cleaned off fats and other connective tissues and then cut into rings 2 to 3 mm wide. In some rings, the endothelium was intentionally removed by gentle rubbing of the intimal surface with forceps to check the endothelium dependent and independent effect [11]. A preload of 1 g was applied to each preparation and incubated for 30 minutes. Changes in isometric tension were recorded and analysed through a force transducer (MLT 0201) coupled with a bridge amplifier (N12128) and PowerLab (ML 846) Data Acquisition System (ADInstruments, Australia).

Rabbit thoracic aorta

As described previously [15], rabbits were euthanized by a blow on the back of head; the thoracic aorta was removed and cut into rings of approximately 2 to 3 mm width and suspended between a pair of stainless steel hooks in 10 mL organ baths, in normal Krebs's solution, maintained at 37°C, and continuously aerated with 5% CO₂ in O₂ (carbogen). Vascular reactivity of the extract was evaluated on the Ca²⁺ influx either through voltage-dependent channels (VDCs) or receptor-operated Ca²⁺ channels (ROCs) and Ca²⁺ release from internal store(s) by inducing contraction through K⁺ (80 mM) and phenylephrine (PE) (1 μM) respectively and then extract and fractions were given cumulatively in order to observe relaxation.

Statistics

Data obtained from the animals and *in vitro* experiments were expressed as the mean ± standard error (± SEM). Statistical difference between the treatments and the control were evaluated by a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using the IBM SPSS software (Version 20, SPSS Inc., Chicago,

IL). Differences were considered significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Results and Discussion

The crude extract *Elaeagnus umbellata* and fractions caused a fall in MAP, (Figure 1A). This suggests that the extract and fractions have a blood pressure lowering effect, which justify its medicinal claim [1, 3]. The ethyl acetate fraction was found potent at the dose of 300 mg/kg (Figure 1A), and

cause fall in BP up to 59 ± 1.00 . To check the possibility of involvement of muscarinic receptors, rats were pre-treated with atropine (1 mg/kg bw). This pre-treatment did not affect the blood pressure lowering effect of the extract and fractions (data not shown). The extract and fractions of *Elaeagnus umbellata* possess antihypertensive effect, the ethyl acetate fraction was found potent at the dose of 300 mg/kg and cause fall in BP up to 83.00 ± 1.00 , (Figure 1A).

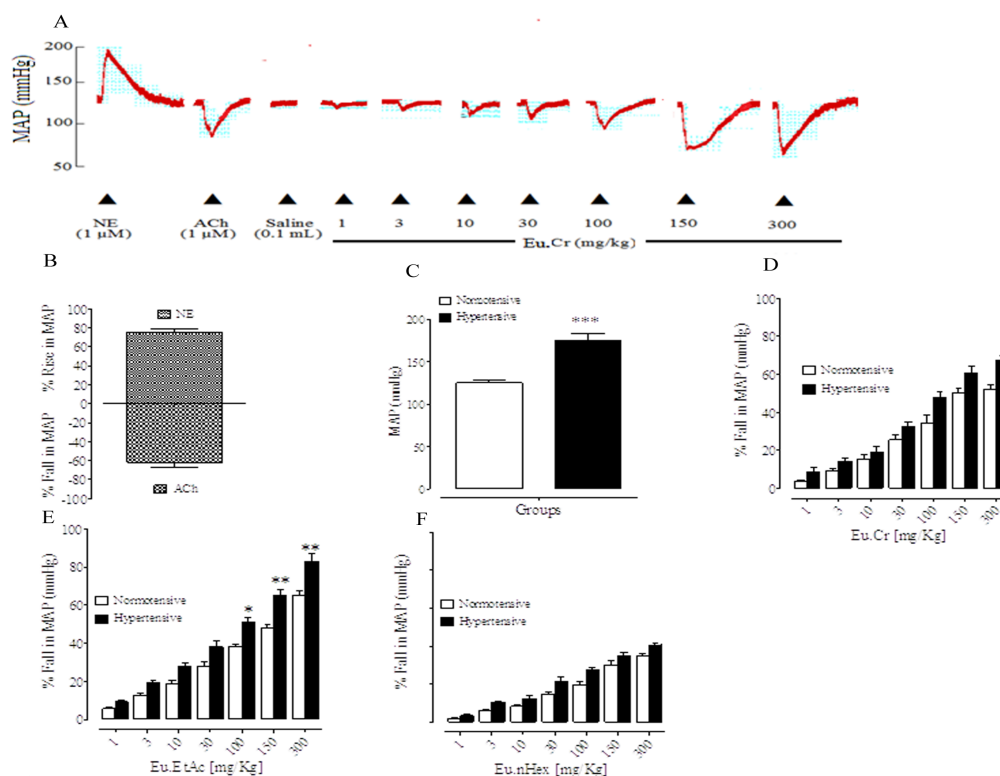


Figure 1.

1A representative tracing showing the effect of norepinephrine (NE), acetylcholine (ACh), and the crude extract of *Elaeagnus umbellata* (Eu.Cr) on mean arterial pressure (MAP) in normotensive rats under anaesthesia; 1B shows the hypertensive and hypotensive effects of NE and ACh, respectively; 1C shows the blood pressure of normotensive and hypertensive rats. 1D - 1F shows the blood pressure lowering effect of the crude extract of Eu.Cr, ethylacetate (Eu.EtAc) and n-hexane (Eu.nHex) fractions, respectively on MAP in normotensive and hypertensive rats, under anaesthesia. Values shown are mean \pm SEM (n= 6 - 7), * $p < 0.05$ and ** $p < 0.01$, represent the significance difference between the % fall in MAP in normotensive and hypertensive rats

Blood pressure is the product of cardiac output and vascular resistance [16]. We explored the vascular component of the antihypertensive effect of the extract and fractions using isolated vascular preparations. To have insight into the role of vascular endothelium-derived relaxing factors, we used isolated rat aorta. Rat aortic rings pre-contracted with PE, cumulative addition of crude extract and ethyl acetate fraction induced endothelium-dependent vasorelaxant effect with EC₅₀ value of 0.328 mg/mL (0.31 - 1.13) in intact endothelium (Figure 2A). Vascular endothelial cells

synthesis and release variety of vasoactive substance including NO [17]. This relaxation was ablated in aortic rings pre-treated with L-NAME, a nitric oxide synthase inhibitor [18]. NO could also directly activate K_{ca} channels of the vascular smooth muscle [19]. Thus, nitric oxide plays a pivotal role in regulating vessel tone. It is known that high salt induces endothelial damage in the high salt-induced hypertensive rats [20]. The endothelium of aortic rings from normotensive rats was deliberately damaged rubbing with forceps. We found that the extract induced the same level of

relaxation in both the aortae i.e from hypertensive rats and damaged endothelium from normotensive rats with EC_{50} value of 2.87 mg/mL (1.14 - 3.47) and 2.91 (1.05 - 3.14) (Figure 2A). This confirms loss of endothelium integrity in both aortae and indicates that the vascular relaxation induced by the crude extract partially mediated by NO. We tested the fractions and found that the ethyl acetate fraction was 13 times more potent than crude extract (Figure 2B). Interestingly, the n-hexane fraction induced a partial (< 50%) relaxation against PE pre-contractions (Figure 2C), suggesting

that the vasorelaxant constituents are equally distributed among the crude and ethyl acetate fraction. To explore the additional mechanism the rat aorta was pre contracted with high K^+ (80mM), the crude extract and fractions induced relaxation (Figure 2E), which suggests that extract and fraction might induced this relaxation through inhibitory effect on the Ca^{2+} movement through voltage-dependent Ca^{2+} channels (VDCs). VDCCs play a central role in the regulation of vascular tone and blood pressure [22].

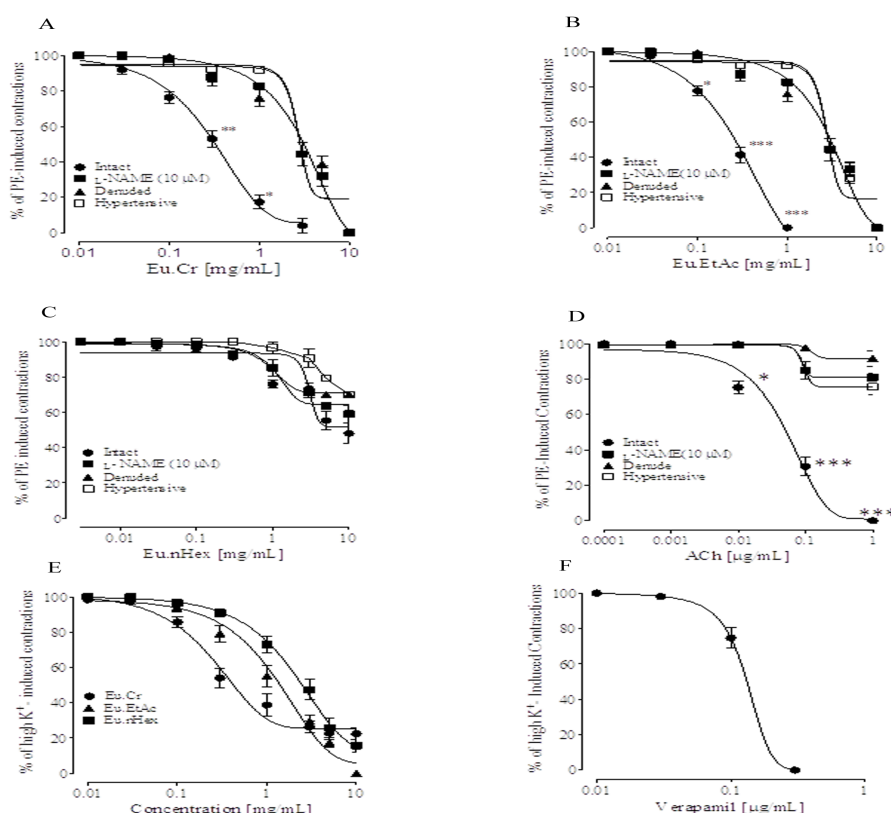


Figure 2.

2A, 2B, 2C, 2D the response of crude extract of *Elaeagnus umbellata* (Eu.Cr), its fractions ethyl acetate (Eu.EtAc), n-hexane (Eu.nHex) and acetyl choline on PE-induced contractions in intact (with and without L-NAME (10 μ M) pre-treatment), denuded aortic rings from normotensive rats and rings from hypertensive rats; 2E a vasodilator effect of Eu.Cr, Eu.EtAc, Eu.nHex; 2F verapamil on high K^+ (80 mM)-induced contractions in isolated rat aorta rings. Values shown are mean \pm SEM (n = 6 - 7), *p < 0.05 and **p < 0.01, ***p < 0.001 represent the significance difference between the relaxation induced by phenylephrine (PE) in intact and denuded aorta.

Further experiments in rat aorta with high K^+ and $CaCl_2$ were required to explore the possible inhibitory effect on VDCs but use of high K^+ and $CaCl_2$ for a long time is erratic. Responses to these agents are not uniform and sustained. Therefore, we used rabbit aorta which is more stable providing long and sustained responses to these agents. In rabbit aortic rings pre-contracted with PE and high K^+ (80 mM), cumulative addition of extract and fractions induced partial relaxation of the high K^+

pre-contraction but complete relaxation of PE pre-contraction (Figure 3 A, E, G), suggesting combined effect on Ca^{2+} movements. We further tested the possibility if they inhibit Ca^{2+} through VDCs. Rabbit aortic rings incubated in Ca^{2+} free/EGTA medium pre-treated with crude extract and ethyl acetate fraction caused shift in the $CaCl_2$ concentration response curves, with suppression of maximum response, similar to verapamil (Fig. 3 B, D, F, H). This indicates that extract and the ethyl

acetate fraction have direct effect on vascular smooth muscle cells and probably inhibit VDCs and induce vascular relaxation. Crude extract and

its fractions inhibit contractile response induced by PE (1 μ M), in Ca^{2+} - free medium, similar to that caused by verapamil.

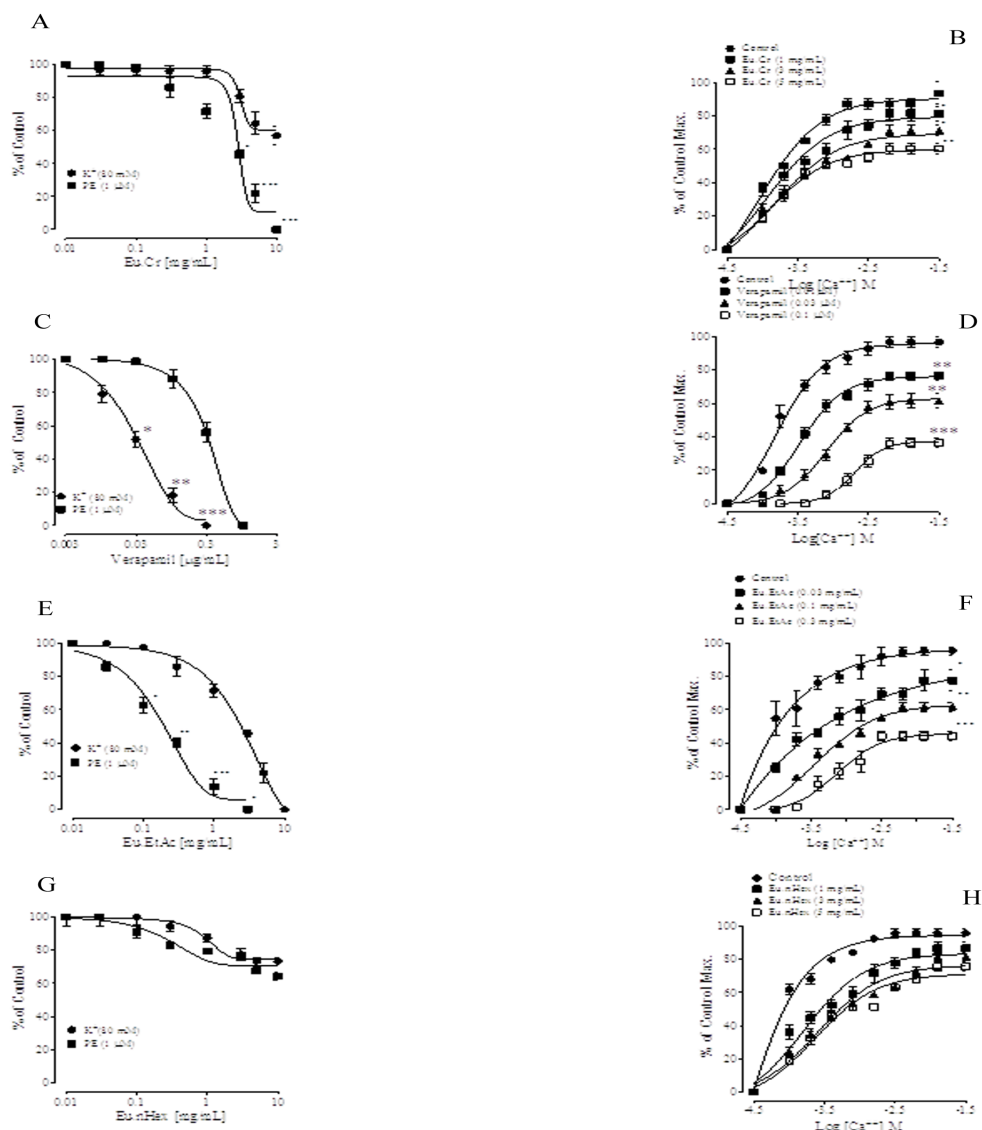


Figure 3.

3A, 3C, 3E, 3G present the concentration-dependent vasodilator effect of the crude extract of *Elaeagnus umbellata* (Eu.Cr), the verapamil, ethylacetate (Eu.EtAc) and n-hexane (Eu.nHex), fractions on phenylephrine (PE) and high K^+ (80 mM) pre-contractions, and 3B, 3D, 3F, 3H respectively, their effect on the Ca^{2+} concentration – response curves, constructed in Ca^{2+} - free medium, in isolated rabbit aorta preparations. Values shown are mean \pm SEM (n = 6 - 7), * p < 0.05 and ** p < 0.01, *** p < 0.001, represent the significance difference between the relaxation induced by phenylephrine (PE) and high K^+ (80 mM).

The fractions ethyl acetate and n-hexane (0.01 - 10.00 mg/mL) also suppressed the transient contractile response of PE (Figure 4). Our findings indicate that the extract and ethyl acetate fraction induce vascular relaxation through NO and inhibitory effect on Ca^{2+} movements and this partly explain the antihypertensive effect of *Elaeagnus umbellata*. The less potent nature of the n-hexane fraction might be due to the presence of

constituents in this particular fraction lacking dominant effect on vascular endothelial and smooth muscle cells.

We did not identify any particular constituents in the extract or fraction that could be considered responsible for the vascular relaxation and or antihypertensive effect. But our preliminary phytochemical analysis indicated presence of alkaloids. Plant derived alkaloids presented

vasorelaxant activity, which is mediated through multiple pathways, such as inhibition of calcium

release from the Ca^{2+} stores [16] and NO pathways [22].

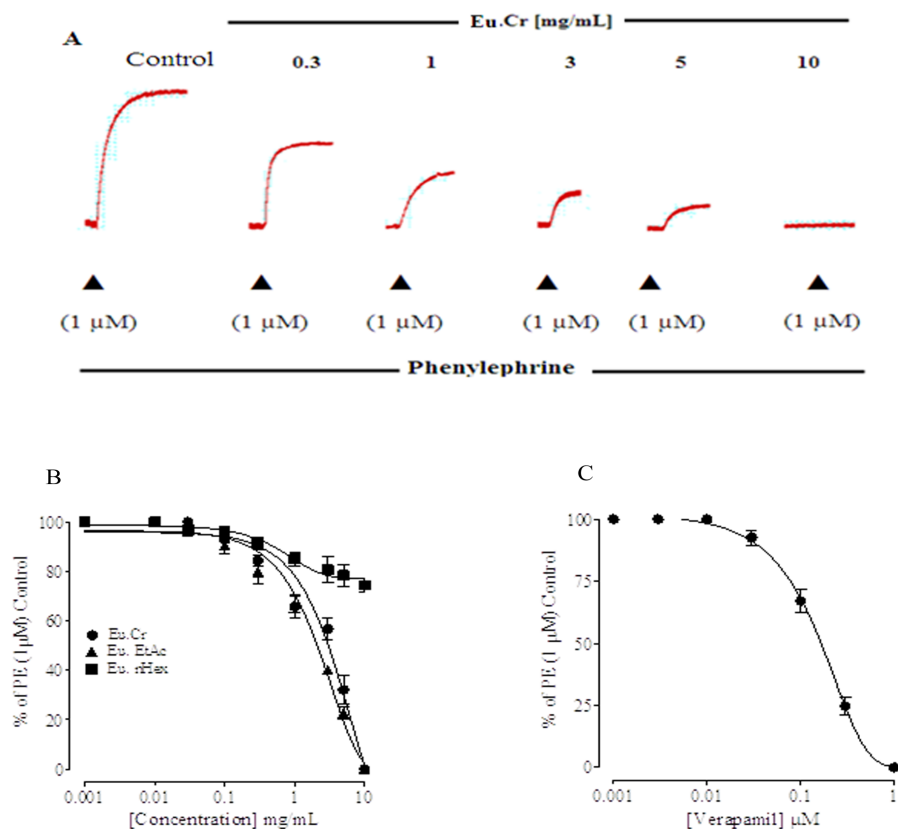


Figure 4.

4A represents a typical tracing showing inhibitory effect of increasing concentrations of the crude extract of *Elaeagnus umbellata* (Eu.Cr) on the initial peak formation of phenylephrine (1 μM) in Ca^{2+} -free medium; 4B shows the combined effect of Eu.Cr, ethylacetate (Eu.EtAc) and n-hexane (Eu.nHex) fractions; 4C the effect of verapamil on the initial peak formation of PE in isolated rabbit aorta preparations, in Ca^{2+} -free medium. Values shown are mean \pm SEM (n = 6 - 7).

We believe that alkaloids from the extract of *Elaeagnus umbellata* might be the active constituents responsible for the cardio vascular effects. However, detailed chemistry is required in order to identify the chemical constituents and to prove the molecular nature of these mechanisms.

Conclusions

The current findings on the cardiovascular activities of *Elaeagnus umbellata* and its fractions indicated that its crude extract and fractions possess unique constituents, which mediate relaxant effect through Ca^{2+} antagonism and NO pathways, which possibly explain the effect in BP. These findings provide a pharmacological base for the medicinal use of *Elaeagnus umbellata* as antihypertensive agent.

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