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Juglone as antihypertensive agent acts through multiple vascular mechanisms

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ABSTRACT

Background: Juglone, a natural phenolic compound obtained from the walnut tree, is known for its wide range of biological activities. However, it has yet to be tested for its effects on hypertension and vascular tone. This investigation was aimed to explore the antihypertensive effect and the nature of vascular reactivity of juglone in rat models.

Methods: Juglone was tested in *in vivo* and *in vitro* experiments in rats. The responses were analyzed and recorded through a PowerLab data acquisition system.

Results: Intravenous injection of juglone significantly decreased the mean arterial blood pressure (MAP) in normotensive and hypertensive rats (Max. fall, 43.50 ± 2.96 vs 49.66 ± 3.28 mmHg). In rats pretreated with Nω-Nitro l-arginine methyl ester ($_L$-NAME), the effect of juglone on MAP was reduced as compared to the control. However, in rats pretreated with atropine the fall in MAP by juglone was not altered. Juglone induced relaxation in the phenylephrine, K^+ (80 mM), and angiotensin II pretreated isolated rat aortic rings. This vasorelaxant effect was reduced with $_L$-NAME pretreatment. Atropine pretreatment did not modify the vasorelaxant effect of juglone. Pre-incubation with juglone attenuated the intracellular Ca^{2+} release by suppressing phenylephrine peak formation and also shifted $CaCl_2$ concentration–response curves (CRCs) to the right. Of note, combined treatment with 4-aminopyridine and barium chloride also reduced juglone-mediated vasorelaxation suggesting a role of K^+-channels as well.

Conclusion: In conclusion, juglone exerts its antihypertensive effect through vasorelaxation, which is mediated by nitric oxide, inhibition of intracellular calcium release and opening of K^+-channels.

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Juglone; antihypertensive effects; vascular reactivity; nitric oxide (NO); Ca^{2+} and K^+-channels

Introduction

High blood pressure is a major risk factor for heart and vascular diseases. A deterioration in nitric oxide (NO) dependent vasorelaxation, overexpression of L- type calcium (Ca^{2+}) channels, inhibition of potassium (K^+) channels, oxidative stress and reactive oxygen species (ROS) are well-established risk factors that can predispose individuals to endothelial damage and ultimately hypertension (1–3). Natural products have been the basis of treatment of human diseases like hypertension (4,5).

Walnut tree parts like hull, root, and leaf are reported for vasorelaxant effect (6,7), antihypertensive (8–10) and antioxidant activity (11). Previous studies have not identified the active constituents responsible for any of these activities and could not reach to conclusive mechanism. In the 1850s the juglone (initially termed "nucin", Latin nux, meaning a nut) was first isolated from the walnut tree (12), and in 1881 the first scientific report on juglone allelopathic effect was published (13). Juglone is a brownish yellow color naphthoquinone and naphthoquinones are included in the sub-class of phenolic compounds (14). Walnut also contains many other phenolic constituents (15). Naphthoquinones improve vascular functions and are also reported for antihypertensive and vasorelaxant activities (16).

Phenolic compounds are the most abundant secondary metabolites of plants that possess diverse biological properties, such as inhibition of endothelial dysfunction (17), antioxidant (18), ca^{2+}channels blockade (19), k^+ channels activation (20) and vasorelaxant activity (21). One of the important phenolic constituents in the walnut is juglone (5-hydroxy-1, 4-naphthoquinone) (22). Juglone is investigated for depressant effect (23), skin diseases (24), antimicrobial (25), antioxidant (26,27) and anticancer actvities (28). However, the role of this important constituent in hypertension is lacking. This study was aimed to explore the role of juglone in managing hypertension and its possible vascular mechanisms.

Materials and methods

Chemicals and standards

Standard drugs like, acetylcholine chloride, atropine sulfate, phenylephrine hydrochloride, potassium chloride, Nω-Nitro l-arginine methyl ester ($_L$-NAME), indomethacin, atropine, verapamil hydrochloride, tetra ethyl amine (TEA), 4-aminopyridine (4-AP) and barium chloride ($BaCl_2$) dimethyl sulfoxide (DMSO) and angiotensin II (Ang II) were purchased from Sigma-Aldrich, St. Louis, MO, USA and ethylene glycol bi's (2-aminoethylether) -*N, N, N′, N′*-tetraacetic acid (EGTA) 97% from Alfa Aesar, Heysham, UK. Pentothal sodium and heparin injections were obtained from Abbot Laboratories, Karachi, Pakistan and F. Hoffmann-La Roche, Basel,

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Switzerland, respectively. The test compound juglone was purchased from Sigma-Aldrich, St. Louis, MO, USA. The stock solutions of the drugs were made in distilled water/DMSO (0.1%) and the subsequent dilutions were prepared fresh on the day of the experiment. Invasive blood pressure and changes in isometric tension were recorded and analyzed through a force transducer (MLT 0201) coupled with a bridge amplifier (N12128) and PowerLab (ML 846) Data Acquisition System (ADInstruments).

Experimental animals and housing conditions

Antihypertensive and vascular reactivity study was conducted on 8–10 week-old Sprague–Dawley (SD) rats (200–250 g), preferably male, were housed at the Animal House of the COMSATS University Islamabad, Abbottabad Campus, maintained at 23–25°C. Experiments performed complies with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (29) and approved by the Ethical Committee of Department of Pharmacy, COMSATS University Islamabad, Abbottabad campus, in its meeting held on 17-06-2013 video notification EC/PHM/07-2013/CIIT/ATD.

Invasive blood pressure measurement

Measurement of mean arterial blood pressure (MAP) in normotensive anesthetized SD rats

The protocol of Shah and Gilani (30) was followed with some modifications. SD rats were anesthetized with an intraperitoneal injection of sodium thiopental (pentothal, 40–100 mg/kg), fixed in a supine position on a dissecting table; a small mid tracheal incision (approximately 1 cm) was made to expose trachea, carotid artery, and right jugular vein. The trachea was cannulated with a polyethylene (PE-20) tubing and cleaned from time to maintain the spontaneous respiration. The carotid artery was cannulated with PE-50 tubing filled with heparinized saline (0.1 mL) to prevent blood clotting. The right jugular vein was also cannulated with a PE-50 tubing to facilitate the intravenous infusions of the normal saline, juglone, and standard drugs. This connection was used for blood pressure recording. The exposed surface was covered with a piece of tissue paper moistened with warm saline. The body temperature of the animal was maintained by using an overhead lamp (30).

Experimental protocol

After 20 to 30 min of the equilibrium period, standard drug; norepinephrine (1 μg/kg) and acetylcholine (1 μg/kg) were used to check the stability of the animals toward hypertensive and hypotensive responses, respectively. Juglone was injected at different doses followed by a flush of normal saline (0.1 ml). To see the involvement of nitric oxide-pathway and muscarinic receptor, SD rats were pretreated with $_{L}$-NAME (20 mg/kg) and atropine (1 mg/kg), respectively. The MAP was allowed to return to the resting level between infusions. Changes in MAP were recognized as the difference between the steady-state values before and the lowest readings after injection. The MAP was calculated by one of the following formulas:

$$\mathrm{MAP} = \mathrm{SBP} + 2(\mathrm{DBP})/3 \text{ or } \mathrm{MAP} = \mathrm{DBP} + 1/3(\mathrm{SBP} - \mathrm{DBP})$$

The percent fall in MAP was calculated as:

$$\mathrm{Control} - \mathrm{Fall}/\mathrm{Control} \times 100$$

Blood pressure measurement in sodium chloride (8%) induced hypertensive anesthetized SD rats

The SD rats were randomly assigned to two groups of six rats each. One group was kept as controlled group receiving normal saline, while the second was given high-salt (8% NaCl) and sodium chloride rich diet for 14 days. The rats were considered hypertensive with MAP above 140 mmHg systolic and 90 mmHg diastolic blood pressure. One day prior to the experiment, the rats were given normal diet and water. Subsequently, the rats were used for *in-vivo* blood pressure measurement as described earlier (31).

Vascular reactivity studies

Tension studies in isolated rat aortic preparations from SD rats

The procedure of Furchgott et al. (32) was followed with some modifications. Thoracic aorta was isolated from SD rats, carefully to avoid any damage to the endothelium. The isolated aorta was cautiously cleaned off from fats and other connective tissues and then cut into rings 2–3 mm wide. The aorta was then transferred into the 10 mL tissue baths containing Kreb's solution at 37°C aerated with carbogen (5% CO_2 in O_2). A preload of 2 g was applied to each preparation and allowed to equilibrate for 30–45 min (32).

The effect of juglone on contraction induced by phenylephrine, K+ (80 mM) and Ang II

The protocols of Chan et al. (33), and Qamar et al. (31) were followed with some modifications. Standard vasoconstrictors like; phenylephrine (1 μM), K^+ (80 mM) and Ang II (5 μM) were used to induce steady-state contractions in rat aortic rings. The juglone was added cumulatively to obtain concentration–response relationship and the relaxation was expressed as percent of agonist-induced contractions. In some aortic rings, endothelium was deliberately damaged with gentle rubbing the intimal surface with forceps and were considered denuded when acetylcholine (0.1 μM) failed to induce relaxation <80% (34).

Determination of the effect of juglone in the presence of $_{L}$-NAME, atropine, and indomethacin

We challenged the endothelium-intact aorta rings with $_{L}$-NAME (10 μM), atropine (1 μM) and indomethacin (1 μM) (35), to investigate the role of nitric oxide (NO), muscarinic receptor and prostacyclin for 20 min prior to pre-contraction with phenylephrine (1 μM). Comparisons were made between the cumulative concentration-response of juglone in aortic rings with and without pre-incubation with the above inhibitors (36).

Effect of juglone on intracellular ca^{2+} stores

Vascular reactivity of the juglone was evaluated on Ca^{2+} influx either through, receptor-operated Ca^{2+} channels (ROCs) and Ca^{2+} release from internal store(s). After the initial phenylephrine peak in normal kreb's solution, the rat aortic rings were exposed to Ca^{2+}-free/EGTA solution for 15 min before the application of phenylephrine (1 μM). The aortic rings without incubation with juglone were considered to be the control group. The juglone (0.03 to 3 μg/mL in organ bath) was used to pre-incubate the aortic rings for 30 min before phenylephrine (1 μM) was added. The rings were then washed three times with a normal Kreb's solution and incubated for at least 40 min for refilling of the intracellular stores. Subsequently, the medium was rapidly replaced with Ca^{2+}-free solution and the rings were incubated for another 15 min. The second contraction was then induced by phenylephrine (1 μM) in the presence of juglone (0.03 μg/mL), which were added 30 min before the application of phenylephrine (1 μM), both contractions were compared. For comparison, similar protocol was also run with verapamil (0.003 to 3 μg/mL) (31,37).

Effect of juglone on calcium channels

To determine the effect of juglone on voltage-dependent Ca^{2+} channels (VDCs), two sets of experiments were carried out, namely the juglone and verapamil groups. Initially, to stabilize and confirm the integrity of tissues, the contraction was produced by K^+ (80 mM). The solution was then replaced with Ca^{2+}-free Krebs' solution containing EGTA (0.2 mM) and control concentration–response curves (CRCs) of $CaCl_2$ (0.01–10.0 mM) (as Ca^{2+}) was obtained. To comprehend the calcium channel blocking activity of juglone, the contractions were recorded for each concentration of Ca^{2+} (0.01–10.0 mM) after pre-incubation of juglone (0.3 to 1 μg/mL). In a similar way, the aortic rings were pre-incubated with verapamil and Ca^{2+}-CRCs were constructed, to see the possible calcium channel blocking effect (33,38,39).

The effect of juglone on rat aorta pre-contracted with phenylephrine in both the absence and presence of k^+-channel blockers

The contraction was evoked with phenylephrine both the absence (control) and presence of potassium channel blockers; 4-aminopyridine (4-AP) (1 mM) (40), barium chloride ($BaCl_2$) (1 mM) (41) and tetraethylammonium (TEA) (5 mM) (42) in independent experiments, which were added to the organ baths 20 min before the phenylephrine-induced contraction. During the sustained phase of the contraction, juglone was cumulatively added to obtain a relaxation curve.

Statistical analysis

Data obtained were expressed as the mean ± standard error mean (SEM) and median effective concentrations (EC_{50}) values with 95% confidence interval (CI). The data were analyzed by using student t-test and two-way ANOVA followed by Bonferroni test, using GraphPad Prism version 8 (Graph Pad, San Diego, CA, USA). Differences were considered significant at $^*p \le 0.05$, $^{**}p \le 0.01$, and $^{***}p \le 0.001$.

Results

Effect of juglone on blood pressure in normotensive and hypertensive SD rats under anesthesia

Effect on mean arterial pressure (MAP)

Intravenous injections of norepinephrine (1 μg/kg) and acetylcholine (1 μg/kg) were administered that induced a rise and fall in mean arterial pressure (MAP) in both normotensive and hypertensive, respectively (Figure 1a–c), this validated the protocol. The juglone produced a dose-dependent fall in MAP in normotensive and hypertensive anesthetized rats after the intravenous administration. The % fall in MAP was 9.01 ± 0.57, 18.67 ± 2.33, 25.67 ± 1.20, 33.01 ± 3.05, 36.02 ± 3.51, 40.67 ± 2.08 and 43.50 ± 2.96 mmHg at different doses tested of 0.003 to 3 mg/kg (Figure 1e). In hypertensive rats, intravenous administration of juglone also caused a fall in MAP with values of 18.01 ± 0.8, 22.67 ± 1.45, 28.33 ± 3.84, 32.67 ± 3.52, 36.67 ± 2.18, 44.34 ± 2.84 and 49.66 ± 3.28 mmHg (Figure 1e). The % fall in MAP of the hypertensive rats was more significant than the normotensive rats at similar doses.

Effects of juglone on MAP in SD rats pretreated with $_L$-NAME and atropine normotensive rats under anesthesia

The experiments were repeated in normotensive rats under anesthesia that were pretreated with $_{L-}$NAME (20 mg/kg) and atropine (1 mg/kg) before the administration of juglone. For comparison, juglone was tested against the normotensive rats without any treatment, % fall in MAP observed was 10.02 ± 1.75, 24.75 ± 32.20 ± 4.80, 37.75 ± 4.34, 41.10 ± 4.26, 44.50 ± 5.25 and 54.75 ± 3.90. In the $_L$-NAME pretreated rats, the fall in MAP induced by juglone was 7.5 ± 0.85, 10.75 ± 1.10, 16.01 ± 0.70, 18.25 ± 0.47, 20.25 ± 1.10, 26.03 ± 1.80 and 30.04 ± 2.27 mmHg. Furthermore, in pretreated atropine the % decline in MAP was 13.75 ± 1.29, 26.75 ± 1.81, 33.50 ± 3.30, 37.25 ± 4.60, 42.01 ± 4.50, 46.50 ± 4.26, 55.75 ± 4.53 mmHg. The fall in MAP to juglone was significantly inhibited in the presence of $_L$-NAME, while not inhibited by atropine as compared to control (Figure 2). Furthermore, from the *in vivo* study we traced the percentage (%) fall in heart rate after the administration of different doses (0.003 to 3 mg/kg) of juglone (Table 1). The maximum decrease in heart rate was observed 37% at 3 mg/kg dose.

Vascular reactivity studies

The effect of juglone against phenylephrine, K^+ (80 mM) and Ang II precontractions

Juglone induced concentration-dependent relaxation against phenylephrine (1 μM), K^+ (80 mM) and Ang II (5 μM) pretreated isolated rat aortic rings with respective EC_{50} values of 1.93 (1.05–2.87), 2.25 (1.30–3.20) and 2.43 μg/mL (1.85–3.01), as shown in (Figure 3a).

Endothelium-dependent and -independent effects

In aortic rings with intact endothelium pre-contracted with phenylephrine (1 μM), cumulative addition of juglone induced a vasorelaxant effect with EC_{50} values of 1.93 μg/mL (1.05–2.07) (Figure 4a). This relaxation of juglone was reduced with the removal of endothelium, with EC_{50} values 2.90 μg/mL

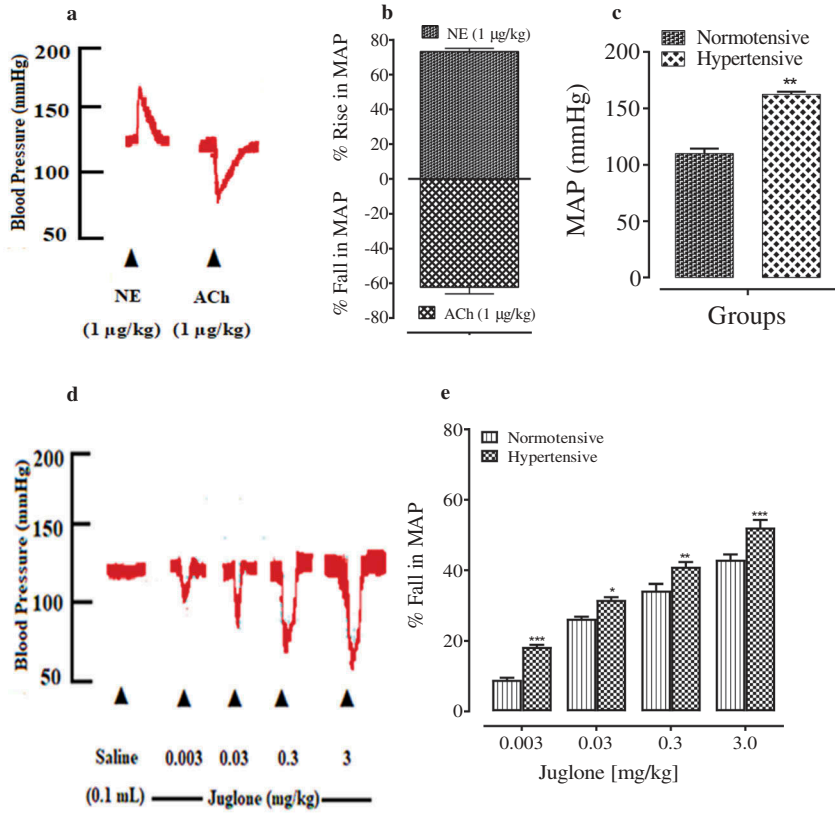

Figure 1. A typical tracing (a) showing the effect of norepinephrine (NE) and acetylcholine (ACH) on mean arterial blood pressure (MAP) and (b) elucidate the percent rise and fall in blood pressure of normotensive rats under anesthesia. (c) Showing the effects of norepinephrine (NE) and acetylcholine (ACH) on MAP in normotensive and hypertensive rats under anesthesia (d) A representative invasive blood pressure measurement tracing shows the blood pressure lowering effect of the juglone in normotensive rat under anesthesia (e) Shows effect of juglone on MAP in normotensive and hypertensive rats, under anesthesia, where; *$p < .05$, **$p < .01$, and ***$p < .001$represent the significant difference between the % fall in MAP on normotensive and hypertensive rats. Bars represent the mean ± SEM for six determinations.

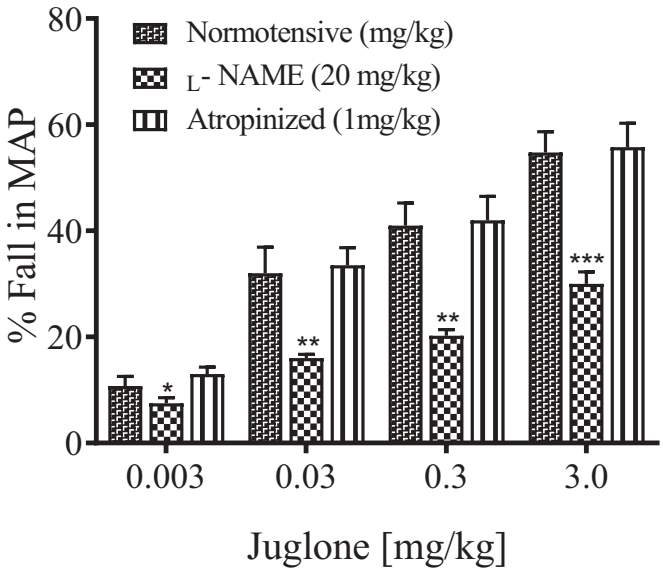

Figure 2. Bar graph shows the comparison of percent fall in MAP by juglone in normotensive, pretreated $_{L}$-NAME and atropinized normotensive anesthetized rats. Where *$p < .05$, **$p < .01$, and ***$p < .001$, represent the significant difference. Bars represent the mean ± SEM for six independent experiments.

(2.15–3.65) (Figure 4a), suggesting the role of endothelium-derived factors. In comparison to the control the vasorelaxant response of juglone to phenylephrine contractions was reduced

Table 1. Shows the percentage (%) decrease in the blood pressure and heart rate after the administration of different doses of juglone.

S. no.	Dose (mg/kg)	BP (%)	Heart rate (%)
1.	Control	100	100
2.	0.003	90	75
3.	0.03	73	62
4.	0.3	64	50
5.	3	55	37

in the intact aortic rings pretreated with $_{L}$-NAME (10 µM), with an EC_{50} value of 6.85 µg/mL (5.20–8.41) (Figure 4a), indicating the role of endothelium-derived nitric oxide (NO). To see the role of endothelium-derived NO-linked muscarinic receptors, intact aortic rings were pretreated with atropine (1 µM). However, this effect did not modify the effect of juglone (Figure 4a). To roll out the involvement of vasorelaxant prostaglandins in the effect of juglone, intact aortic rings were pretreated with indomethacin. This pretreatment also did not modify the effect of juglone (Figure 4a).

Effect of juglone on intracellular ca^{2+} stores

In a series of experiments designed to show the effect of juglone on the transient contractile response induced by phenylephrine (1 µM). Pretreatment of the aortic rings with juglone (0.03–3.0 µg/mL) attenuated the phenylephrine peak

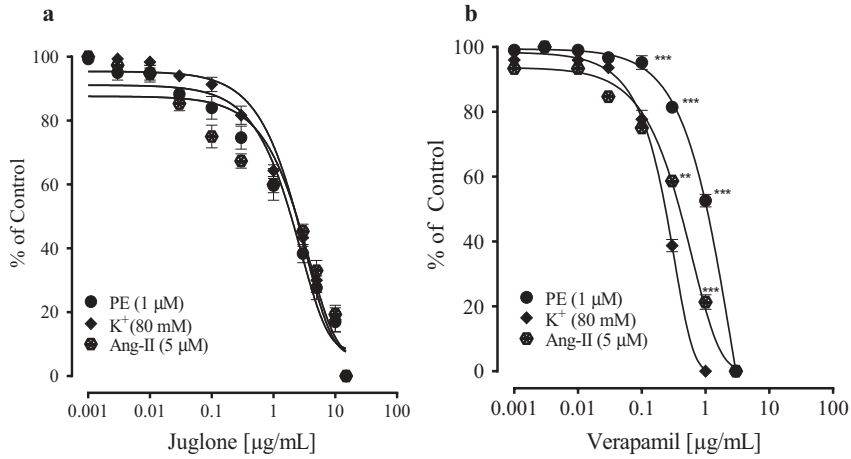

Figure 3. The graphs show the (a) concentration-dependent vasorelaxant effect of juglone and (b) verapamil on phenylephrine (PE), K^+ and angiotensin II (Ang-II) precontractions in rat aortic rings suspended in normal Krebs solution. Where $^{**}p < .01$, and $^{***}p < .001$, represent the significant difference. Each point represents mean ± SEM of six (6) determinations.

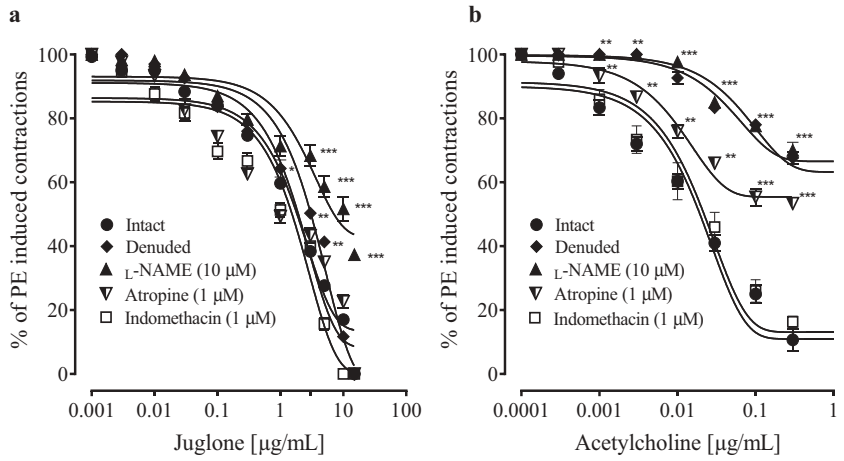

Figure 4. The response of (a) juglone and (b) acetylcholine on phenylephrine pre-contractions in intact, denuded and pretreated; Nω-nitro l-arginine methyl ester ($_L$-NAME), atropine and indomethacin rat aortic rings. Where $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$, represent the significant difference. The values shown are mean ± SEM (six determinations).

formation in Ca^{2+}-free/EGTA Kreb's solution, similar to that caused by verapamil (Figure 5a,b).

Effect of juglone on calcium channels

Pre-incubation of rat aortic rings with juglone (0.3–5.0 µg/mL) significantly inhibited the $CaCl_2$ (0.01–1 mM) response curves in Ca^{2+}-free/EGTA Krebs solution and shifted the Ca^{2+} CRCs to the right, with suppression of maximum response (Figure 6a) similar to verapamil (Figure 6b).

The effect of juglone on rat aorta pre-contracted with phenylephrine in the absence and presence of k^+- channel blockers

To investigate the possibility that the vasorelaxant effects of juglone are mediated via K^+ channels activation, various K^+ channel blockers such as, 4-AP (K_V channels blocker, a predominant blocker of voltage-gated K^+ channels), $BaCl_2$ (a K_{ir} blocker) and TEA (K_{Ca} channels blocker, a blocker of big Ca^{2+}-activated K^+ channels), were used. The vasorelaxant effect of juglone was significantly attenuated at initial doses by pre-treatment with 4-AP (1 mM) and $BaCl_2$ (1 mM). However, the effect of juglone was not modified with aortic rings pretreated with TEA (5 mM) (Figure 7). The EC_{50} values for control, 4-AP (1 mM), $BaCl_2$ (1 mM) and TEA (5 mM) were 0.51 (0.29–0.74), 26.5 (23.5–29.4), 36.8 (31.9–41.7) and 0.69 µg/mL (0.50–0.81), respectively (Figure 7).

Discussion

The present study shows the antihypertensive effect of juglone in normotensive and hypertensive rats. When tested in anesthetized normotensive rats, intravenous administration of juglone caused a dose-dependent fall in MAP. However, the effect was more significant in the high salt-induced hypertensive rats. The natural source of juglone, walnut tree is reported, reducing blood pressure through the possible release of nitric oxide (6,7). To see the role of endogenous nitric oxide in the antihypertensive effect of juglone, rats were pre-treated with $_L$-NAME, this pretreatment significantly reduced

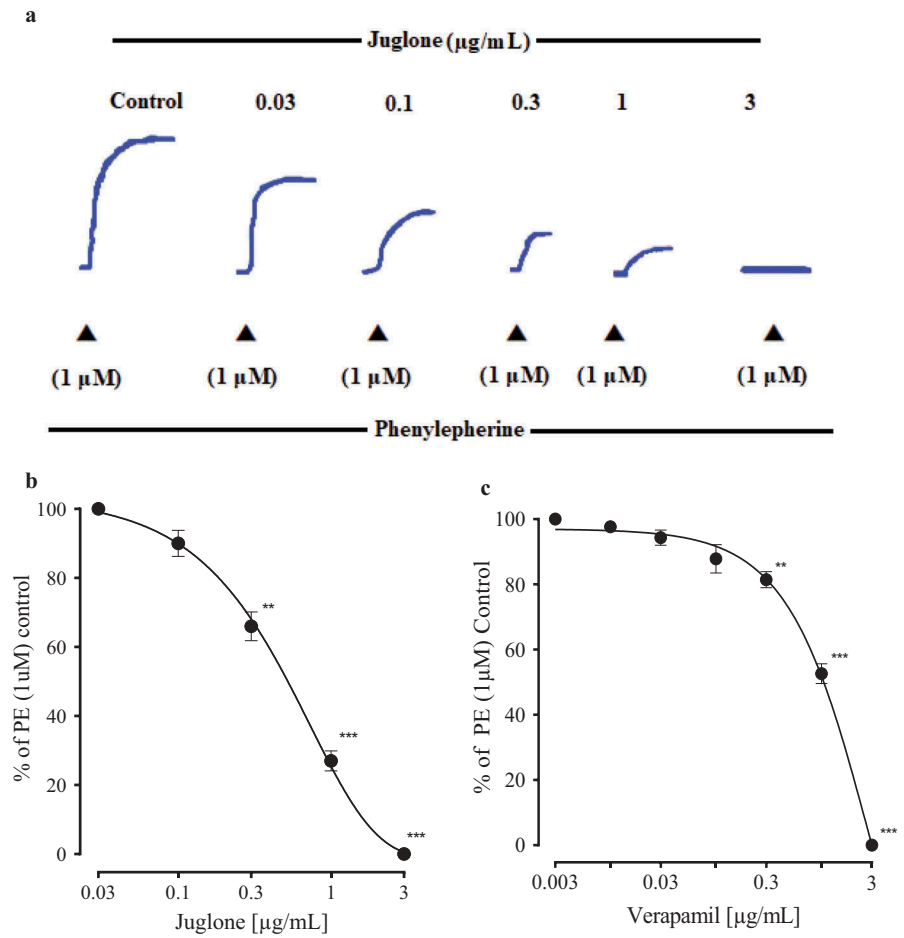

Figure 5. A typical tracing (a) showing inhibitory effect of increasing concentrations of the juglone on the initial peak formation of phenylephrine (PE)-induced contractions in Ca^{2+}-free/EGTA medium. (b) The effect of juglone (c) and verapamil in isolated rat aorta preparations. Where $^{**}p < .01$, and $^{***}p < .001$, represent the significant difference. Each value represents the mean ± SEM of six determinations.

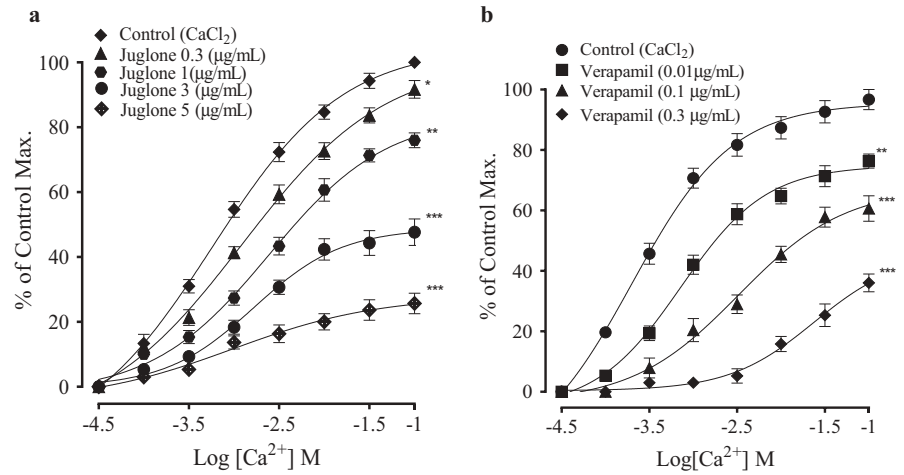

Figure 6. The graphs shows the (a) vasorelaxant effect of juglone and (b) verapamil on the Ca^{2+}-CRC constructed in Ca^{2+}-free/EGTA medium, in isolated rat aorta preparations. Where $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$, represent the significant difference. The values shown are mean ± SEM (six determinations).

(>50%) the fall in MAP to juglone. These findings suggest that the fall in MAP to juglone is partly due to the involvement of NO. The NO release in blood vessels is linked with muscarinic receptor activation (43). It was hypothesized that juglone might have stimulated vascular muscarinic receptors prior to the release of NO. To test this hypothesis, rats were pretreated atropine, a muscarinic receptor antagonist (30,44). This pre-treatment did not modify the effect of juglone on MAP, indicates that juglone releases NO without having an effect on vascular muscarinic receptors. Moreover, according to the

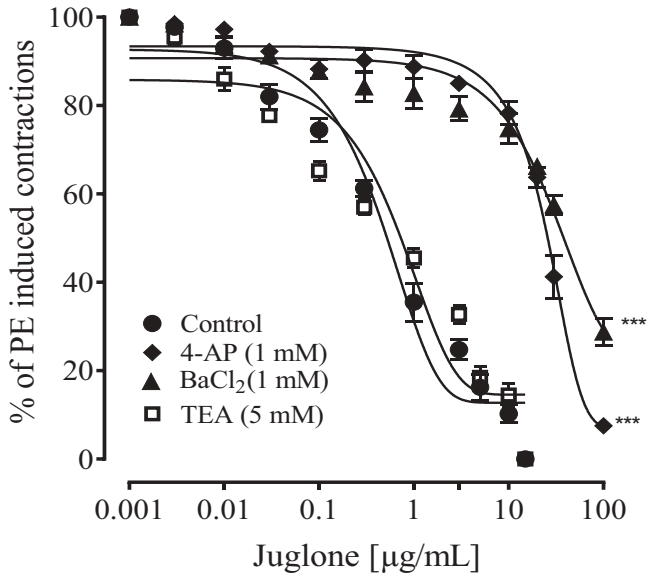

Figure 7. The response of juglone (µg/mL) on phenylephrine (1 µM) precontractions in intact and pretreated; 4-AP (1 mM), $BaCl_2$ (1 mM) and TEA (5 mM) rat aortic rings. Where ***$p < .001$, represent the significant difference. The values shown are mean ± SEM (six determinations).

in vivo data, juglone also produced fall in heart rate that could be due to the calcium channels blocking (CCB) activity of juglone, like verapamil which also effect the heart rate (45), however, further studies are required to trace out the underlying mechanism.

Usually, effect on MAP in normotensive rats is not considered a true representative as antihypertensive. For these agents must also be evaluated in hypertensive model. To achieve this objective, normotensive rats were made hypertensive by giving high salt for 14 days. After 14 days, MAP was found significant high. Thus, confirmed as hypertensive rats (46). In the high-salt hypertensive rats, intravenous administration of juglone also induced fall in the MAP. This effect in the hypertensive rats was more significant the normotensive rats, this was exciting finding on juglone as an antihypertensive agent. To have insight into the vascular mechanisms of the antihypertensive effect of juglone, further in vitro studies were carried in rat aorta.

Vascular architecture is constituted by two important kinds of cells; endothelial and smooth muscle. The vascular endothelial cells have a pivotal role in vascular homeostasis by modulating the vascular smooth muscle tone through the release of a variety of substances (47). NO is an important regulator of arterial blood pressure and the vascular function (48). However, different channels and receptors on the smooth muscles also mediate important vascular functions; the Ca^{2+}-channel blockade and opening of K^+-channels in the vascular smooth muscle cells also provide an important mechanism to dilate arteries (41). Isolated rat aorta from the normotensive rats was used to evaluate the effects of juglone on endothelial and smooth muscle cells. Different mediators such as phenylephrine, high K^+ and Ang II were used to differentiate the effect of juglone on NO synthesis, calcium movement through a membrane-bound voltage-dependent calcium channels or release from the internal Ca^{2+} store. Isolated aorta from normotensive rats with intact endothelium was precontracted with phenylephrine, cumulative addition of different concentrations of juglone induced vasorelaxation. The relaxation to juglone was attenuated with pretreatment of the arotic rings with $_L$-NAME, a nitric oxide inhibitor (48), this finding suggested that vasorelaxation to juglone was partly mediated through NO. Nitric oxide is one of the important vasodilatory mediators of endothelial origin that induces vascular smooth muscle relaxation through activation of guanylyl cyclase and formation of cGMP (49). In vascular endothelial NO-pathway is linked with muscarinic receptors (M_3), to see if effect of juglone linked to M_3 receptors coupled to NO pathway, aortic rings with intact endothelium were pretreated with atropine, a muscarinic receptors antagonist (31). This pretreatment did not change the effect of juglone, indicates that vascular muscarinic receptors activation is not involved and suggests that direct activation of the $_L$-NAME sensitive nitric oxide synthase pathway has played a role. Vascular endothelium synthesizes variety of vasoactive substances other than NO, including vasodilator prostaglandins such as prostacyclin (50). Rat aortic with intact endothelium were pretreated with indomethacin, a prostaglandin inhibitor (51). Interestingly, indomethacin pretreatment did not modify vasorelaxant effect of juglone. These findings suggest that the endothelium-dependent vasodilatory effect of juglone is predominantly mediated through activation of nitric oxide synthase that led to the formation of NO. NO is produced mainly as a result of endothelial NO synthase (eNOS) in the endothelium and neuronal NO synthase (nNOS)-containing perivascular nerves. Important for peripheral vasodilation, eNOS plays the role and probably in controlling peripheral vascular resistance (PVR) (52).

The other aspect of the vascular effect of juglone was studied on smooth muscle cells. As observed previously, juglone inhibited phenylephrine-induced contractions, suggesting a possible inhibitory effect of Ca^{2+} release from the internal store. Phenylephrine is known to cause aortic contraction by increasing the intracellular Ca^{2+} concentration through the release of Ca^{2+} from sarcoplasmic reticulum (SR) and influx through ROCs (53,54). To confirm this possibility, rat aortic rings were pretreated with different concentrations of juglone in Ca^{2+} free/EGTA medium (31). Interestingly, this pretreatment suppressed phenylephrine individual contractions compared to control, indicating the inhibitory effect of juglone on Ca^{2+} release from the internal Ca^{2+} store, similar to verapamil, a calcium entry blocker (33). Thus, it seems likely that the vascular effect of juglone involved a reduction of IP_3-dependent Ca^{2+} release from sarcoplasmic reticulum (SR) sensitive to phenylephrine. This finding encouraged us to see if juglone also has effect on Ca^{2+} moments through membrane associated channels. Some rat aortic rings were precontracted with high K (80 mM) and juglone was tested on induced contractions. High K^+ (80 mM) induced contraction depends on the influx of Ca^{2+} into the cells through voltage-dependent Ca^{2+} channels (54,55). It is apparent that an agent inhibits high K^+-induced contractions could be a possible Ca^{2+} entry blocker (56). Juglone inhibited K^+ (80 mM)-induced vascular smooth muscle contraction concentration-dependently and significantly reduced the Ca^{2+}-induced contraction in aortic rings, similar to verapamil. These findings suggest that juglone also has an inhibitory effect on Ca^{2+} entry through VDCs, this possibility was further tested. Rat aortic rings were suspended in Ca^{2+} free/EGTA medium and $CaCl_2$ concentration–response curves (CRCs) were obtained in

duplicate. Pre-incubation of the aortic rings with different concentrations of juglone induced a rightward shift with suppression of maximum response, in the $CaCl_2$, similar to verapamil, indicating that juglone also inhibits Ca^{2+} entry through VDCs.

Rat aortic cells are known to have angiotensin receptors (57) and play an important role in maintaining vascular tone by regulating immediate vasoconstriction in the cardiovascular (58). To see if juglone has an inhibitory effect on angiotensin-II (Ang-II) receptors, rat aortic rings were precontracted with angiotensin-II. Interestingly, cumulative addition of juglone induced inhibitory effect against angiotensin-II precontractions, thus suggesting a possible inhibitory effect on angiotensin-II receptors also that needs further molecular level investigation. Ang II induces vasoconstriction via multiple pathways, including the activation of the angiotensin type 1 receptor (AT_1R) in smooth muscle cells, which lead to the activation of phospholipase C (PLC) and the increase of cytoplasmic Ca^{2+} concentrations (59,60). So the vasorelaxant effect produced by juglone after the Ang II vasoconstriction might be mediated through inhibiting the elevation in intracellular Ca^{2+}. However, our findings could possibly explain this effect due to the inhibitory effect of juglone on the release of intracellular Ca^{2+}.

These findings on the vascular reactivity of juglone did not identify a dominating mechanism related to the vascular smooth muscles. Therefore, further insight into other vascular aspects was investigated. Vascular K^+ channel activation is known to play an important role in the regulation of vascular tone. There exist different types of K^+ channels in the vascular smooth muscles. These include calcium-activated K^+ channels (K_{Ca}), K^+ voltage-gated channels (K_v) and inward rectifying K^+ channels (Kir). To see if these K^+ channels have played a role in the vasorelaxant effect of juglone, aortic rings were precontracted with phenylephrine and effect was reproduced in the presence of different K^+ channel blockers. TEA, blocker of K_{Ca} channels (20) was without effect on the vasorelaxant effect of juglone, however, $BaCl_2$, a blocker of Kir channels (61) and 4-aminopyridine, blocker of Kv channels (62) significantly inhibited relaxation to juglone. These exciting findings on juglone indicate that predominate endothelium-independent vasorelaxant mechanism is the activation or opening of Kv and Kir channels. Moreover, past work on $BaCl_2$ has revealed that lower concentrations have shown similar effects. For example, Tyml et al. (63) used 1 μM in capillaries of frog, Ellis et al. (64) used 30 μM in mouse isolated aorta and small mesenteric artery, while others (41,65,66) used 1 mM to block potassium channels (Kir) in rat aorta. So, in comparison with frog capillaries and mouse aorta and small mesenteric artery, we used 1 mM for rat aorta, which is a larger blood vessel in diameter.

The findings on the potential role of potassium channels in the juglone-mediate relaxation may be the result on action at TRP (Transient receptor potential cation channel) receptors (67). Juglone is reported to activate TRPA1 channels, and possibly might interact with the other TRP channels associated with intracellular and extracellular calcium entry (68). The subsequent Ca^{2+} sparks by TRP receptors agonist activate potassium channels leading to vascular smooth muscle membrane potential hyperpolarization and vasorelaxation (69). So, the findings on the potential role of TRP receptors and potassium channels in the juglone-mediate relaxation are also novel and interesting.

Our finding on the vascular mechanisms of juglone indicated that it acts through multiple pathways, including NO release (dominate endothelium-dependent) and K^+ channel activation in addition to antagonistic effects on Ca^{2+} movements (dominate endothelium-independent). These synergistic vasodilatory mechanisms explain the antihypertensive effect of juglone observed in high salt-induced hypertensive rats.

Conclusion

In conclusion, this study has identified juglone an important natural phenolic compound as a potential antihypertensive agent. The antihypertensive effect of juglone is the outcome of vasodilatation that is mediated through endothelium-dependent (NO pathway) and endothelium-independent (K^+ channel activations) mechanisms, in addition to the inhibitory effect on Ca^{2+} movements. Further electrophysiological studies would provide more insight into the cellular aspects of these mechanisms.

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Conflicts of Interest

The authors declare no conflict of interest

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