Punica granatum attenuates Angiotensin-II induced hypertension in Wistar rats

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Abstract: Acute subcutaneous administration of angiotensin-II (Ang II-150 μg/kg) causes a rise in blood pressure in normal Wistar rats. Administration of Punica granatum juice extract (PJ- 100 mg/kg and 300 mg/kg; p.o.) for 4 weeks in angiotensin-II treated rats significantly (P<0.05) reduced the mean arterial blood pressure and vascular reactivity changes to various catecholamines. PJ treatment significantly (P<0.05) decreased the serum levels of ACE and the levels of thiobarbituric acid reactive substances (TBARS); while enzyme activity of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSH) in kidney tissue showed a significant elevation in PJ treated Ang II induced hypertensive rats. The cumulative concentration response curve (CCRC) of angiotensin-II was shifted towards right in rats treated with PJ using isolated strip of ascending colon. The results suggest that Punica granatum juice extract could prevent the development of high blood pressure induced by angiotensin-II probably by combating the oxidative stress and antagonizing the physiological actions of Ang II.

Keywords: Angiotensin II, Punica granatum, flavonoids, oxidative stress, colon.

1. Introduction
Hypertension and resulting end-organ damage such as stroke, heart failure, myocardial infarction, and renal disease rank among the leading causes of death in industrialized nations. [1] The octapeptide hormone angiotensin- II (Ang I to 8; Ang II) is considered to be the main effector of renin–angiotensin system (RAS) in the control and regulation of blood pressure.[2] Most of the physiological actions of Ang II in the cardiovascular system are thought to be mediated by the activation of AT₁ receptors.[3] It has been reported that there is a direct correlation between state of Ang II receptors in the diencephalon and development of hypertension; that is, upregulation of Ang II receptors have been linked to induction of deoxycorticosterone (DOCA) induced hypertension and genetically induced hypertension.[3, 4] Angiotensin has been identified as major modulator of endothelial function. Endothelial cells produce vasoconstrictor substances such as endothelin-I and vasodilator substances such as nitric oxide.[5] Angiotensin-II also activates superoxide anion production and due to interaction of superoxide anion with NO, the net effective NO availability appears to be reduced.[6] All the components of the RAS, including angiotensinogen, prorennin, renin, angiotensin-converting enzyme (ACE), and the angiotensin receptors are present not only in the circulation but also in certain tissues including the heart, kidney, and brain.[7] The human body produces reactive oxygen species (ROS) such as superoxide anion \( \text{O}_2^- \), hydroxyl radicals (\( \cdot\text{OH} \)), and peroxo radicals (ROO·) by many enzymatic systems through oxygen consumption. [8] In small amounts these ROS can be beneficial as signal transducers [9] and growth regulators. [10] However during oxidative stress, large amounts of these ROS can be produced and may be dangerous because of their ability to attack numerous molecules, including proteins and lipids. [11] Damages
mediated by free radicals result in the disruption of membrane fluidity, protein denaturation, lipid peroxidation, oxidative DNA, and alteration of platelet function which have generally been considered to be linked with many chronic health problems such as cancer, inflammation, aging and atherosclerosis.\[13\] The rind of *Punica granatum* fruit (commonly called pomegranate) is used as an astringent, digestive, cardiotoxic, stomachic and highly effective in chronic diarrhoea and dysentery, dyspepsia, colitis, piles and uterine disorders.\[13, 14\] Pomegranate juice administered to patients caused a significant drop in blood pressure.\[15\] Pomegranate is rich in antioxidant of polyphenolic class which includes tannins and anthocynins\[16\] and flavonoids\[17, 18\]. Content of soluble polyphenols in pomegranate juice varies within the limits of 0.2–1.0%, depending on variety and include mainly tannins, ellagic tannins, anthocyanins, catechins, gallic and ellagic acids.\[19, 20\] There are many evidences that flavonoids interact with various biological system.\[21\] Flavonoid intake appears inversely related with mortality from coronary heart disease in epidemiological studies.\[22\] Despite the fact that *Punica granatum* juice has antioxidant properties, its antihypertensive activity against Ang II induced hypertension has not been studied. In view of this, the present study was designed to investigate if oral administration of PJ extract for 4 weeks attenuates Ang II induced hypertension.

2. Materials and methods

2.1 Preparation of extract

1 Kg of Pomegranate fruits were purchased from local market. The seeds were isolated and were ground to obtain juice. The juice was air dried and concentrated under reduced pressure to obtain 32 g, corresponding to a yield of 3.2% w/w. The total flavanoid content of pomegranate juice extract was found to be 455.7± 6.33 mg rutin equivalent/ g of extract.\[23\] The total phenolic content of pomogranate juice extract was found to be 94.15± 9.93 mg gallic acid equivalent/ g of extract.\[24\] An appropriate concentration of the extracts was made in distilled water. The phytoconstituents present in the crude extract are flavonoids, tannins and anthocynins.\[25\]

2.2 Experimental Animals

Male albino rats (Wistar strain) weighing between 150-200 g were obtained from Serum Institute, Pune. Animals were housed into groups of five under standard laboratory conditions of temperature 25 ± 1°C with free access to food (Hindustan Lever, India) and water. The experiments were performed during the light portion (09-14 h). The experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, and approved by the Institutional Animal Ethical Committee.

2.3 Drugs and chemicals

Adrenaline (Adr), Nor adrenaline (NA), Phenylephrine (PE), Angiotensin II (Ang II), 5- Hydroxytryptamine (5-HT), Urethane, thiobarbituric acid (TBA) and 5, 5'-dithiobisnito benzoic acid (DTNB) were purchased from Sigma-Aldrich, Mumbai. All chemicals for sensitive biochemical assays were obtained from Sigma Chemicals Co. India and Hi media Chemicals, Mumbai, India. Distilled water was used for biochemical assays. All drug solutions were freshly prepared in saline before each experiment. PJ extract was dissolved in distilled water and administered orally.

2.4 Experimental Design

A total of 30 animals were randomized and divided into six groups of five each.

**Group I:** Control: Animals received 1ml of distilled water, p.o for 4 weeks.

**Group II:** Animals received PJ (100mg/kg, p.o.) for 4 weeks.

**Group III:** Animals received PJ (300mg/kg, p.o.) for 4 weeks.

**Group IV:** Animals received Angiotensin II (150µg/kg, s. c.) 2 hours prior to vascular reactivity.

**Group V:** Animals received PJ (100mg/kg, p.o.) for 4 weeks and Angiotensin II at the end of treatment schedule 2 hours prior to vascular reactivity.

**Group VI:** Animals received PJ (300mg/kg, p.o.) for 4 weeks and Angiotensin II at the end of treatment schedule 2 hours prior to vascular reactivity.\[26\]

2.5 Measurement of Blood Pressure by invasive (direct) method and recording of vascular reactivity

After completion of treatment schedule rats from each group were anesthetized with urethane (120mg/100gm). Femoral vein was cannulated with fine polyethylene catheter for administration of drugs. Tracheostomy was performed and blood pressure was recorded from left common carotid artery using pressure transducer by direct method on Chart data system (PowerLab/4SP, ADInstruments, Australia). Heparinised saline (100 IU/ml) was filled in the transducer and in the fine polyethylene catheter cannulated to the carotid artery to prevent clotting. After 30 min of stabilization, heart rate, mean arterial blood pressure (MABP) and vascular reactivity to Adr (1µg/kg), NA (1µg/kg), PE (1µg/kg), Ang II (25 ng/kg) and 5-HT (1µg/kg) were recorded.

2.6 Antioxidants study

The kidneys of the rats from individual group was dissected out, washed with ice-cold saline and weighed. Ten percent homogenate was prepared in 0.1 M Tris–buffer, pH 7.4. The homogenate was centrifuged at 15,000 x g for 20 min. The supernatants were used for measuring activity of enzymes, superoxide dismutase (SOD) and catalase (CAT),
glutathione reductase (GSH) and thiobarbituric acid reactive substances (TBARS).

2.6.1. Superoxide dismutase (SOD) activity
The assay of SOD was based on the ability of SOD to inhibit spontaneous oxidation of adrenaline to adrenochrome. To 0.05 ml supernatant, 2.0 ml of carbonate buffer and 0.5 ml of EDTA were added. The reaction was initiated by addition of 0.5 ml of epinephrine and the autooxidation of adrenaline (3X 10^{-4} M) to adrenochrome at pH 10.2 was measured by following the change in OD at 480 nm. The change in optical density every minute was measured at 480 nm against reagent blank. The results are expressed as units of SOD activity (U/g wet tissue). One unit of SOD activity induced approximately 50% inhibition of adrenochrome.

2.6.2. Catalase (CAT) activity
The Catalase activity assay was based on the ability of CAT to induce the disappearance of hydrogen peroxide. The reaction mixture consisted of 2 ml phosphate buffer (pH 7.0), 0.95 ml of hydrogen peroxide (0.019 M) and 0.05 ml supernatant in final volume of 3 ml. Absorbance was recorded at 240 nm every 10 sec for 1 min. One unit of CAT was defined, as the amount of enzyme required decomposing 1 µmol of peroxide per min, at 25°C and pH 7.0. The results were expressed as units of CAT activity (U/g wet tissue).

2.6.3. Estimation of Reduced glutathione (GSH)
Reduced glutathione was determined by the method of Ellman. 1 ml of homogenate was added to 1 ml of 10% trichloroacetic acid (TCA) and centrifuged. 1.0 ml of supernatant was treated with 0.5 ml of Ellman’s reagent (19.8 mg of 5, 5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 1.0% sodium citrate) and 3 ml of phosphate buffer (pH 8.0). The color developed was measured at 412 nm. The results were expressed as nM/mg of wet tissue of GSH activity.

2.6.4. Estimation of lipid peroxidative indices
Lipid peroxidation as evidenced by the formation of thiobarbituric acid reactive substances (TBARS) was measured by the method of Nechaus and Samuelsson. 0.1 ml of homogenate (Tris-HCL buffer, pH 7.5) was treated with 2 ml of 1:1:1 ratio TBA-TCA-HCl reagent (Thiobarbituric acid 0.37%, 0.25N HCl and 15% TCA) and placed in water bath at 105°C for 30 min at 37°C with shaking. The enzymic reactions were terminated by addition of 0.25 ml of 1N HCl; HCl was added before the serum in zero-time control assays. The hippuric acid formed by action of the ACE on HHL is extracted from acidified solution into 1.5 ml of ethyl acetate by vortex mixing. After a brief centrifugation, 1 ml aliquots of each ethyl acetate layer was transferred to a clean tube and heated at 120°C for 30 min. The hippuric acid was then re-dissolved in 1 ml distilled water and the amount formed was determined from its absorbance at 228 nm.

2.9 Statistics
The mean ± SEM values were calculated for each group. One-way ANOVA followed by Dunnett’s multiple comparison tests were used for statistical analysis. Values of p<0.05 were considered statistically significant.

3. Results
3.1 Mean Arterial Blood Pressure (MABP) and vascular reactivity
The effects of various treatment groups on mean arterial blood pressure and vascular reactivity to various drugs are shown in Fig.1 and Fig.2 respectively. Angiotensin II treatment (150µg/kg, s. c., 2 hours prior to vascular reactivity) was associated with a significant increase (p<0.05) in MABP. Chronic treatment with PJ (100 mg/kg and 300 mg/kg) significantly reduced the elevation of blood pressure in Angiotensin II treated rats in invasive blood pressure measurements. Treatment with PJ (100 mg/kg, p.o.) alone did not produce any significant change when compared to vehicle treated animals.

3.2 Antioxidant enzymes
Table 1 shows the levels of antioxidant enzymes SOD, CAT, GSH and TBARS in various treatment groups. The levels of SOD, CAT, and GSH enzymes were significantly (p<0.05) decreased and those of TBARS were significantly (p<0.05) increased in kidney tissue of Ang II treated hypertensive rats when compared to control rats. The levels of antioxidant enzymes- SOD, CAT and GSH were significantly (p<0.05) increased and those of TBARS were significantly (p<0.05) decreased in kidney tissue of Ang II treated...
hypertensive rats that received pomegranate juice (100 mg/kg/day and 300 mg/kg/day, p.o.) for 4 weeks as compared to Ang II treated rats.

3.3 In-vitro study
Chronic administration of PJ (100 and 300 mg/kg/day, p.o.) for 4 weeks in Ang II treated rats significantly (p<0.05) shifted the cumulative concentration response curve (CCRC) of Ang II to the right with suppression of maxima as compared to CCRC of Ang II treated rats on isolated ascending colon (Fig. 3).

3.4 Serum ACE activity
The serum ACE activity levels in PJ (100 mg/kg and 300 mg/kg, p.o.) were significantly (P<0.05) lower than vehicle treated control rats (Fig 4). Pre-treatment with PJ (100 mg/kg/day and 300 mg/kg/day, p.o.) for 4 weeks in Ang II treated rats significantly (P<0.05) lowered serum ACE activity as compared to Ang II treated group (Fig 4.).

4. Discussion
The renin-angiotensin system (RAS) with its effector hormone -angiotensin II is a key regulator of blood pressure (BP). Several actions leading to an increase in BP are elicited by Ang II via the angiotensin AT₁ receptor (AT₁R), including vasoconstriction, renal sodium reabsorption (directly or through the release of aldosterone), vasopressin release, and facilitation of sympathetic nerve activity. Ang II via the AT₁R also significantly contributes to end-organ damage. Interference with the RAS, by AT₁R blockade or ACE inhibition, is a potent way of reducing BP and preventing end-organ damage. [33]
The pressor responses to Adr, NA, PE, Ang II and 5-HT were significantly (p<0.05) increased in Ang II treated hypertensive rats as compared to control rats. The pressor responses to Adr, NA, PE, Ang II and 5-HT were significantly (p<0.05) reduced in case of Ang II treated rats that received PJ extract (100 and 300 mg/kg/day, p.o.) for 4 weeks as compared to only Ang II treated rats. The pressor responses to Adr, NA, PE, Ang II and 5-HT were significantly (p<0.05) reduced in rats that received PJ extract (300 mg/kg/day, p.o.) for 4 weeks as compared to control rats. The basal arterial blood pressure was not altered in case of PJ (100 mg/kg/day and 300 mg/kg/day, p.o.) treated rats as compared to control rats. Thus sympathetic over activity as manifested by elevated vascular reactivity to various drugs further exemplifies the role of angiotensin in the development of hypertension. In our study, we have observed that there was a significant correlation between the reduction in serum ACE activity and vascular reactivity to various drugs and thus reduction in ACE activity may contribute to lowering of blood pressure. It is known that reactive oxygen species (ROS) contribute to the pathogenesis of numerous cardiovascular diseases including hypertension, atherosclerosis, cardiac hypertrophy, heart failure and retinosis, NAD(P)H oxidase being the predominant source of ROS. [34] Activation of this enzyme leads to a variety of intracellular signaling events that ultimately promote endothelium dysfunction, vascular smooth muscle cell proliferation, pro-inflammatory genes expression and reconstruction of the extracellular matrix. [35] Ang II, via activation of the AT₁ receptor, stimulates NAD(P)H oxidases activity in vascular smooth muscle cells increasing superoxide anion formation and nitric oxide inactivation, effects associated with the pathogenesis of hypertension. [36, 37]
Furthermore, many Ang II effects seem to be mediated by enhanced ROS production. [38]
In humans, hypertension is also considered a state of oxidative stress that can contribute to the development of atherosclerosis [39] and other hypertension-induced organ damage. [40] SOD, CAT and GSH are the three primary antioxidant enzymes among the endogenous systems for removal of reactive oxygen species. [41, 42] The three enzymes are also the first set of defences for the body against oxidant-induced cytotoxic challenges. Assessment of antioxidant activities and lipid peroxidation byproducts in hypertensive subjects indicate an excessive amount of ROS and a reduction of antioxidant mechanism activity in both blood as well as in several other cellular systems. [43]
Present study reveals that there is a significant decrease in antioxidant enzyme levels of SOD, CAT, GSH and increase in the level of TBARS in kidneys of rats treated with angiotensin-II. This is an indication of oxidative stress generated by angiotensin-II. Pre-treatment with pomegranate juice (PJ) restored the antioxidant enzyme level and decreased TBARS level, which in turn indicate the protective effect of PJ against oxidative stress. Increased serum ACE activity is associated with enhanced susceptibility to lipid peroxidation [44] and hence the inhibitory effect of pomegranate juice on serum ACE activity can further contribute to an antioxidant environment.
Several lines of evidence support the hypothesis that Ang II stimulates the production and release of ET. [45, 46] The reduced vascular response to angiotensin-II in PJ treated rats may indicate inhibition of endothelin-1 receptors. Moreover by shifting the CCRC of Ang II towards the right by PJ extract (100 mg/kg and 300 mg/kg/day, p.o.) indicate inhibitory effect on Ang II receptors. Thus, pomegranate juice significantly reduced mean arterial blood pressure, vascular reactivity changes to various drugs, and prevented oxidative damage in angiotensin model of hypertension. The antioxidant activity, serum ACE inhibition activity and blockade of angiotensin receptor may be partly responsible for its antihypertensive action.
Acknowledgements
The authors are grateful to Prof. V.M. Aurangabadkar, Principal, MGV’s Pharmacy College, Nashik for providing laboratory facilities.

Table 1. Effect of PJ extract (100mg/kg/day, 300 mg/kg/day, p.o., for 4 weeks) on antioxidant enzymes and lipid peroxidation index in Ang II treated rats

<table>
<thead>
<tr>
<th>Sr.no.</th>
<th>Treatment Groups</th>
<th>SOD (U/mg of wet tissue)</th>
<th>CAT (×10⁻³ U/mg of wet tissue)</th>
<th>GSH (nM/mg of wet tissue)</th>
<th>TBARS (μM/mg of wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>124.2 ± 5.968</td>
<td>34.32±1.59</td>
<td>72.57±4.24</td>
<td>1.42±0.039</td>
</tr>
<tr>
<td>2</td>
<td>PJ (100)</td>
<td>193.6 ± 9.357</td>
<td>74.6±1.99</td>
<td>63.97±1.96</td>
<td>0.25±0.013</td>
</tr>
<tr>
<td>3</td>
<td>PJ (300)</td>
<td>250.5 ± 19.04</td>
<td>95.84±1.03</td>
<td>74.66±3.09</td>
<td>0.15±0.0085</td>
</tr>
<tr>
<td>4</td>
<td>Ang II</td>
<td>85.57 ± 4.407*</td>
<td>17.85±0.79*</td>
<td>45.99±2.44*</td>
<td>1.81±0.049*</td>
</tr>
<tr>
<td>5</td>
<td>PJ (100) + Ang II</td>
<td>171.5 ± 6.849#</td>
<td>45.77±1.99#</td>
<td>49.63±1.91</td>
<td>0.95±0.024#</td>
</tr>
<tr>
<td>6</td>
<td>PJ (300) + Ang II</td>
<td>215.7 ± 4.155#</td>
<td>62.7±1.21#</td>
<td>86.8±1.63#</td>
<td>0.49±0.019#</td>
</tr>
<tr>
<td></td>
<td>F (5,24)</td>
<td>38.53</td>
<td>353.37</td>
<td>33.62</td>
<td>526.54</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM, n=5. All data are subjected to One-Way ANOVA followed by Dunnett’s test. * p<0.05 when compared to Control and # p<0.05 when compared to Ang II group.

Fig. 1-Effect of PJ extract (100mg/kg/day, 300 mg/kg/day, p.o., for 4 weeks) on MABP in Ang II treated rats

Values are expressed as mean ± SEM, n=5. All data are subjected to One-Way ANOVA followed by Dunnett’s test. * p<0.05 when compared to Control and # p<0.05 when compared to Ang II group.
Fig. 2- Effect of PJ extract (100mg/kg/day, 300 mg/kg/day, p.o., for 4 weeks) on vascular reactivity changes to various drugs: Adrenaline (Adr) 1µg/kg, Nor adrenaline (NA) 1µg/kg, Phenylepherine (PE) 1µg/kg, Angiotensin-II (Ang-II) 25 ng/kg, 5-Hydroxytryptamine (5-HT) 1µg/kg.

Values are expressed as mean ± SEM, n=5. All data are subjected to One-Way ANOVA followed by Dunnett’s test. * p<0.05 when compared to Control and # p<0.05 when compared to Ang II group.

Fig. 3- Effect of PJ extract (100mg/kg/day, 300 mg/kg/day, p.o., for 4 weeks) on CCRC of Angiotensin-II on isolated rat ascending colon in Ang II treated rats.

Values are expressed as mean ± SEM, n=5. All data are subjected to One-Way ANOVA followed by Dunnett’s test. * p<0.05 when compared to Control and # p<0.05 when compared to Ang II group.
Serum ACE activity

Fig. 4- Effect of PJ extract (100mg/kg/day, 300 mg/kg/day, p.o., for 4 weeks) on Serum ACE activity in Ang II treated rats

Values are expressed as mean ± SEM, n=5. All data are subjected to One-Way ANOVA followed by Dunnett’s test. * p<0.05 when compared to Control and # p<0.05 when compared to Ang II group.

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