Implication of *Punica granatum* Leaves in Experimental Cardiac Hypertrophy

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The present study has been designed to explore the beneficial effect of *Punica granatum* leaves in experimental cardiac hypertrophy in rats. *Punica granatum* L., (Family: Punicaceae) is used in Indian Unani medicine for treatment of diabetes mellitus, cytotoxicity and in wound healing owing to its anti-oxidant activity. The male wistar rats were anaesthetized with thiopentone sodium and were subjected to Partial Abdominal Aortic Constriction (PAAC) for 4 weeks. The treatment with ethanolic extract of leaves of *P. granatum* (EPG) (200 mg/kg and 400 mg/kg) was started three days before surgery and it was continued for 4 weeks after surgery. The development of left ventricular (LV) hypertrophy was assessed by measuring ratio of LV weight to body weight (LVW/BW), LV wall thickness (LVWT), LV protein content, LV collagen content and LV RNA concentration. Further, mean arterial blood pressure (MABP) was recorded. Moreover, oxidative stress in heart was measured by CK-MB and LDH estimation. The PAAC significantly increased the ratio of LV weight to body weight, LV wall thickness, LV protein content, LV collagen content and LV RNA concentration. Further PAAC significantly increased MABP and oxidative stress. The EPG (400mg/kg) markedly attenuated PAAC induced increase in LV hypertrophy, MABP and LV oxidative stress. These results implicate the role of *Punica granatum* leaves in PAAC induced oxidative stress and pathological cardiac hypertrophy.

**Keyword:** Aortic Constriction, Cardiac Hypertrophy, *Punica granatum*.

1. INTRODUCTION: Pomegranate (*Punica granatum* L.) (*P. granatum*) belongs to the Punicaceae family. It is one of the important and commercial horticultural fruits which are generally very well adapted to the Mediterranean climate. It is native to India, Pakistan and Afghanistan and also cultivated in some parts of USA (California), China, Japan and Russia. Pomegranate fruits are consumed fresh or processes as juice, jellies and syrup for industrial production [4,5]. Different parts of its tree (leaves, fruits and bark skin) have been used traditionally.
for their medicinal properties and for other purposes such as in tanning. It is proved to have high antioxidant activity [6] and good potency in the treatment of heart failure [7].

Cardiac hypertrophy is a major predictor of progressive heart disease and an adverse prognosis. It is recognized as an adaptive process to a variety of physiological and pathological conditions like ischemic heart disease, hypertension and heart failure. Hence, it is a well-established risk factor for cardiovascular mortality in patients [8,9]. The induction, progression, and subsequent detrimental effects of cardiac hypertrophy is characterized by an increment in cardiomyocyte size, increased protein synthesis and changes in the organization of sarcomeric structure [9].

The management of heart failure without any side effects is still a challenge to the medical system. Recent years has witnessed a renewed interest in plants as pharmaceuticals because they synthesize a variety of secondary metabolites with antioxidant potential which can play a major role in protection against molecular damage induced by reactive oxygen species. Hence, compounds with antioxidative properties would be useful agents for prevention of cardiac failure. The role of PPAR agonists in various cardiovascular complications such as vascular endothelial dysfunction, myocardial ischemia reperfusion-induced injury, hypertension, and hypertension-induced cardiac hypertrophy has been explored [15]. Moreover, PPAR-dual agonists such as punica granatum recently have been reported to exhibit beneficial effect in diabetes and vascular endothelial dysfunction [9,10]. However, the effect of PPAR dual agonists in cardiac hypertrophy still remains unexplored. Hence, the present study has been designed to investigate the usefulness of P. granatum flower extract in cardiac hypertrophy in rats.

2. MATERIALS AND METHODS
a. Plant material
The flowers of P. granatum were collected in and around Trichy, Tamil Nadu, India. The flowers (500 g) were shade dried, powdered and then soaked in 1500 mL of 95% ethanol overnight. After filtration, the residue obtained was resuspended in 95% ethanol for 48 h and filtered. The filtrates obtained were combined and the solvent was evaporated in a rotavapour at 40-50 °C under reduced pressure. A semisolid brown powder of the extract was obtained (20% w/w) and stored until further use. A known amount of the residual extract was suspended in distilled water and was orally administered during the experiment.

b. Experimental animals
Male Wistar albino rats weighing 200 to 230 g were used in the present study. They were maintained on rat feed and water ad libitum and were exposed to a 12-hour light and 12-hour dark cycle. The Institutional Animal Ethics Committee approved the experimental protocol and care of the animals was carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg. No. 10/2010/CPCSEA).

c. Phytochemical screening
Chemical tests were carried out on the ethanolic extracts using the standard procedures to identify the presence of glycosides, saponins, flavonoids, alkaloids, tannins, triterpenids, phytosterols, carbohydrates, fats, proteins and volatile oils [7,8].

d. Experimental Design
The present study comprised six groups with each comprising of 12 to 14 animals. In Group 1 (sham operated), surgery was performed to expose the abdominal aorta but it was not constricted. In Group 2 (PAAC group), surgery was performed and rats were subjected to partial abdominal aortic constriction. In Group 3 (sham operated low treatment group), rats were subjected to surgery without aortic banding and were treated with ethanolic extract of Punica granatum (200 mg/kg per day orally) for three days before surgery and it was continued for 4 weeks after surgery. In Group 4 (sham operated high treatment group), rats were subjected to surgery without aortic banding and were treated with...
ethanolic extract of *Punica granatum* (400 mg/kg per day orally) for three days before surgery and it was continued for 4 weeks after surgery. In Group 5 (treatment low dose), rats were administered ethanolic extract of *Punica granatum* (200 mg/kg per day orally) for three days before PAAC and it was continued for 4 weeks after surgery. In Group 6 (treatment high dose), rats were administered ethanolic extract of *Punica granatum* (400 mg/kg per day orally) for three days before PAAC and it was continued for 4 weeks after surgery.

e. **Morphologic Assessment of Cardiac Hypertrophy**
At the end of the four weeks, the rats were euthanized and hearts were excised and washed with cold saline. The left ventricular weight including interventricular septum and right ventricular weight was noted separately and expressed as milligrams per gram of body weight. The left ventricle was divided into three parts and wall thickness of each slice was noted at eight different parts using an ocular micrometer. The mean value of all three slices was taken and expressed in millimeters [18].

f. **Estimation of Collagen Content**
The left ventricular collagen content was estimated biochemically in terms of hydroxyproline concentration as previously described [5, 14]. The hydroxyproline content was expressed as milligrams per gram dry weight of left ventricle.

g. **Biochemical Assessment**
The left ventricle was stored at –80°C in liquid nitrogen for quantitative estimation of biochemical parameters. The left ventricle was homogenized and protein content was determined spectrophotometrically at 750 nm by Lowry's method [21] and expressed as mg/g of left ventricular weight.
The RNA was extracted from homogenized left ventricular tissues using method of Chomczynski and Sacchi [22]. RNA concentration was estimated spectrophotometrically at 260 nm. One absorbancy unit at 260 nm in a 1 cm light path cuvette was assumed to be equal to 40 μg/mL of RNA. The purity of RNA was assessed by determining the ratio of absorbance at 260 and 280 nm and the ratio was more than 1.8.
The DNA was extracted from homogenized left ventricular tissue using method of Ausubel et al [22]. The concentration of DNA was determined spectrophotometrically at 260 nm.

h. **Measurement of Mean Arterial Blood Pressure**
The mean arterial blood pressure (MABP) in carotid artery of anesthetized rats was recorded using a pressure transducer (BIOPAC System, Goleta, CA) just before morphologic and biochemical studies [18].

i. **Assessment of Oxidative Stress**
Left ventricle from freshly excised heart was minced and homogenized in 0.1 M Tris HCl buffer (pH 7.4, 1:10 w/v) using a Teflon homogenizer. The clear supernatant of homogenate was used to estimate CK-MB and LDH after centrifugation at 800 g for 10 minutes.

j. **Drugs and Chemicals**
Atorvastatin was obtained as a kind gift from Ranbaxy Lab. Ltd, Gurgaon, India. Folin-Ciocateu’s Phenol Reagent, Tris buffer, agarose and Chloramine T were purchased from Loba Chemie, Coimbatore, India. CK-MB kit was purchased from labkit, Spain and LDH kit from Merck Ltd., Mumbai, India. All other reagents used in the present study were of analytical grade.

k. **Statistical Analysis**
The results were expressed as mean ± standard deviation. The data obtained from various groups were analyzed using one-way analysis of variance followed by Tukey's multiple range test. The P value <0.05 was considered to be statistically significant.

3. **RESULTS**
There was no significant change in body weight of rats subjected to sham surgery and partial abdominal aortic constriction with or without the *Punica granatum* treatment.
a. Effects of Ethanolic extract of *Punica granatum* leaves Treatment on Morphologic and Haemodynamic Parameters

No significant change in body weight was observed in rats subjected to partial abdominal aortic constriction (PAAC) (Table 3). PAAC resulted in a significant increase in ratio of left ventricular weight to body weight (LVW/BW) and left ventricular wall thickness (LVWT) as compared to the control group and sham group. However, treatment with *Punica granatum* (200 and 400 mg/kg per day orally) significantly attenuated PAAC-induced increase in LVW/BW and LVWT in a dose-dependent manner (Table 3). PAAC significantly increased Mean arterial blood pressure (MABP) which was markedly attenuated in a dose dependent manner by EMC treatment (Table 1).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham Operated</th>
<th>PAAC</th>
<th>PAAC +EPG(200mg/kg)</th>
<th>PAAC+EPG(400mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>269.4±10.53</td>
<td>267.7 ±11.62a</td>
<td>257.4 ±12.24b</td>
<td>256.2 ±9.62b</td>
</tr>
<tr>
<td>LVW/BW (mg/g)</td>
<td>1.89±0.07</td>
<td>2.88±0.06a</td>
<td>2.34±0.06b</td>
<td>1.92±0.04b</td>
</tr>
<tr>
<td>LVWT (mm)</td>
<td>2.18±0.07</td>
<td>3.77±0.10a</td>
<td>2.68±0.07b</td>
<td>2.34±0.06b</td>
</tr>
<tr>
<td>MABP (mmHg)</td>
<td>108.2±2.7</td>
<td>165.1±3.6a</td>
<td>142.04±3.1b</td>
<td>120.2±2.6b</td>
</tr>
<tr>
<td>Protein Content</td>
<td>121.5±5.3</td>
<td>175.7±4.2a</td>
<td>135.3±3.2b</td>
<td>127.5±3.1b</td>
</tr>
<tr>
<td>Collagen Content</td>
<td>1.67±0.06</td>
<td>4.62±0.07a</td>
<td>3.17±0.07b</td>
<td>2.52±0.06b</td>
</tr>
<tr>
<td>RNA Conc.</td>
<td>2.75±0.03</td>
<td>3.42±0.05a</td>
<td>3.14±0.02b</td>
<td>2.88±0.02b</td>
</tr>
</tbody>
</table>

PAAC, partial abdominal aortic constriction; EPG, Ethanolic extract of *Punica granatum*; MABP, mean arterial blood pressure; BW, body weight; LVW, left ventricular weight; LVWT, left ventricular wall thickness. Values are mean ± SEM a: p<0.05 vs sham control; b: p<0.05 vs PAAC control

Table 2. Effect of *Punica granatum* treatment on CK-MB and LDH levels

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham Operated</th>
<th>PAAC</th>
<th>PAAC +EPG(200mg/kg)</th>
<th>PAAC+EPG(400mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK-MB (IU/L)</td>
<td>86.4±0.07</td>
<td>199.9±0.06a</td>
<td>144.5±0.06b</td>
<td>112.6±0.04b</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>81.7±0.07</td>
<td>160.1±0.10a</td>
<td>121.8±0.07b</td>
<td>96.2±0.06b</td>
</tr>
</tbody>
</table>

PAAC, partial abdominal aortic constriction; EPG, Ethanolic extract of *Punica granatum*. Values are mean ± SEM a: p<0.05 vs sham control; b: p<0.05 vs PAAC control

b. Effect of Ethanolic extract of *Punica granatum* leaves Treatment on Biochemical Parameters

PAAC significantly increased protein content and RNA concentration in left ventricle. *Punica granatum* (200 and 400 mg/kg per day orally) treatment significantly attenuated PAAC induced increase in protein content and RNA concentration. (Table 1)

c. Effect of Ethanolic extract of *Punica granatum* leaves Treatment on Left Ventricular Collagen Content

A significant increase in left ventricle collagen content was observed in the rats subjected to PAAC as compared with rats in the sham treated group. The treatment with *Punica granatum* significantly attenuated PAAC-induced increase in collagen content in a dose-dependent manner (Table 1).

d. Effect of Ethanolic extract of *Punica granatum* leaves on CK-MB and LDH

PAAC produced significant increase in CK-MB and LDH levels in the left ventricle. *Punica*
*Punica granatum* (200 and 400 mg/kg per day orally) significantly reduced PAAC induced increase in CK-MB and LDH levels (Table 2).

4. DISCUSSION
The partial abdominal aortic constriction (PAAC) model used in this study [16, 17] involves placing a suture on the aorta below the diaphragm leading to pressure overload induced left ventricular hypertrophy. PAAC subjected for 4 weeks produced significant cardiac hypertrophy as witnessed by increased ratio of left ventricular (LV) weight to body weight, LV wall thickness, LV protein content, LV collagen deposition and LV RNA concentration [19-22]. *Punica granatum* treatment significantly attenuated PAAC induced increase in LVW/BW, LVWT, LV protein content, LV RNA concentration and collagen deposition in a dose-dependent manner. Cardiac stress or injury results in increase in cardiac marker enzymes. Cardiac marker enzymes are the enzymes which are very sensitive to any pathophysiological changes to the heart and they increase during cardiac disorders and diseases. As CK-MB and LDH are very good marker enzymes to detect the cardiac abnormalities, these have been estimated in our present study. [18, 22] PAAC induced cardiac hypertrophy has been noted to increase CK-MB and LDH levels in left ventricle. Moreover, *Punica granatum* attenuated PAAC induced increase in CK-MB and LDH levels perhaps due to PPAR agonist action. The abdominal aortic constriction may be initially responsible to increase MABP, which has been observed to return to the normal value after about one and a half-hour of PAAC. However, MABP has been noted to increase gradually and attain peak level after 3-4 wk of PAAC. The marked increase in MABP in PAAC model may be due to pathological cardiac hypertrophy as reported recently [25,26]. The PAAC induced increase in MABP has been noted to be attenuated by *Punica granatum* treatment. It suggests that PAAC induced cardiac hypertrophy may be responsible to increase MABP. *Punica granatum* is reported as PPAR dual agonist and is able to inhibit hypertrophy probably through PPARα/γ dual agonistic mechanism. PPARα and PPARγ downregulation is responsible for the progression of cardiac hypertrophy which is observed by increased expression of fetal genes, increase in number of inflammatory cytokines, increased oxidative stress and decreased fatty acid oxidation [15-20]. Treatment with EPG activates PPARα/γ and is able to reverse cardiac hypertrophy which is shown by decreased oxidative stress, cytokines and increased fatty acid oxidation. Hence, in the present study, we have focused on the effects of *Punica granatum* on cardiac hypertrophy in rats. To the best of our knowledge, it is the first study to explore the effect of PPAR- α / γ dual agonist in PAAC-induced cardiac hypertrophy. Hence, on the basis of this discussion, it may be concluded that *Punica granatum* exhibits pleiotropic cardiac effects in PAAC-induced cardiac hypertrophy in a dose-dependent manner possibly through its PPAR (Peroxisome proliferator activated receptor) dual agonist action.

5. REFERENCES
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