Chrysin pretreatment improves mitochondrial enzymes and angiotensin converting enzymes in L-NAME induced hypertensive rats

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Abstract
Hypertension is one among the important factors that causes cardiovascular disorders. N\textsuperscript{\textregistered}-nitro-L-arginine methyl ester (L-NAME) induces hypertension by blocking nitric oxide (NO) synthesis. Aim of present study was to investigate the effects of chrysin is one of major flavnoids, on L-NAME-induced hypertensive rats. Induces hypertension in adult male wistar rats weighing 180-220 g by oral treated of L-NAME (40 mg/kg/day) dissolved in drinking water daily for 8 weeks. Experimental rats were oral treated with chrysin (25 mg/kg b.w). Both the systolic and diastolic blood pressure of control and experimental rats were measured by tail cuff plethysmography system.

In our studies results showed an increase in the levels of systolic and diastolic blood pressure, heart, liver, kidney, body weight, plasma, and aortic Angiotensin converting enzymes (ACE), Sodium (Na\textsuperscript{+}), Chloride (Cl\textsuperscript{-}) levels in L-NAME treated rats. At the same time in L-NAME treated rats, there was a decrease in the levels of potassium (K\textsuperscript{+}), Plasma and heart- aortic nitrite/nitrate level, mitochondrial enzymes in liver such as Isocitrate dehydrogenase (ICDH), ß-ketoglutarate dehydrogenase (ß-KGDH), Succinate dehydrogenase (SDH) and Malate dehydrogenase (MDH). Chrysin treatment prevented the increase in systolic and diastolic blood pressure in the L-NAME-treated rats. Blood pressure (BP) reduction was interrelated with a reduction in Na\textsuperscript{+}, Cl\textsuperscript{-}, ACE activity and increased K\textsuperscript{+}, plasma and heart, aortic nitrite/nitrate levels. In contrast, L-NAME had opposite effects on mitochondrial liver enzymes, electrolytes, ACE and NO by treatment of chrysin.

Hence, the present findings might suggest that chrysin improve the balance between circulating nitric oxide and rennin-angiotensin system and beneficial effects on cardiovascular tissue through its ACE inhibitor activity.

Key words: Angiotensin II, Chrysin, hydroxyproline, ß-ketoglutarate dehydrogenase, nitric oxide, renin-angiotensin system.

Abbreviations: ACE - Angiotension converting enzymes; BP - Blood pressure; eNOS - endothelial nitric oxide synthase; ICDH - isocitrate dehydrogenase; L-NAME - N\textsuperscript{\textregistered}-nitro-l-arginine methyl; SDH - succinate dehydrogenase.

Introduction
Hypertension is acknowledged to be a ‘silent killer, which causes no signs and symptoms for so many years, even decades, until it finally damages certain vital organs [1]. Both Dyslipidemia and hypertension are the major risk factors for cardiovascular disease [2]. Hypertension is one of the risk factor responsible for cardiovascular diseases to accounts for about 54% of deaths through stroke and 47% of deaths.
through coronary heart disease in adults worldwide [3]. Changes in lifestyle and dietary habits in modern worlds may affect blood pressure (BP) and increases cardiovascular risk factors. Studies have reported that nitric oxide (NO) and angiotensin II (Ang II) are the primary key factors that regulate BP and cardiovascular tissue structures [4]. NO has been proved to have important role in the maintenance of normal BP and body fluid homeostasis [5]. Is one of the biologically active molecules that are produced from L-arginine by nitric oxide synthase (NOS). There exist three isoforms of these enzymes: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) [6]. NO helps to maintain vascular tone and is a regulator of platelet activation inhibitor of endothelial cell stimulation [7]. The chronic administration of NOs inhibitors provides an experimental animal model for hypertension [8]. Bioavailability of NO can be enhanced by inhibition of oxidative stress, and therefore the agents with antioxidant properties inactivating free radicals, increase NO bioavailability [9]. Endothelial dysfunction exhibits a reduction in NO bioavailability that may also cause cardiovascular remodeling through the activation of the RAS to produce Ang II. Treatment of hypertension with ACE inhibitors may inhibit the formation of Ang II and thus, suppress vasoconstriction, oxidative stress and lower BP [10]. The RAS or the rennin angiotensin-aldosterone system (RAAS) is a major endocrine/paracrine system that plays a vital role in BP regulation, fluid and electrolyte homeostasis. The RAAS regulates BP via angiotensin release and blood electrolyte content through release of aldosterone [11]. The ACE is a carboxypeptidase is involved in the conversion of angiotensin I (Ang I) into the biologically active Ang II [12]. ACE is important in the production of Ang II. Treatment with ACE inhibitors in rats with L-NAME induced hypertension was reported not only to reduce BP but also to prevent the progression of cardio renal remodeling [13].

Plant polyphenolic compounds the flavanoids consist of number of classes, as flavanols, flavones and flavans. A naturally occurring flavones, Chrysin (5, 7-dihydroxy flavone structure shown in Fig. 1) contained in flowers blue passion flower (Passiflora caerulea), Indian trumpet flower, as well as in edible of mushrooms [14], honey and propolis [15]. At the same time it possess antioxidant capacity, anti-inflammatory activity, anti-allergic, anti-cancer, antiestrogenic, anxiolytic [16], antihypertensive properties [17]. Chrysin has tyrosinase inhibitory activity, moderate aromatase inhibitory activity, and also inhibits estradiol-induced DNA synthesis. C-iso-prenylated hydrophobic derivatives of chrysin are potential P-glycoprotein modulators in tumour cells [18]. The earlier study showed that chrysin has antihypertensive effects, and reduces hepatic, renal damages and endothelial dysfunction in L-NAME induced hypertensive rats [19]. The present study aimed to evaluate the effect of chrysin on Electrolytes, mitochondrial liver enzymes, ACE, nitric oxide metabolites (nitrite and nitrate) in the L NAME induced hypertensive rats against the control and unsupplemented groups.

Materials and Methods

Chemicals: Chrysin and L-NAME was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade and obtained from E-Merck or HIMEDIA, Mumbai, India.

Animals: All the animal handling and experimental procedures were approved by the Institutional Animal Ethics Committee of Bharathidasan University and animals were cared

Fig. 1. Chemical structure of chrysin (5,7 dihydroxyflavone)
for in accordance with the Indian National Law on Animal Care and Use. Male Wistar rats (180-220 g) were purchased from the Indian Institute of Science, Bangalore, India. Rats were housed in plastic cages with filter tops under controlled conditions of a 12 h light-dark cycle, 50% humidity and temperature of 28°C. All rats received a standard pellet diet (Lipton Lever Mumbai, India) and water ad libitum.

**Induction of L-NAME induced hypertension:** L-NAME (40 mg/kg B.W) was dissolved in drinking water and given to rats at an interval of 24 h for 8 weeks. Mean arterial blood pressure (MAP) was measured using tail cuff method. MAP measurements were performed during the time of 1-8 weeks [16].

**Blood pressure measurements:** Systolic and diastolic blood pressures were determined by the tail-cuff method (IITC, model 31, Woodland Hills, CA, USA). The animals were placed in a heated chamber at an ambient temperature of 30-34°C for 15 minutes and from each animal one to nine blood pressure values were recorded. The lowest three readings were averaged to obtain a mean blood pressure. All recordings and data analyses were done using a computerized data acquisition system and software.

**Study design:** Animals were divided into four groups of six rats each and all were fed the standard pellet diet. The rats were grouped as given below.

- **Group I:** Control.
- **Group II:** Normal + Chrysin (25 mg/kg of B.W) after 4th week.
- **Group III:** L-NAME induced hypertension (40 mg/kg of B.W).
- **Group IV:** L-NAME induced hypertension (40 mg/kg of B.W) + Chrysin (25 mg/kg of B.W) after 4th week.

Chrysin (25 mg/kg of B.W) was administered orally once in a day in the morning for 4 weeks. Chrysin dose (25 mg/kg of B.W) based on our previous study. The compound was suspended in 2% dimethyl sulfoxide solution and fed by intubation. After 8 weeks, the animals were sacrificed by cervical dislocation. The blood was collected in clean dry test tubes and allowed to coagulate at ambient temperature for 30 minutes. Serum was separated by centrifugation at 2000 rpm for 10 minutes. The blood, collected in a heparinized centrifuge tube, was centrifuged at 2000 rpm for 10 minutes and the plasma separated was removed by aspiration and was used for estimations.

**Biochemical estimation and Heart weight and collagen content:** The Heart, aorta and kidney was dissected out and then weighed. Heart weight-to-body weight ratio was calculated. Hydroxyproline concentration was estimated in heart and aorta samples and the determined from standard curve and expressed as mg/g dry weight [20].

The electrolytes such as sodium (Na+) and chloride (Cl-) along with potassium (K+) were analyzed by AVL 9180 Electrolyte analyzer (ROCHE-USSR). Methodology is based on the ion selective electrode measurement principle to precisely determine the measurement values Burtis and Ashwood [21].

The activities of isocitrate dehydrogenase (ICDH), succinate dehydrogenase (SDH), malate dehydrogenase (MDH), α- ketoglutarate dehydrogenase (α-KGDH) were assayed by the methods of silambarasan et al., [22] respectively.

**Nitric oxide metabolites level:** Nitrite/nitrate (stable NO metabolites) in the aorta and heart samples were measured based on the Griess reaction Green et al., [23] in which a chromophore with a strong absorbance at 550 nm is formed by reaction of nitrate with a mixture of naphthyl ethylene diamine and -sulfanilamide. The nitrate was reduced to nitrite by 30 minutes incubation with nitrate reductase in the presence of NADPH. The amount of nitrite/nitrate present in the aorta and heart sample was estimated from the standard curve obtained. Nitrite/nitrate levels were expressed as nmol/mg protein.
ACE assay: ACE (USCN Life, West Lake, and Wuhan, China) activity was analyzed in cardiac samples using commercially available kits following the manufacturer’s instructions. The colored end product of this enzyme was measured by a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm [24].

Statistical analysis: Statistical analysis were analysed by one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT) using a commercially available Software Package for the Social Science (SPSS) software package version 11.0. Results were expressed as mean ± S.D. for six rats in each group. For all the statistical tests, values of P < 0.05 were statistically significant.

Results

Table 1 shows the effect of chrysin on the weight of heart, aorta and kidney weights, and heart weight-to-body weight ratio in control and L-NAME induced hypertensive rats. L-NAME induced hypertensive rats had significantly increased heart, aorta and kidney weights, heart weight-to-body weight ratio and hydroxyproline weights. Treatment with chrysin (25 mg/kg) significantly (P < 0.05) reduced the heart, aorta and kidney weights and weight-to-body weight ratio.

Fig. 2 shows effect of chrysin in cardiac and aortic hydroxyproline in experimental rats. In cardiac and aorta hydroxyproline concentration levels are increased in L-NAME induced hypertension as compared to control. Supplementation of chrysin significantly (P < 0.05) reduces in cardiac and aorta levels of hydroxyproline in group IV as compared to group I. There is no significant difference between group I and II.

Table 2 shows the effect of chrysin on the levels of plasma electrolytes such as Na⁺, K⁺ and Cl⁻ in control and L-NAME hypertensive rats. L-

![Graph showing hydroxyproline levels in heart and aorta in various experimental groups.](image)

Columns are mean ± S.D. for six rats in each group. Columns not sharing common superscript are significant with each other at P<0.05 (Duncan’s multiple range test).

Fig. 2. Effect of chrysin in hydroxyproline levels in heart and aorta in various experimental groups
NAME rats had significantly (P < 0.05) increased Na\(^+\), Cl\(^-\) levels and decreased K\(^+\) levels. Supplementation with chrysin significantly (P < 0.05) brought back these values towards near to normal levels. No significant differences between Group I and II.

Activities of ICDH, SDH, MDH, and á-KGDH were significantly (P < 0.05, Table 3) decreased in the liver mitochondria of L-NAME rats. Oral treatment with chrysin significantly (P < 0.05, Table 3) increased the activities of these enzymes when compared to untreated L-NAME rats. No significant difference between group I and II.

Plasma and heart, aortic nitrite/nitrate level was significantly (P < 0.05) reduced in L-NAME rats whereas chrysin treatment for 4 weeks significantly (P < 0.05) increased the above to normal (Fig. 3.A.B.). There is no significant change of group I and II.

L-NAME hypertension significantly (P < 0.05) enhanced the activity of ACE in heart and aorta compared with control and this increase was attenuated by chrysin treatment (Fig. 4.A.B.).

Discussion

In previous studies it was reported that in L-NAME treated rats there was a significant increase of heart, kidney, liver and aorta weights [25]. Our studies also supported that L-NAME induced hypertensive rats has significantly increased heart, aorta, and kidney weights. Supplementation of chrysin dose (25 mg/kg) significantly reduces the aortic, renal and cardiac hypertrophy that might be due to the BP lowering effect of chrysin. Because it has been also proved to reduce BP and antihypertensive effects on our previous study [18].

Cardiac and aorta tissues hydroxyproline concentration levels are increased in L-NAME
induced hypertension as compared to control rats. Cardiac hypertrophy is propositional with increased BP, increased fibrosis, collagen deposition, and reduced cardiac function. Hydroxyproline is major component of collagen and its concentration is a quantitative index of fibrosis. During heart failure the collagen get deposited in heart causing stiffening of the heart walls, impaired relaxation, impaired filling, and reduced cardiac output [26]. In aortic wall components, it is shown that collagen with highest elastic modulus and, therefore, appears as major determinant of aortic stiffness. In our proposed study we have determined the level of hydroxyproline which is the collagen marker in aorta as wells as cardiac tissue. Chrysin has the capability of preventing the accumulation of collagen in aorta. At the same time the elevated cardiac weight in hypertension was evidently suppressed by supplementation of chrysin. The antihypertensive potential of chrysin is main reason for reducing pressure load with induced hypertrophy. Treatment of L-NAME significantly elevates BP and ACE levels which cause cardiovascular system and kidneys injuries that might be leads to aggravation of hypertension. Renal NO synthesis plays an important role in acute and chronic regulation of sodium balance. In experimental rats maintained on high salt diet, the expression of all three NOS is forms are increased in the inner medulla. An important characteristic of renal body fluid feedback control system is the pressure naturituresis or the ability of the kidneys to play central role in reducing BP by altering the renal excretion of salt and water such as sodium balance, extracellular fluid volume and BP homeostasis. In addition to playing a major role in long term BP adjustment, the sodium transporters along the nephron are very dynamic, even responding quickly to normal fluctuations of BP. Increased ANG II and pressure-induced oxidative stress alter mitochondria and electrolyte

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Columns are mean ± S.D. for six rats in each group.
Columns not sharing common superscript are significant with each other at P<0.05 (Duncan’s multiple range test).

**Fig. 4. A.** Effect of chrysin in ACE in plasma in various experimental groups

Columns are mean ± S.D. for six rats in each group.
Columns not sharing common superscript are significant with each other at P<0.05 (Duncan’s multiple range test).

**Fig. 4. B.** Effect of chrysin in ACE in Heart and aorta in various experimental groups.

Chrysin improvement of mitochondrial enzymes and ACE in L-NAME hypertension
transport efficiency, which together reduce kidney oxygen tension and can cause tissue hypoxia. In our studies plasma electrolytes such as Na⁺, K⁺ and Cl⁻ are kept in control and in L-NAME hypertensive rats. It is shown that L-NAME rats has significantly (P < 0.05) increased Na⁺, Cl⁻ levels and decreased K⁺ levels. The electrolytes Na⁺, K⁺ play critical role in the normal regulation of blood pressure. These electrolytes have has main impact in the control of arterial resistance [27]. Number of research suggests that intracellular sodium overload and potassium depletion are important for pathophysiology of hypertension [28]. As previously reported increased level of Na⁺ and Cl⁻ along with a decreased level of potassium results with chronic inhibition of NOS by L-NAME [29] is observed in our studies. Administration of chrysin brought back these parameters to near normal values similar to control rats.

On the same time NO synthase substrate, it should be remembered that arginine participates in other metabolic functions, including α-ketoglutarate metabolism and polyamine synthesis. (Fumarase catalyzes the reversible conversion between fumarate and L-malate in the tricarboxylic acid cycle in mitochondria. L-malate can be converted to oxaloacetate, aspartate, argininosuccinate, and L-arginine, the substrate of nitric oxide (NO) synthase.). Our studies have shown that activities of ICDH, SDH, MDH, and α-KGDH, which were significantly (P < 0.05), decreased in the liver mitochondria of L-NAME rats. It was already explored that under oxidative stress conditions the functions of several key tricarboxylic acid cycle enzymes were perturbed [30]. Giribabu et al., [31] reported that Vitis vinifera seed extract has ability for preventing the decrease in ICDH, SDH and MDH in liver homogenates respective to decrease in liver oxidative stress in diabetes. In our study is also accepted the earlier report, chrysin, it is observed to increase the activities of liver mitochondrial enzymes and which might be the effect of oxidative stress.

Hypertension is one of the most important risk factors associated with the development of vascular diseases. The modulation of vascular tone results from NO synthesis and release by endothelial cells [32]. The use of NO is not limited but in other cellular events, such as vascular smooth muscle cell proliferation [33]. Chronic inhibition of NO synthase persuaded by administration of L-NAME resulting in induction of hypertension, hypertrophy, cardiac remodelling [34]. Treatment with L-NAME induces hypertension and provides an experimental model to study hypertension. It has been shown that ventricular hypertrophyn L-NAME model of hypertension increase the chance of fibrosis and left ventricle hypertrophy [35].

In this study on treating with chronic inhibition of NOS by nitric oxide deficient hypertensive rats we observed the significant decrease of plasma, heart and aorta NOx levels. Moreover, our findings are in consistent with several other reports which showed the association between chronic NO deficiency and hypertension [36]. It shown that chrysin protects NO from free radicals, increasing the bioavailability of NO and also increase the activity of eNOS resulting in decreased BP. There are no significant changes between group I and II. With antioxidant property, chrysin is believed to reduce oxidative damage and lower cardiovascular diseases as shown in our studies.

RAAS is important physiological role in BP control and sodium volume homeostasis. In this study, it is observed that plasma, heart and aortic ACE activity are increased in L-NAME induced hypertensive rats. ACE converts Ang I to Ang II. In general the reduction of ACE activity leads to a reduction in blood pressure because of the reduction in Ang II synthesis. Ang II acts as a potent vasoconstrictor. Ang II exacerbates oxidative stress by increasing the production of superoxide [37]. Increased ACE activity and oxidative stress was observed in hypertensive animals. Increased plasma Ang II availability was reported in L-NAME-treated rats because of
Table 1. Estimation of heart, kidney, aorta weights and heart weight-to-body weight ratio levels in various experimental groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control+25 mg chrysin</th>
<th>L-NAME induced hypertension</th>
<th>L-NAME+25 mg chrysin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Weight (mg)</td>
<td>821.12± 31.28a</td>
<td>819.24 ± 32.12a</td>
<td>1099.57 ± 42.68 b</td>
<td>861.57 ± 39.29 c</td>
</tr>
<tr>
<td>Kidney weight (mg)</td>
<td>872.39± 35.81a</td>
<td>869.41 ± 34.20a</td>
<td>1486.92 ± 49.11b</td>
<td>901.14 ± 38.24 c</td>
</tr>
<tr>
<td>Aorta weight (mg)</td>
<td>98.59± 5.19a</td>
<td>97.41 ± 4.76a</td>
<td>121.85 ± 9.59b</td>
<td>103.23 ± 5.27 c</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>3.08 ± 0.19a</td>
<td>3.06 ± 0.18a</td>
<td>5.24 ± 0.32b</td>
<td>3.14 ± 0.17c</td>
</tr>
<tr>
<td>LVW/BW (mg/g)</td>
<td>1.21 ± 0.06a</td>
<td>1.20 ± 0.06a</td>
<td>1.58± 0.05b</td>
<td>1.25 ± 0.05c</td>
</tr>
<tr>
<td>RVW/BW (mg/g)</td>
<td>0.43 ± 0.05a</td>
<td>0.42 ± 0.06a</td>
<td>0.31 ±0.04b</td>
<td>0.41 ± 0.05c</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. for six rats in each group. Values not sharing common superscript are significant with each other at P < 0.05 (Duncan’s multiple range test).

Table 2. Effect of chrysin on Electrolytes of control and L-NAME induced hypertensive rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control+25 mg chrysin</th>
<th>L-NAME induced hypertension</th>
<th>L-NAME+25 mg chrysin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma sodium (mmol/L)</td>
<td>146.23 ± 8.12a</td>
<td>145.12 ± 7.27a</td>
<td>169.55 ± 9.63b</td>
<td>150.67 ± 6.38c</td>
</tr>
<tr>
<td>Plasma potassium (mmol/L)</td>
<td>4.81 ± 0.39a</td>
<td>4.82 ± 0.40a</td>
<td>2.39 ± 0.19b</td>
<td>4.52 ± 0.40c</td>
</tr>
<tr>
<td>Plasma chloride (mmol/L)</td>
<td>99.53 ± 4.27a</td>
<td>98.11 ± 4.57a</td>
<td>127.27 ± 8.16b</td>
<td>104.17 ± 6.75c</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. for six rats in each group. Values not sharing common superscript are significant with each other at P < 0.05 (Duncan’s multiple range test).

endothelial NO synthase (eNOS) inhibition [38]. The increase of BP and ACE levels further causes pathological injuries to the cardiovascular system and kidneys, which in turn leads to aggravation of hypertension [39]. In current study, chrysin have reduced plasma, cardiac and aortic ACE activity in L-NAME received rats. This result suggests that the antioxidant properties of chrysin proportional to the reduction of the enzyme activity. The effect of chrysin on ACE in this study in accordance with Kataoka et al., [40] has reported that anti-inflammatory and antioxidant effects of calcium channel blockers might be a result of the inhibition of the local RAS.

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**Conclusion**

The present study demonstrated that chrysin attenuates the hypertension and thereby protects cardiovascular function against damages from endothelial dysfunction through its ACE inhibitor activity. Chrysin at a dose of 25 mg/kg regulates the L-NAME-induced BP and in chain shows a reduction in plasma electrolytes, cardiac and aortic hydroxyproline cardiac, aortic and plasma ACE activity. Chrysin also increases mitochondrial enzymes, plasma, heart, aortic nitrite/nitrate concentration. On the basis of these results, we suggest that chrysin may be a useful therapeutic drug treatment for cardiovascular disease and hypertension.

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**Conflict of interest statement**

None declared

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