Histological, Immunohistochemical and Biochemical Study on the Possible Cardioprotective and Antihypertensive Role of the Flavonoid in Unilateral Renal Artery Ligation of Adult Albino Rats

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Abstract

The blood pressure lowering effect of herbal-rich diet is a necessary dietary lifestyle measure now included the guidelines for the management of arterial hypertension. Flavonoids represent a major class of plant polyphenolics, they have antioxidant effects and show a wide range of pharmacological activities. This study was to clarify the antihypertensive effect of the flavonoid extracted from the *Origanum vulgare*.

Forty five adult male albino rats were classified into three main groups as follows: group I: control. Group II: hypertension was induced by ligation of left renal artery. Group III: hypertension was induced and given OV as 100 mg/kg body weight daily for eight weeks. Blood samples were collected and cardiac sections were stained with H&E and immunohistochemical staining for caspase 3 and Bcl-2.

The body weight of control, hypertensive and treated hypertensive groups showed a significant weight gain. Significant increase in creatinine and cholesterol in untreated in contrast to other groups. There was significant reduction of blood pressure (BP) on using OV in contrast to other groups. The hypertension group induced myocardial apoptosis were significantly decreased with OV compared with other groups. Caspases 3 and Bcl2 immunostaining in OV-treated rats were significantly decreased compared with other groups.

The supplementation of OV herbetic extract (flavonoid) in hypertensive rats partially prevented such occurred changes, suggesting its possible protective effect against the risk of the progression of cardiovascular diseases during hypertension.

Keywords: Hypertension; Cardiac muscle; Flavonoids; Bcl-2; Activated caspases 3

Introduction

Renal artery stenosis is associated with an increased incidence of cardiac death, myocardial infarction and stroke. In the Cardiovascular Health Study the presence of renovascular disease increased the risk of short-term adverse cardiovascular outcomes by approximately 2-fold [1].

High blood pressure is one of the major risk factors for developing cardiovascular diseases, including coronary disease, stroke, peripheral artery disease; renal disease and heart failure [2]. Efforts to reduce the prevalence of hypertension have focused on non-pharmacologic approaches that lower blood pressure. The lifestyle measures that are widely agreed to lower blood pressure or cardiovascular risk associated with hypertension include smoking cessation, weight reduction, physical exercise, reduction of excessive alcohol intake and dietary measures such as a reduction in sodium, an increase in potassium and a decrease in saturated and total fat intake [3]. An increase in fruit and vegetable intake has also been included in the guidelines for the management of arterial hypertension in recent years [3]. Earlier epidemiological studies suggested that vegetarians tend to have lower blood pressures than non-vegetarians. The blood pressure lowering effects of vegetables and a fruit-rich diet were later confirmed in randomized intervention studies [4,5]. Because high blood pressure is the major cause of stroke, the blood pressure lowering effect could be one of the major mechanisms contributing to a reduced risk of stroke with increased fruit and vegetable intake [6]. The specific components present in fruits and vegetables that reduce blood pressure are not fully characterized. It was originally suggested that an increase in the ratio of polyunsaturated to saturated fats, fiber, calcium and magnesium intake, and a decrease in the intake of protein and vitamin B12 might be responsible. However, the reduction in blood pressure produced by these substances in trials of dietary supplements has typically been small and inconsistent. The presence of multiple antioxidants in these foodstuffs has also been suggested to be involved [7]. Among these plant antioxidants, polyphenolics have received considerable attention in recent years as compounds with potential beneficial effects for cardiovascular health. Flavonoids represent the major class of polyphenolics, which, in addition to their antioxidant effects, show a wide range of pharmacological activities [8]. The term flavonoid describes several thousand plant-derived compounds sharing a common skeleton of phenylchromane [7]. *Origanum vulgare* extract is a complex mixture extracted from leaves which contain different phytochemical groups such as alkaloids, saponins, coumarins, sterols, terpenes, tannins, Anthraquinonine derivatives, Volatile oil and...
flavonoids [9,10]. Also have different pharmacological activities not only as preventive but also as a treatment of some diseases with full relief of their symptoms [11]. The current research studies introduced many antihypertensive agents with limited adverse side effects, however the recognition of herbal remedies as another therapeutic alternatives (anti-thrombin activities) have been considered as an important goal for the pharmaceutical industries through their components [12]. Origanum vulgare displayed anti-platelet properties in human, has a role in controlling blood sugar and also inhibits abnormal cell growth, it also helps in maintaining healthy cholesterol and blood pressure [13] and is widely distributed through different regions as Morocco, Russia and Egypt [14].

The main pharmacological effects of Origanum vulgare are being anti-oxidant, anti-thrombin and potent anti-hypertensive through maintaining renal function, creatinine clearance and inhibiting mechanical forces acting on arterial wall so preventing excess hardening and thickening [12,15].

The aim of this work was to study the effect of flavonoid on cardiac muscle of induced hypertensive rats through histological, immunohistochemical and biochemical examination.

**Material and Methods**

**Materials**

**Plant extraction:** Origanum vulgare obtained from Secum Pharmaceutical Company (24 Mohamed Hassan El Gamal St. 6th Zone, Nasr City, Cairo, Egypt).

The aerial part (leaves) of the plant material (500 g) was cleaned of foreign material, grinding and kept soaked for three days in the aqueous ethanol (30:70) with occasional shaking, at room temperature then cover it by aluminum foiled paper and put it in dark place. The filtration was carried out using a muslin cloth and then through Whatman qualitative grade 1 filter paper. This procedure was repeated twice and then all the filtrates obtained were combined and concentrated on a rotary evaporator (RE-111, Buchi, Flawil, Switzerland) accompanied with B-700 recirculation chiller and a water bath model 461 at 45°C under vacuum. Finally, the extract change to a thick pasty mass called crude extract was partitioned several times with ethyl acetate (3 × 500 ml), the combined n-hexane extract was concentrated under vacum. Finally, the extract change to a thick pasty mass called crude extract was partitioned several times with ethyl acetate (3 × 500 ml), the combined n-hexane extract was concentrated under vacuo at 40°C to dryness (18 g). The remaining aqueous extract subjected to concentration ethanolic extract was then suspended in distilled water (300 ml) and filtered. The water soluble portion was defatted with n-hexane (3 × 500 ml), the combined n-hexane extract was concentrated under vacuo at 30 Centigrade (30°C) to dryness (27 g). The defatted crude extract was partitioned several times with ethyl acetate (3 × 500 ml). The combined ethyl acetate extract was concentrated under vacuo at 40°C to dryness (18 g). The remaining aqueous extract subjected to freeze drying to afford a residue (20 g).

Then 1 g of each extract (Ethanolic extracts, Ethyl acetate extracts, and n-hexane extracts) was dissolved or suspended in 250 ml distilled water separately to give different extracts of concentration of 4 mg/ml (100 mg/kg body weight) [9,10].

**Animals:** Forty five adult male albino rats were used in this study which has been carried out in the animal house of the Faculty of Medicine, Zagazig University. They were housed in a temperature-controlled and light controlled room (12 h light/dark cycle), with free access to food and water. They were housed in three groups in plastic cages. Rats were fed ad libitum. The rats were randomly collected and divided into 3 groups (15 animals each), the first group was considered as a control, the second as hypertensive untreated while the third group as hypertensive treated with Origanum vulgare.

**Methods**

**Measurement of blood pressure and biochemical studies:** All 45 animals were weighed before starting the study and initial blood samples were collected from the vein of the neck for creatinine.

The first group followed up through the period of study by measuring the systolic blood pressure and biochemistry every 2 weeks for 8 weeks (period of study).

In the second group, The animals were anesthetized with intraperitoneal injection of thiopental sodium (Thiopental; Eipico) at a dose of 80 mg/kg body weight then hypertension was induced by ligation of left renal artery to raise systolic BP to more than 150 mmHg and they were randomly assigned on a daily placebo (water) during the 8 weeks, starting from the raised systolic BP point, via a stomach tube. Systolic blood pressure was measured every 2 weeks till the end of study by a tail cuff plethysmographic noninvasive methods (Letica LE 5100, panlab, Barcelona, Spain).

The third group in which hypertension was induced as mentioned above and systolic pressure was above 150 mmHg were treated with Origanum vulgare given orally by gastric intubation for 8 weeks.

After 8 weeks of starting the study, the aorta was exposed, cannulated with polyethylene catheter and connected to a blood pressure monitor transducer 8/38 to monitor the mean arterial pressure and then blood samples were collected from the aortic cannula in heparinized tubes for final analytical assays for creatinine. The rats were finally weighed and killed with an overdose of phenobarbital.

**Light microscopic studies:** The heart specimens obtained were fixed in 10% formol saline, processed, and embedded in paraffin sections. Sections were cut into 5 μm thicknesses using a microtome, mounted on slides, and subjected to the following techniques: H&E stain for histological assessment [16].

**Immunohistochemical study:** Immunostaining was performed using the avidin biotin peroxidase technique for localization of activated caspases 3 and Bcl-2. Paraffin sections mounted on coated slides were deparaffinized and treated with 0.01 m citrate buffer for 10 minutes to unmask antigens21. Then sections were incubated in H2O2 for 10-15 min until color intensity was reached. Sections were counterstained with hematoxylin. Sections treated with the same antibody (dilution 1:1000) for 1 hour and then washed by buffer and rinsed from the slide by immersing in cold buffer for 2 changes of 5 minutes. Denatured heat-reduced tissue sections, 3–4 μm thick, were mounted on positively charged slides and heated at 60°C for 30 min. They were then deparaffinized and rehydrated through a series of xylene and alcohol before staining. Antigen retrieval was carried out with microwave treatment in 10 mmol/l citrate buffer (Neo-Markers; Thermo Fisher Scientific Inc., Rockford, Illinois, USA).

Slides were then incubated overnight at 4°C with polyclonal rabbit anti-Bax (dilution 1:50; Dako Cytomation Norden A/S, Glostrup, Denmark,which it is the blocking solution). Excess antigen was rinsed from the slide by immersing in cold buffer for 2 changes of 5 to 10 minutes each. Then the slide were incubated with secondary antibody (dilution 1:1000) for 1 hour and then washed by buffer and substrate was added. DAB substrate chromogen solution was applied and incubated for 10-15 min until color intensity was reached. Sections were counterstained with hematoxylin. Sections treated with the same protocol in the absence of the primary antibodies served as negative controls [17].
**Morphometric analysis:** In each group, five different caspase-3 and Bcl-2 stained sections were examined (n=5). Five high-power fields (400X) were measured per section and their mean was achieved. Measurements were taken using a Leica Q win software program installed on a PC (Dell, Round Rock, Texas, USA) connected to a microscope (Leica Microsystem, Heerbrugg, Switzerland). Data were presented as mean ± SEM.

The following measurements were taken:
1. Mean area % of caspases 3 immunoreactivity.
2. Mean area % of Bcl-2 immunoreactivity.

**Statistical Analysis**

The findings in this study were recorded as mean, standard error of mean (SEM). Student’s t test was used and both t and probability (p) values were estimated. The results were considered significant when the two tailed p value was less than 0.05 [18,19].

**Results**

**Body weight**

The recorded results of the body weight of control animals ranged from 195 to 218 gm with a mean of 207.4 ± 2.2 while the final body weight ranged 213 to 240 gm with a mean of 223.5 ± 2.6 gm. In untreated hypertensive rats, the initial body weights ranged 198-232 gm with a mean of 214.5 ± 3.2 gm and the final body weight ranged 180-215 gm with a mean of 197.6 ± 4.2 gm.

In treated hypertensive group, the initial body weights ranged 200-225 gm with a mean of 192.7 ± 2.4 gm while the final body weights ranged 218-243 gm with a mean of 229.5 ± 2.6 gm. A highly significant increase in body weight (P<0.02) was noticed in all groups (Table 1).

**Blood pressure and biochemical results**

**Blood pressure estimation:** In control group the initial systolic BP ranged 113–145 mmHg with a mean of 130 ±5 mmHg and final values ranged 128–144 mmHg with a mean of 136 ± 6 mmHg.

In untreated hypertensive rats the initial systolic BP ranged 155–178 mmHg with a mean of 163 ± 6 mmHg while the final values ranged 168–194 mmHg with a mean of 186 ± 8 mmHg. In treated group the initial systolic BP ranged 156–171 mmHg with a mean of 166 ± 6 mmHg and the final values after 8 weeks ranged 125–156 mmHg with a mean of 138 ± 4 mmHg. There was a significant increase in BP in untreated group (P=0.02) and a significant reduction in BP in treated group (P<0.05) (Table 2).

**Serum creatinine:** The initial creatinine levels in control rats ranged from 0.4–1.2 mg/dl with a mean of 1 ± 0.15 mg/dl and final levels after 8 weeks ranged 0.5–1.2 mg/dl with a mean of 0.9 ± 0.1 mg/dl.

In untreated hypertensive group, the initial creatinine values ranged 0.4–1.4 mg/dl with a mean of 0.9 ± 0.09 mg/dl while the final values after 8 weeks ranged 1.6–2.6 mg/dl with a mean of 2.08 ± 0.12 mg/dl while in treated group, the initial values ranged 0.5–1.1 mg/dl with a mean of 0.8 ± 0.06 mg/dl while the final values ranged 0.5–1.3 mg/dl with a mean of 0.9 ± 0.07 mg/dl. A highly significant increase in creatinine levels (p<0.02) was found in untreated hypertensive group in contrast to treated and control groups (Table 3).

**Histological results**

**H&E stain:** Light microscopic examination of cardiac muscle of the control group showed a normal myofibrillar structure with striations, branched appearance and continuity with adjacent myofibrils. Myocytes exhibited an acidophilic sarcoplasm and central oval single nuclei (Figure 1). Longitudinally arranged fibers were cylindrical with

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<table>
<thead>
<tr>
<th>Control rats</th>
<th>Untreated hypertension</th>
<th>Treated hypertensive</th>
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<tbody>
<tr>
<td>Initial systolic BP</td>
<td>Follow up systolic BP</td>
<td>Final systolic BP</td>
</tr>
<tr>
<td>130 ± 5</td>
<td>138 ± 7</td>
<td>128 ± 5</td>
</tr>
<tr>
<td>163 ± 6</td>
<td>168 ± 5</td>
<td>182 ± 8</td>
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<tr>
<td>166 ± 6</td>
<td>158 ± 5</td>
<td>146 ± 6</td>
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A significant increase in BP in untreated group (P=0.02) and a significant reduction in BP in treated group (P<0.05).

**Table 2:** Means ± SD of initial, during follow up and final measurements of systolic blood pressure (mmHg) in all groups of rats.

<table>
<thead>
<tr>
<th>Mean ± SD</th>
<th>Control rats</th>
<th>Hypertensive</th>
<th>Treated hypertensive</th>
</tr>
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<tbody>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.9 ± 0.1</td>
<td>2.08 ± 0.12</td>
<td>0.9 ± 0.07</td>
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A highly significant increase in creatinine levels (p<0.02) was found in untreated hypertensive group in contrast to treated and control groups.

**Table 3:** Means ± SD of serum creatinine (mg/dl) in control, untreated hypertensive and treated hypertensive rats.

central oval vesicular nuclei and faint striations. Cardiac myocytes were connected to make fibers through intercalated discs (Figure 2). The interfiber spaces were narrow and contained blood vessels and fibroblasts (Figures 1 and 2).

The cardiac muscle fibers of group II (hypertensive group) showed separation of myofibers with increase in the interfiber spaces and extravasated RBCs (Figure 3). In addition, large areas of hemorrhage could also be detected [4]. Focal degeneration was evident in this group, degenerated cardiac myocytes appeared swollen. They were either pale with vacuolated cytoplasm (especially around the nucleus) or darkly stained nuclei (Figures 4 and 5). Focal mononuclear cellular accumulation could also be detected in many areas (Figure 5).

Treated group, showed a histological pattern nearly similar to the control group. Most of the cardiac muscle fibers appeared cylindrical, with central oval nuclei. However, many wide interfiber spaces were still detected (Figure 6). Nevertheless, some areas showed pale vacuolated cardiac muscle fibers and others revealed extravasated blood in the wide interfiber spaces (Figure 7).

Immunohistochemical results

**Bcl2 expression:** Positive immunohistochemical staining of bcl2 demonstrated brown cytoplasmic staining (index for the antiapoptotic effect). Cardiac sections stained with anti-Bcl2 antibodies showed a strong positive immunostaining for Bcl2 in the cytoplasm of most cardiac cells of the control group (group I) (Figure 8). In the hypertensive group (group II), few cardiac cells showed moderate immunoreactivity for Bcl2 (Figure 9) treatment with OV (group III) resulted in a marked
Morphometric and statistical results

**Mean area% of Bcl2 immunoreactivity**: There was a significant decrease in the mean area% of Bcl2 immunoreactivity in group II (1.73 ± 1.30) when compared with the control group (5.10 ± 2.56), with a $P$ value (0.006). In group III, the mean area% of Bcl2 (6.32 ± 1.35) was significantly increased as compared with that of group II ($P$ value 0.001). No significant difference was found between group III and control group I ($P$ value 2.001) (Table 1).

**Mean area% of caspase 3 immunoreactivity**: There was a significant increase in mean area% of caspase 3 immunoreactivity in group II (12.32 ± 1.93) compared with the control group (5.10 ± 2.12), with a $P$ value (0.001). In group III, the mean area% of caspase 3 (7.12 ± 1.81) was significantly decreased as compared with group II ($P$ value 0.039), but still significantly increased as compared with the control group ($P$ value 0.021) (Table 4).

**Discussion**

Hypertension is a risk factor for many cardiovascular diseases. Control of hypertension by dietary anti-hypertensive agents is an important strategy to manage this risk factor. Here, we have analyzed the effects of the dietary flavonoid *Origanum vulgare* on cardiac muscle in a model of hypertension. A significant weight gain was recorded in our study in all groups of experimental animals which didn't agree with aguila [20] who didn't record any changes in body weight of experimental induced hypertensive animals on long-term intake of edible oils.

In the present work unilateral renal artery ligation was performed to induce hypertension. Deteriorated renal function was confirmed in the present work by the presence of statistically significant increase in Bcl2 immunoreactivity as many cardiac cells showed a positive reaction for Bcl2 (Figure 10).

**Caspase-3 expression**: Positive immunohisto-chemical staining of caspase-3 demonstrated as brown cytoplasmic staining (index for the degree of nuclear apoptosis). Cardiac sections stained with anti-caspase 3 antibodies showed weak immunostaining in the cytoplasm in the control group (Figure 11). Hypertensive group (group II) showed marked increase in caspase 3 expression in the cytoplasm of many cardiac cells that showed a strong reaction (Figure 12). In the OV-treated group (group III), the expression of caspase 3 by cardiac cells was markedly reduced as compared with (group II) (Figure 13).
Hypertension accelerated the aging process of both kidney and heart like hypertrophic changes which led to structural destruction [32] while in the present study OV has reduced and alleviated pathological damage in both kidney and aorta which coincided with Lermioglu [33] who related these effects due most likely to reduced toxins released in hypertensive rats treated by OV. Different antihypertensive medications are now available in the market with different biological effects in hypertensive human subject. However very little data are published about the histopathological abnormalities resulted from using of such extracts and also no enough references were reported to deal with histological effect of Origanum vulgare on heart of the experimental animals. Myocardial cell death via necrosis and apoptosis is the primary feature of ischemia and reperfusion due to hypertension. Reducing cardiocyte loss through suppression of cell death is a logical strategy to protect cardiomyocytes [34]. Caspases, a family of cysteine proteases that cleave to aspartate residues, are central to the execution of apoptosis [35]. During the execution phase of apoptosis, initiator caspases activate effector caspases and ultimately cleave a set of proteins, disassembling the cell [36]. Some studies suggest that caspase-3 may be the main cause of cells apoptosis [34]. Apoptosis via activation of distinct signaling pathways involves mitochondria, mitochondrial regulatory proteins, and caspase activation [34,36]. Caspase activation may be regulated directly or indirectly by members of the Bcl-2 family of proteins. Bcl-2 proteins play a critical role in regulating apoptosis by promptly impacting caspase activation on multiple levels. The main site of action of anti-apoptotic Bcl-2 proteins appears to be the mitochondrion, which resides in the outer mitochondrial wall and regulates apoptosis by inhibiting the release of cytochrome c [34]. Bax, one of the pro-apoptotic proteins, originates in the cytosol but translocates to the mitochondrion and forms a pro-apoptotic complex with Bcl-2. In particular, the balance of Bcl-2 and Bax expression has been suggested as an important factor in determining the extent of apoptosis [35]. Therefore, in the present study, the expression of Bcl-2 family was examined immunohistochemically to elucidate the involvement of Bcl-2 in hypertension induced apoptosis. Examination of the cardiac muscle of the hypertensive rats that were stained immunohistochemically for caspase3 revealed intense positive immunoreaction in the cardiac myocytes that statistically were confirmed by comparison with that observed in the control. In hypertensive rats, weak immunostaining for Bcl-2 was demonstrated in most of cardiac cells, this result was in accordance with that observed by other authors who suggest that hypertensin induced apoptosis was induced by a down regulation of Bcl-2 and this was concomitant with activation of caspase-3 which mediates apoptosis [37,38]. On the other hand, it was documented that hypertensin induce apoptosis in cardiac muscle via activation of Bax expression and Bcl-2 suppression [39]. Cardiomyocyte loss due to multiple mechanism of death, including apoptosis [40]. Besides a reduction in the number of cardiomyocytes, apoptosis may contribute to heart failure through different pathways [41]. Thus, apoptosis may be one of the mechanisms involved in the loss of contractile mass and function in hypertensive cardiomyopathy. It is well known that apoptosis is associated with activation of caspases that mediate the cleavage of vital and structural proteins. Communal et al. [42] have reported recently that caspase-3 cleaved cardiac myofibrillar proteins, resulting in an impaired force/CA relationship and myofibrillar ATPase activity. In this regard, Laugwitz et al. [43] have demonstrated that caspase activation is associated with cleavage of myofilaments oxidant stress is an important factor inducing cardiomyocyte apoptosis. Thus, antioxidant therapy, whether through administration of additional antioxidants or by boosting innate antioxidant mechanisms, has been shown to be a viable approach in reducing cardiac apoptosis in experimental conditions [44]. In this study, a concomitant administration of Origanum Vulgare in the hypertensive group significantly reduced the hypertensive -induced apoptotic changes in cardiac muscles of rats, but not completely recovering the histopathological changes to normal. Also, we observed diminished immunostaining for Bcl-2 protein when compared with

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean area % of Bcl2</th>
<th>P value</th>
<th>Mean area % of caspase 3</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>5.10 ± 2.56</td>
<td>0.006* vs. II 2.43 vs. III</td>
<td>5.10 ± 2.12</td>
<td>0.001* vs. II 0.027* vs. III</td>
</tr>
<tr>
<td>Group II</td>
<td>1.73 ± 1.30</td>
<td>0.006* vs. I 0.001* vs. II 12.32 ± 1.93</td>
<td>0.001* vs. I 0.39 vs. III</td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>6.32 ± 1.35</td>
<td>0.288 vs. I 0.001* vs. II</td>
<td>7.12 ± 1.81</td>
<td>0.021* vs. I 0.039 vs. II</td>
</tr>
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*The mean difference is significant at a P value ≤ 0.05

Table 4: Mean area % of Bcl2 and caspase 3 immunoreactivity in cardiac myocytes of the all groups.
those of treated group. Origanum Vulgari was reported to have antioxidative effect [45].

Spriodin et al they found that *Origanum vulgare* and Melissa officinalis extracts present the most effective antioxidant capacity in scavenging DPPH radicals.

They reported that oxidative stress plays a role in mediating apoptosis through mitochondrial dysfunction. This damage may change mitochondrial membrane permeability, which leads to the release of cytochrome c into the cytosol and commits the cell to apoptosis [46,47].

In conclusion, our results demonstrate that *Origanum vulgare*, possesses antihypertensive effect against hypertensive rats, which is evidenced by a considerable decrease in blood pressures, renal function. Our results also revealed that the cardiac damage was decreased by *Origanum vulgare* supplementation. Further studies are needed to find out the exact mechanism of action of *Origanum vulgare*.

**References**


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