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Original article

Effect of ethyl acetate fraction of *Melothria maderaspatana* leaf on membrane bound ATPases in DOCA-salt induced hypertensive rats

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Summary. More recently, research has been focused on the use of medicinal plants or plants derived compounds for treatment of many diseases including hypertension. *Melothria maderaspatana* commonly used in traditional medicine for hypertension and numerous preclinical studies have shown wide range of pharmacological activities, including antioxidant, anti-inflammatory, anticancer and antidiabetic activities. The present study was designed to investigate the effect of ethyl acetate fraction of *Melothria maderaspatana* (EAFM) leaf on membrane bound ATPases in uninephrectomized deoxy-corticosterone acetate (DOCA)-salt hypertensive rats. Hypertension was induced in uninephrectomized rats by weekly twice subcutaneous injection of DOCA (25 mg/kg) and 1% NaCl in drinking water for six weeks. EAFM was administered orally once a day for 6 weeks. In the present study, administration of DOCA-salt significantly increased the systolic and diastolic blood pressure while treatment with EAFM, reverted the abnormal level of blood pressure significantly to near sham-operated control rats. The activities of Na^+-K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase were significantly decreased in DOCA-salt induced hypertensive rats when compared to that of sham-operated control rats. Administration of EAFM to hypertensive rats restored the abnormal membrane bound ATPases activity toward the sham-operated control rats. Thus, results indicate that administration of EAFM is having good blood pressure control and to protect deranged activity of membrane-bound ATPases in DOCA-salt induced hypertensive rats. Further detailed investigation is necessary to discover *M. maderaspatana* mechanism of action.

Key words: Deoxy-corticosterone acetate, salt, hypertension, membrane bound ATPases, *Melothria maderaspatana*

Introduction

Hypertension is a major risk factor for cardiovascular disease and, although most hypertension have no obvious direct cause, dietary salt (or sodium) has been suggested to be an important etiological factor (1). In addition to its effects on blood pressure, excess salt intake has been demonstrated to have blood pressure-independent effects on the heart and blood vessels (2). The administration of a synthetic mineralocorticoid derivative, DOCA, in combination with salt loading in the diet to young adult Wistar rats following surgical removal of one kidney induces hypertension with cardiovascular remodeling characteristic of human volume-overload induced hypertension, especially hypertrophy, fibrosis, conduction abnormalities and endothelial dysfunction (3). Membrane ATPases may play an important role in ionic gradients between the

intra cellular/extra cellular compartments of the cell. Membrane Na^+-K^+-ATPase plays an important role in active transport of Na^+ and K^+ ions across the membrane. Similarly, Ca^{2+}-ATPase is clearly linked with Ca^{2+} pump and transport of Ca^{2+} while Mg^{2+} activated ATPase is distributed in all cell compartments. The Mg2 ions forms Mg^{2+}-ATPase complex, which is the substrate for the enzyme. Mg^{2+}-ATPase is to control the intra cellular Mg^{2+} concentration, changes which can modulate the activity of Mg^{2+} dependent enzymes and regulate rates of protein synthesis and cell growth (4). A decrease in the enzymatic activity of Na^+-K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase has been described in various diseases including hypertension (5). Abnormal lipid peroxides affect the activity of membrane bound ATPases and their level was decreased due to the excessive production of thiobarbituric acid reactive substances (6). Recent study has been found that the increased level of free radicals affect the cellular membrane lipids and reduced the activity of membrane bound ATPases in essential hypertension (7).

Melothria maderaspatana (Linn.) Cogn. Syn. *Mukia maderaspatana*, *Cucumis maderaspatana* or *Mukia scabrella* (Family: Cucurbitaceae) is a monoecious plant having scandent or prostrate stems, very hispid, leaves variable in size, densely covered with white hairs. It is widely recommended in the southern part of Sri Lanka for alleviation of various forms of liver disorders (8). *M. maderaspatanas* has been reportedly found to exhibit anti-inflammatory (9), antidiabetic (10) and anticancer (11) activities. In our earlier study, we investigated the ethanolic extract of *Melothria maderaspatana* and showed it to had antihypertensive activity in DOCA-salt-induced hypertensive rats (12). In a further study we fractionated the ethanolic extract of *Melothria maderaspatana* through silica gel (100-200 mesh) column using chloroform, ethyl acetate and methanol (solvents with increasing polarity), test the antihypertensive effect on DOCA-salt-induced hypertensive rats and identified compounds from the active fraction by GC-MS analysis. Among the three fractions, EAFM alone significantly lowered the systolic and diastolic blood pressure. By GC-MS analysis, phytochemicals such as coumarin, vallinic acid, p-coumaric acid, gallic acid, caffeic acid, and ferulic acid were identified in EAFM (13). Ferulic acid has been shown to lower arterial blood pressure in spontaneously hypertensive rats (14). No study has been conducted on the effect of EAFM on membrane bound ATPases in DOCA-salt induced hypertensive rats. Hence, in the present study we examined the effects EAFM on membrane bound ATPases in DOCA-salt hypertensive rats.

Materials and methods

Experimental animals

Male albino Wistar rats weighing 200-230 g were purchased from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, and maintained in an air-conditioned room (25 ± 1 °C) with a 12 h light/12 h dark cycle. Feed and water were provided *ad libitum*. Animal handling and experimental procedures were approved by the Institutional Animal Ethics Committee, Annamalai University (Registration Number: 66/1999/CPCSEA, Proposal No. 459) and animals were cared for in accordance with the Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA, 2004).

Preparation of leaf fractions by column chromatography

Melothria maderaspatana leaf powder was purchased from the local herbal market (Vinayaga herbals), Chidambaram, Cuddalore district, Tamil Nadu, India. Leaves of *M. maderaspatana* were collected from the same local herbal market and the plant was botanically authenticated. A voucher specimen (AU-6054) of the plant has been deposited at the Herbarium of the Department of Botany, Annamalai University, Annamalainagar, Tamil Nadu. The leaf powder was sieved and kept in a freezer until use. 100 g of dry fine powder was suspended in 300 mL of ethanol for 72 h. The extract was filtered using a muslin cloth and concentrated at 40±5 °C. The crude ethanolic extract (CEEM) was further fractionated through silica gel (100-200 mesh) column using chloroform, ethyl acetate and methanol (solvents with increasing polarity) and dried at room temperature. The chloroform (CFM), ethyl acetate

(EAFM) and methanolic (MFM) fractions were dissolved in 0.5% dimethyl sulphoxide (DMSO) at different concentrations and used for testing antihypertensive effects in uninephrectomized DOCA-salt hypertensive rats. Of the three fractions, EAFM alone significantly lowered the systolic and diastolic blood pressure (13). The active fraction of EAFM was used in this study.

Method of uninephrectomy

Animals were anesthetized by an intraperitoneal injection of ketamine [75 mg/kg body weight (BW)]. A small patch of skin above the left kidney was shaved and cleaned and iodine-based antiseptic was applied. A 1-cm incision was made at the midscapular region. The kidney was freed from the surrounding tissues and gently pulled out. The adrenal gland, which is attached loosely to the anterior pole of the kidney by connective tissue and fat, was gently freed by tearing the attachments, and was put back into the abdominal cavity. The renal artery and ureter were tied by silk thread, severed and then the kidney was removed. The muscle and skin layers were closed separately using a chromic sterile absorbable suture. After a 1-week recovery period the animals were used for further experiments.

Experimental induction of hypertension

Uninephrectomized animals were given twice-weekly subcutaneous injections of DOCA (25 mg/kg BW) in dimethyl formamide (vehicle) solution and salt was administered by substitution of 1% NaCl solution for drinking water *ad libitum* throughout the experimental period.

Experimental protocol

The animals were divided into six groups of six animals each. EAFM or nifedipine was suspended in 0.5% DMSO and administered by intubation (p.o.) once a day, between 9 a.m. and 10 a.m., for 6 weeks.

Group 1: Sham-operated control (0.5% DMSO only)
Group 2: DOCA + 1% NaCl
Group 3: DOCA + 1% NaCl + EAFM (30 mg/kg BW of 0.5% DMSO)
Group 4: DOCA + 1% NaCl + EAFM (60 mg/kg BW of 0.5% DMSO)
Group 5: DOCA + 1% NaCl + EAFM (120 mg/kg BW of 0.5% DMSO)
Group 6: DOCA + 1% NaCl + nifedipine (20 mg/kg BW of 0.5% DMSO)

After 6 weeks, the systolic and diastolic blood pressures were determined by the tail-cuff method. Among the three different doses of EAFM, 60 mg gave a maximum improvement of blood pressure. So, the dose of 60 mg used for further study.

Experimental protocol for further study

The animals were divided into five groups of six animals each. EAFM or nifedipine was suspended in 0.5% DMSO and administered by intubation (p.o.) once a day, between 9 a.m. and 10 a.m., for 6 weeks.

Group 1: Sham-operated control (0.5% DMSO only)
Group 2: Sham-operated control + EAFM (60 mg/kg BW of 0.5% DMSO)
Group 3: DOCA + 1% NaCl
Group 4: DOCA + 1% NaCl + EAFM (60 mg/kg BW of 0.5% DMSO)
Group 5: DOCA + 1% NaCl + nifedipine (20 mg/kg BW of 0.5% DMSO)

After 6 weeks, the animals were anaesthetized, using ketamine (intramuscular injection), and killed between 8 a.m. and 9 a.m. by cervical dislocation. Erythrocytes and tissues (kidney and heart) were collected for the measurement of membrane bound ATPases such as Na^+/K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase. We have prepared Tris buffer using redistilled water. Tissues were homogenized in Tris buffer and redistilled water was used throughout the experiment to avoid interference prior to phosphorous estimation in the assay of ATPases.

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 environment temperature of 30-34°C for 15 min and 1-9 blood pressure values

were recorded from each animal. The mean blood pressures were calculated based on three different readings. All recordings and data analyses were done using computerized data acquisition system and software.

Biochemical assays

Estimation of total protein

Total protein was estimated by the method of Lowry et al., (15). The standard ranging from 0.2-1.0 ml containing 20-100 μg of protein respectively was taken in different test tubes. The volume in each test tube was made up to 1ml with distilled water and 1 ml of water was taken as blank. 5 ml of alkaline copper reagent was added to each tube and mixed thoroughly. The test tubes were allowed to stand at room temperature for 10 minutes. 0.5 ml of folins ciocalteau reagent was added to each tube rapidly and mixed thoroughly. After incubation at room temperature the colour developed was read against blank at 680 nm and 0.1 ml of the sample was treated similarly. The level of total protein was expressed as mg/g of wet tissue.

Estimation of $Na^{+}K^{+}$-ATPase

Na^{+}/K^{+}-ATPase was assayed according to the method of Bonting (16). To 1 ml of tris buffer, 0.2 ml of each of $MgSO_4$, KC1, NaCl, EDTA were added and equilibrated at 37°C for 10 minutes and the enzyme reaction was initiated by the addition of 0.1 ml of sample. The assay medium was then incubated for one hour at 37 °C. The reaction was arrested by addition 1 ml of 10% TCA. The tubes were centrifuged and the phosphorous content in the supernatant was estimated according to the method of Fiske and Subbarow, (17) using commercial diagnostic kit (Qualigens diagnostics, India). The activity of Na^{+}/K^{+}-ATPases was expressed as μmoles of phosphorous liberated/hr/mg protein.

Estimation of Ca^{2+}-ATPase

Ca^{2+}-ATPase was assayed by the method of Hjerten and Pan (18). The incubation mixture contained 0.1 ml each buffer, $CaCl_2$ATP and water. After equilibrating the tubes at 37°C the reaction was initiated by the addition of 0.1 ml of sample. The contents were incubated at 37 °C for half an hour. The reaction was arrested by addition 1 ml of cold 10% TCA. The tubes were centrifuged and phosphorous content in the supernatant was estimated by Fiske and subbarow method (17) using commercial diagnostic kit (Qualigens diagnostics, India). The activities of Ca^{2+}-ATPases were expressed as μmoles of phosphorous liberated/hr/mg protein.

Estimation of Mg^{2+}-ATPase

The activity of Mg^{2+}-ATPase was assayed by the method of Ohinishi et al., (19). The incubation mixture contained 0.1 ml of 375 mM Tris-HCl buffer (pH 7.6), 0.1 ml of 25 mM $MgCl^2$, 0.1 ml of 10 mM ATP, 0.1 ml water and 0.1 ml of sample. The contents were incubated for 15 min at 37°C and the reaction was arrested by adding 0.5 ml of 10% TCA. The tubes were centrifuged and the phosphorous content in the supernatant was determined by Fiske and Subbarow method (17) using commercial diagnostic kit (Qualigens diagnostics, India). The activities of these ATPase were expressed as μmoles of phosphorous liberated/hr/mg protein.

Statistical analysis

Statistical evaluation was performed using a one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) using SPSS version 10.0. The significance level was set at $P<0.05$.

Results and discussion

The administration of mineralocorticoid together with salt loading in the diet to young adult Wistar rats following surgical removal of one kidney induces hypertension. We chose this model because the hypertension produced is characteristic of human volume-overload induced hypertension with cardiac remodeling especially hypertrophy, fibrosis, conduction abnormalities and endothelial dysfunction (20). Table 1 shows the effect of EAFM at different concentrations (30, 60 and 120 mg/kg BW) on systolic and diastolic blood pressure on sham-operated and DOCA-salt hypertensive rats for 6 weeks. The systolic and diastolic blood pressure significantly increased in DOCA-salt hypertensive rats and treatment with

EAFM significantly restored the blood pressure values. Since EAFM at a dose of 60 mg/kg BW gave the maximum improvement in blood pressure, it was fixed as the optimum dose for further work. The extract of EAFM has been found to contain phytochemicals such as coumarin, vallinic acid, p-coumaric acid, gallic acid, caffeic acid and ferulic acid (13). Several epidemiological studies have shown a significant inverse association between dietary phenolic compounds and long-term mortality from coronary heart disease (21, 22). *In-vivo* biological actions of ferulic acid, one of the phenolic compounds found in EAFM, may support the observed antihypertensive effect of EAFM. Ferulic acid has been shown to lower arterial blood pressure in spontaneously hypertensive rats (14). A recent study suggests that cardiac and renal injury can be avoided or minimized by reducing oxidative stress through increased intake of antioxidants (23). A previous study reported that antioxidants such as vitamins and superoxide dismutase normalize the endothelial dysfunction and improve vascular remodeling in experimental hypertension (24). Raja et al., (25) reported that *M. maderaspatana* possesses antioxidant properties *in vitro*. Engelhard et al., (26) reported that natural antioxidants from tomato extract reduce blood pressure in patients with grade 1 hypertension. Thus, the antihypertensive effect of EAFM may be due to enhanced antioxidant potential.

Changes in membrane lipid composition and enzymatic properties of membrane bound enzymes are shown to occur in hypertension. Figures 1, 2 and 3 show the activity of Na^{+}-K^{+}-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase in erythrocytes and tissues (kidney and heart) of sham-operated and DOCA-salt hypertensive rats. The activities of Na^{+}-K^{+}-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase were shown to decreased significantly on DOCA-salt induced hypertensive rats. Treatment with EAFM significantly increased these enzymes activity to near sham-operated control rats. Thus decreased activities of Na^{+}-K^{+}-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase could be due to the enhanced lipid peroxidation by free radicals in DOCA-salt induced animals. Oxidative stress plays a role in the pathogenic mechanism of essential hypertension (27). Lipid peroxidation can alter the cellular structure of membrane-bound enzymes by changing the membrane phospholipids fatty acids composition. Recent studies has been documented that the administration DOCA-salt, provides a reliable animal model of oxidative and inflammatory stress in the cardiovascular systems (28). Besides endothelin-1 concentrations were elevated in the DOCA-salt rat, which also increased NADPH oxidase-induced superoxide production, also contributing to the endothelin-1-induced vasoconstriction (29). In consistent with our previous finding the increased levels of lipid peroxidations on DOCA-salt induced rats (30) that may involve the decreased activity of Na^{+}-K^{+}-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase. Abnormal lipid peroxides affect membrane bound ATPases activities and their levels were decreased due to the excessive production of thiobarbituric acid reactive substances (6). The detailed mecha-

Table 1. Effect of ethyl acetate fraction of Melothria maderaspatana leaf (EAFM) on the systolic and diastolic blood pressure in sham-operated and uninephrectomized DOCA-salt hypertensive rats

Name of the groups	Systolic blood pressure (mm Hg)		Diastolic blood pressure (mm Hg)	
	0 week	6th week	0 week	6th week
Sham-operated control	120.57 ± 5.89	125.35 ± 7.58 [a]	84.58 ± 4.96	88.34 ± 5.22 [a]
DOCA-salt + 1% NaCl	117.74 ± 7.24	215.68 ± 10.67 [b]	83.66 ± 4.56	172.53 ± 9.15 [b]
DOCA-salt + 1% NaCl + EAFM (30 mg/kg BW)	121.64 ± 6.86	180.54 ± 9.26 [c]	85.37 ± 5.68	145.64 ± 8.46 [c]
DOCA-salt + 1% NaCl + EAFM (60 mg/kg BW)	120.31 ± 6.73	129.78 ± 8.49 [a]	80.17 ± 4.81	92.86 ± 5.48 [a]
DOCA-salt + 1% NaCl + EAFM (120 mg/kg BW)	118.64 ± 6.20	176.48 ± 9.48 [c]	83.17 ± 5.55	140.35 ± 7.98 [c]
DOCA-salt + 1% NaCl + nifedipine (20 mg/kg BW)	117.54 ± 6.58	132.17 ± 7.22 [a]	84.54 ± 5.39	94.68 ± 5.96 [a]

Values are means ± SD for six rats in each group; Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT)

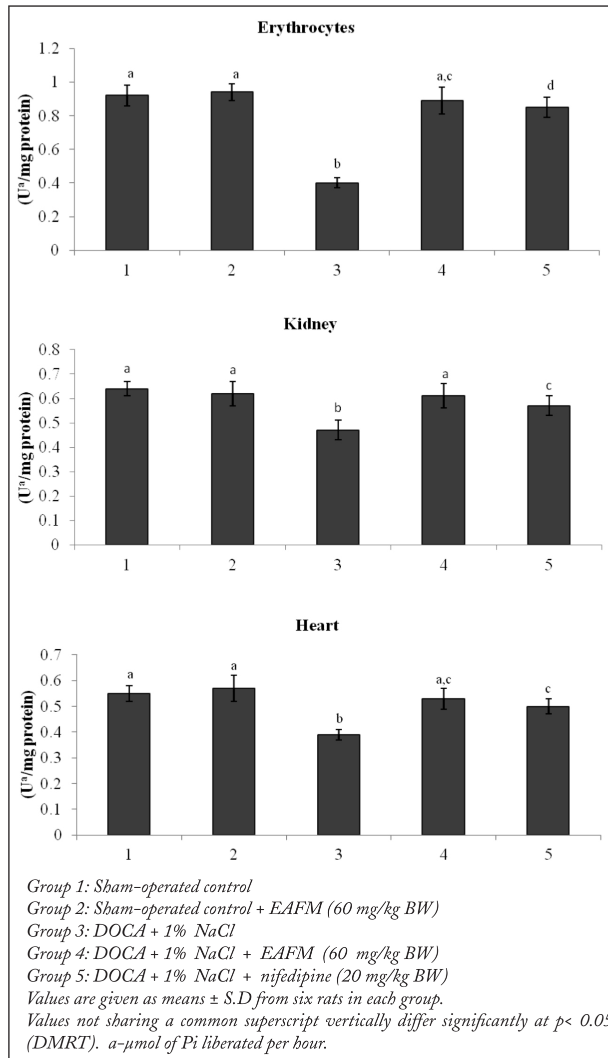

Figure 1. Effect of EAFM on $Na^{+/}$ K^{+}-ATPase in the erythrocytes and tissues of sham-operated and uninephrectomized DOCA-salt hypertensive rats

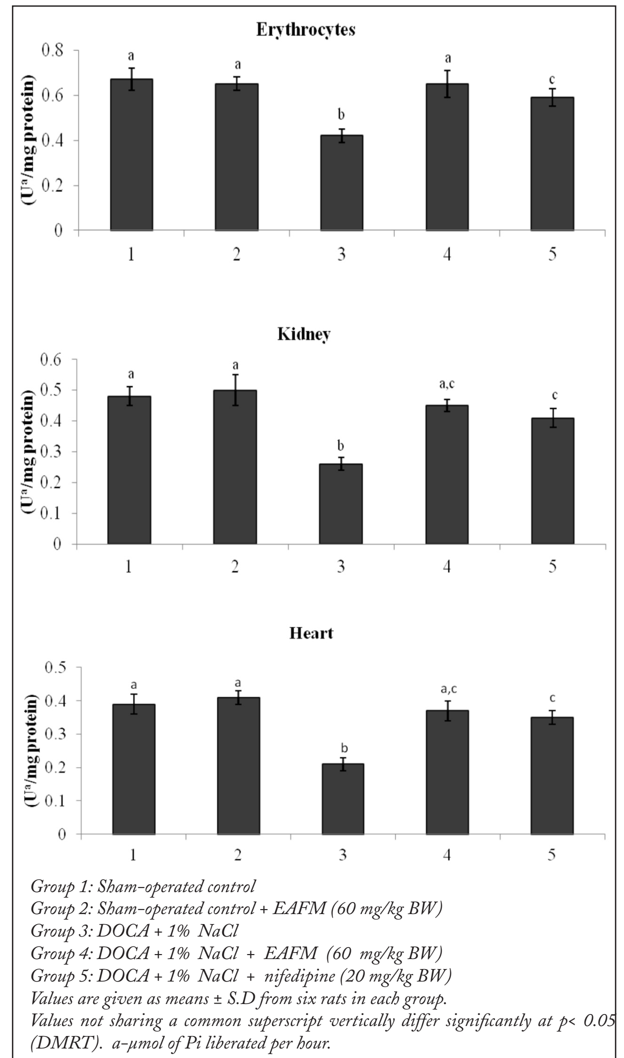

Figure 2. Effect of EAFM on Ca^{2+}-ATPase in the erythrocytes and tissues of sham-operated and uninephrectomized DOCA-salt hypertensive rats

nism of reduced membrane bound ATPases associated with membrane damages on DOCA-salt induced hypertensive rats should be studied in future.

Dietary antioxidants can prevent excess oxidative membrane damage, restore mitochondrial and other cellular membrane functions and reduce essential hypertension. Recent studies demonstrated that treatment with antioxidants or superoxide dismutase mimetics may lower blood pressure and improve vascular structure and function in experimental and human hypertension (31, 32). In the present study, we observed increased activity of Na^{+}-K^{+}-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase in erythrocyte and tissues in administration of EAFM to DOCA-salt hypertensive rats. Antioxidants prevent the peroxidation of membrane lipids, thus preserved effect of abnormal lipid peroxidation to improve membrane integrity and membrane bound ATPases activity, which in turn leads to enhance in cellular homeostasis (33). In our previous report has been shown that the extract of *M. maderaspatana* to protect against the enhanced peroxidation of membrane lipids and decreased antioxidant status of DOCA-salt induced hypertensive rats (30). Thus, increased activity of Na^{+}-K^{+}-ATPase, Ca^{2+}-ATPase and

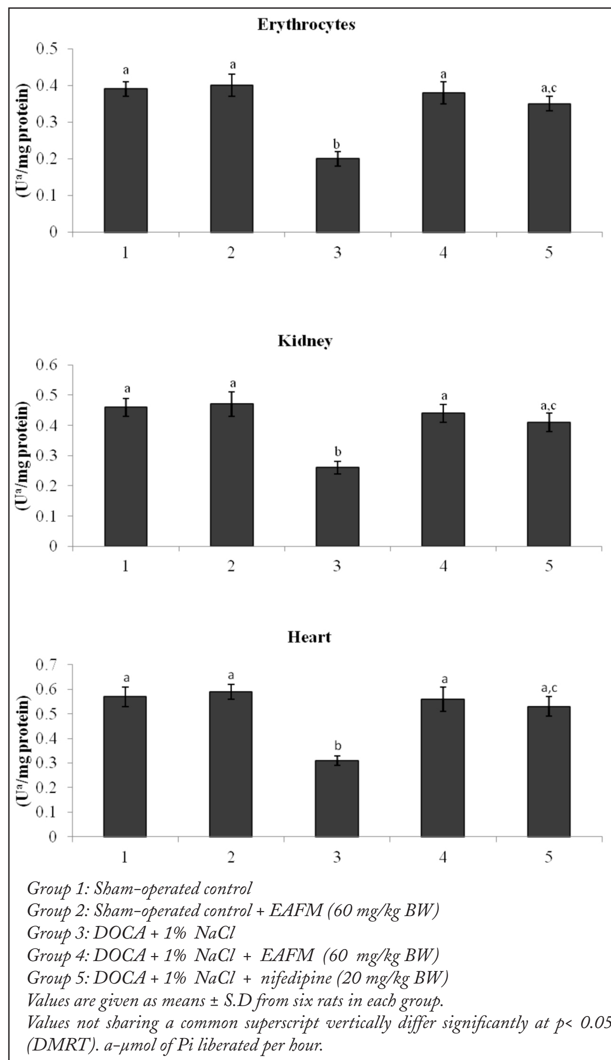

Figure 3. Effect of EAFM on Mg^{2+}-ATPase in the erythrocytes and tissues of sham-operated and uninephrectomized DOCA-salt hypertensive rats

Mg^{2+}-ATPase due to decreased peroxidation of membrane lipids by free radicals and enhanced antioxidant status of this extract.

In conclusion, our study has shown that administration of EAFM to DOCA-salt induced hypertensive rats significantly increased the activity of membrane-bound ATPases in erythrocytes, and in kidney, and heart tissue. This could be due to decrease in enhanced oxidative stress and an increase in antioxidant defense system. Our previous study found that the administration of *M. maderaspatana* to hypertensive rats significantly decreased their enhanced oxidative stress and increased their antioxidants status (30). Further detailed investigation is necessary to discover *M. maderaspatana* mechanism of action and establish its therapeutic potential in the treatment of hypertension and hypertensive complications.

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