Available online at www.jpsscientificpublications.com
Volume – 1; Issue - 3; Year – 2015; Page: 280 – 289

Indo – Asian Journal of Multidisciplinary Research (IAJMR)
ISSN: 2454-1370

THE FLAVONOID TROXERUTIN AMELIORATES HYPERTENSION, OXIDATIVE STRESS AND LIPID METABOLISM IN L-NAME HYPERTENSIVE RATS

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Abstract

The present study was undertaken to assess the antihypertensive and antihyperlipidemic effect of troxerutin (TX) on N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) induced hypertension in male wistar rats. Hypertension was induced by oral administration of L-NAME (40 mg/kg body weight [bw]) dissolved in drinking water daily for 4 weeks. Rats were treated with different doses of TX (25, 50 and 100 mg/kg bw). Hypertension was manifested by considerably increased diastolic blood pressure. L-NAME treated rats showed significant increase in the levels of thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH), total cholesterol (TC), triglycerides (TG), free fatty acids (FFA), phospholipids (PL) and significant decrease in the levels of non-enzymatic antioxidants such as reduced glutathione (GSH), vitamin C and vitamin E in plasma. TX supplementation throughout the experimental period significantly restored all the above alterations. The effect at a dose of 100 mg/kg bw of TX was more pronounced than that of the other two doses (25 and 50 mg/kg bw). No significant effect was observed in control rats treated with TX (100 mg/kg). These results of the present study conclude that TX acts as a protective agent against hypertension, oxidative stress and hyperlipidemia in L-NAME induced hypertensive rats.

Key words: Tixerutin, Wistar rats, Hypertension and Oxidative stress.

1. Introduction

Cardiovascular diseases (CVD) remain the leading cause of mortality worldwide (Quam *et al*., 2006). In humans, hypertension and hyperlipidemia are frequent causes of CVD and major risk factors for atherosclerosis; the presence of both conditions accelerates atherosclerosis (Kwon *et al*., 1998). Hypertension affects more than 600 million people and results in 13% of total deaths globally, and it is estimated that there will be 29% of the world's adult with hypertension by 2025 (Mittal and Singh, 2010). Epidemiological studies provide a large body of evidence for the independent relationship between lipid profile and cardiovascular risk (Seventh report, 2003). Recent evidence indicates that oxidative stress, as the main mechanism is responsible for cardiovascular complications such as hypertension and alteration in lipid metabolism (Prahalathan *et al*., 2012a; Prahalathan *et al*., 2012b).

Oxidative stress, originally described as an altered balance between the production of free radicals and antioxidant defenses, is an important phenomenon in different physiological and pathological processes (Chang and Wu, 2006). Reactive oxygen species (ROS) has been

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Received: 30.06.2015; *Revised*: 25.07.2015;
Accepted: 22.08.2015.

implicated in the pathogenesis of vascular diseases, including atherosclerosis, apoptosis and hypertension (Veeramani *et al*., 2011). The generated ROS induce lipid peroxidation, a type of oxidative deterioration in polyunsaturated fatty acids (PUFAs), which has been linked with altered membrane structure and enzyme inactivation (Kumar *et al*., 2011). Nitric oxide (NO) is one of the smallest biologically active molecules that are produced from L-arginine by nitric oxide synthase (NOS) (de-Belder and Radomski, 1994). NO synthesis and release by endothelial cells play an important vascular relaxation effect, contributing to the modulation of vascular tone (Mori *et al*., 2006). Chronic inhibition of NO synthesis by the administration of L-NAME (N^{ω}-nitro-L-arginine methyl ester hydrochloride) inhibits NOS activity and hence NO biosynthesis, leading to hypertension, atherosclerosis, cardiac remodeling and lipid metabolism alterations (Sanada *et al*., 2003, Khedara *et al*., 1996).

In recent years, the prevention of cardiovascular diseases has been associated with ingestion of fresh fruits, vegetables or plants rich in natural antioxidants (Retelny *et al*., 2008). The polyphenolic compounds were shown to have beneficial effects in preventing cardiovascular alterations in NO-deficient hypertension (Pechanova *et al*., 2004). Flavonoids are the most abundant polyphenolic compounds present in fruits, vegetables and plant-derived beverages such as tea and red wine (Dixon and Steele, 1999). Several epidemiological studies have reported an inverse correlation between flavonoid consumption and CVD risk (Hollman *et al*., 2010). Troxerutin (TX), a trihydroxyethylated derivative of the natural bioflavonoid rutin is present in tea, coffee, cereal grains and a variety of fruits and vegetables. TX possesses a variety of biological activities, such as vasoprotective, anti-oxidative, anti-inflammatory property (Fan *et al*., 2009). However, no scientific investigation has so far been conducted on the antihypertensive and antihyperlipidemic activity of TX in L-NAME induced hypertensive rats. Therefore, present study was designed to determine the dose-dependent effect of chronic administration of TX on L-NAME induced hypertension in albino Wistar rats.

2. Materials and Methods

Animals and chemicals

Healthy male albino Wistar rats (180-220g), were obtained from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, India. They were housed (3 rats/cage) in polypropylene cages (47 × 34 × 20 cm) lined with husk, renewed every 24 h and maintained in an air-conditioned room (25 ± 3 °C) with a12 h light/12 h dark cycle. Feed and water were provided *ad libitum* to all the animals. The whole experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India and approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (Reg No. 160/1999/CPCSEA, Proposal number: 925), Annamalai University, Annamalainagar.

L-NAME and troxerutin were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All other chemicals used in this study were of analytical grade obtained from Merck and Himedia, India.

L-NAME induced hypertensive animal model and troxerutin treatment

Animals were given L-NAME in drinking water at a dosage of 40 mg/kg bw for 4 weeks. Troxerutin was dissolved in water (vehicle) and administered to rats orally everyday using an intragastric tube for a period of 4 weeks.

Experimental protocol

Different doses of troxerutin (25, 50 and 100 mg/kg/body weight (bw)) were assessed to find out the antihypertensive effect in L-NAME-induced hypertension.

Group I : Control+vehicle
Group II : Control+ troxerutin (100 mg/kg bw)
Group III : L-NAME control (40 mg/kg bw)
Group IV : L-NAME+ troxerutin (25 mg/kg bw)
Group V : L-NAME+ troxerutin (50 mg/kg bw)
Group VI : L-NAME+ troxerutin (100 mg/kg bw)

During the experimental period, body weight gain was measured everyday. After the completion of experimental period, the rats were anaesthetized and sacrificed by cervical dislocation. Blood samples were collected into heparinized tubes and centrifuged at 1000 × *g* for 10 min and the plasma was separated by aspiration.

Blood pressure measurement

Before commencement of the experiment, animals were trained with the instrument for measuring blood pressure. In all groups of animals, diastolic blood pressure was measured every week during the entire period of the study noninvasively using a tail cuff method (IITC, model 31, USA) according to standard procedures. Values reported are the average of lowest three readings. All the recordings and data analyses were done using a computerized data acquisition system and software.

Estimation of lipid peroxidation products

The levels of thiobarbituric acid reactive substances (TBARS) in plasma was estimated by the method of Niehaus and Samuelson (1968). A total of 0.5 ml of plasma was diluted with 0.5 ml of double distilled water and mixed well, and then 2.0 ml of thiobarbituric acid (TBA)–trichloroacetic acid (TCA) - hydrochloric acid (HCL) reagent was added. The mixture was kept in boiling water bath for 15 min. After cooling, the tubes were centrifuged for 10 min and the supernatant was taken for measurement. The absorbance was read at 535 nm against reagent blank.

Estimation of plasma lipid hydroperoxides (LOOH) was done by the method of Jiang *et al*. (1992). Fox reagent (0.9 ml) was mixed with 0.1 ml of plasma and incubated for 30 min at room temperature. The color developed was read at 560 nm.

Determination of non-enzymatic antioxidants

Reduced glutathione (GSH) in the plasma was estimated by the method of Ellman (1959). 0.5 ml of plasma was pipetted out and precipitated with 2.0 ml of 5% TCA. A total of 2.0 ml of supernatant was taken after centrifugation and 1.0 ml of Ellman's reagent and 4.0 ml of 0.3 M disodium hydrogen phosphate were added. The yellow color developed was read at 412 nm.

Vitamin C in the plasma was estimated by the method of Roe and Kuether (1943). To 0.5 ml of plasma, 1.5 ml of 6% TCA was added and allowed to stand for 5 min and centrifuged. To the supernatant, 0.3 g of acid washed Norit was added, shaken vigorously and filtered. A total of 0.5 ml of the filtrate was taken and 0.5 ml of dinitrophenylhydrazine (DNPH) was added, stoppered and placed in water bath at 37 °C for exactly 3 h, removed, placed in ice-cold water and added 2.5 ml of 85% sulphuric acid. The contents of the tubes were mixed well and allowed to stand at room temperature for 30 min. The color developed was read at 540 nm.

The level of Vitamin E in the plasma was estimated by the method of Baker *et al*. (1980). To 0.5 ml of plasma, 1.5 ml of ethanol was added, mixed and centrifuged. The supernatant was evaporated at 80 °C and to the precipitate, 3.0 ml of petroleum ether, 0.2 ml of 2, 2′ dipyridyl solution and 0.2 ml of ferric chloride were added. Afterwards, all the tubes were mixed well and kept in dark for 5 min and 4.0 ml of n-butanol was added. The red color developed was read at 520 nm.

Extraction of lipids

Total lipids were extracted from plasma according to the method of Folch et al. [26] using chloroform: methanol mixture (2:1, v/v). Plasma was mixed with cold chloroform–methanol (2:1, v/v) and the contents were extracted after 24 hours. The extraction was repeated four times. The combined filtrate was washed with 0.7% of potassium chloride (0.1 N) and the aqueous layer was discarded. The organic layer was made up to a known volume with chloroform and used for the analysis of lipids.

Estimation of total cholesterol

The levels of total cholesterol (TC) were estimated by the method of Zlatkis *et al*. (1953). Lipid extract of 0.5 ml was evaporated to dryness. To this, 5.0 ml of ferric chloride-acetic acid reagent was added. The tubes were mixed well and 3.0 ml of concentrated sulphuric acid (H_2SO_4) was added. A series of standards containing cholesterol in the range 3–15µg were made up to 5.0 ml with the reagent and a blank containing 5.0 ml of the reagent were prepared. The absorbance was read after 20 minutes at 560 nm.

Estimation of triglycerides

The content of triglycerides (TG) was estimated by the method of Fossati and Prencipe (1982). Lipid extract of 0.5 ml was evaporated to dryness. To this, 0.1 ml of methanol was added followed by 4.0 ml of isopropanol. About 0.4 g of alumina was added to all the tubes and shaken well for 15 minutes. It was centrifuged and then accurately 2.0 ml of the supernatant was transferred to appropriately labeled tubes. The tubes were placed in a water bath at 65°C for 60 minutes for saponification after adding 0.6 ml of the saponification reagent followed by 0.1 ml of sodium metaperiodate and 0.5 ml of acetyl acetone reagent. After mixing, the tubes were kept in a water bath at 65°C for an hour. A series of standards of concentration 8–40µg triolein were treated similarly along with a blank containing only the reagents. All the tubes were cooled and read at 405 nm.

Estimation of free fatty acids

Free fatty acid (FFA) level was estimated by the method of Falholt *et al*. (1973). An aliquot (0.5 ml) of the lipid extract was evaporated to dryness. To this, 1.0 ml of phosphate buffer, 6.0 ml of extraction solvent, and 2.5 ml of copper (Cu-TEA) reagent were added. All the tubes were shaken vigorously for 90 seconds and were kept aside for 15 minutes. Then the tubes were centrifuged and 3.0 ml of the upper layer was transferred to another tube containing 0.5 ml of diphenylcarbazide solution and mixed carefully. The absorbance was read at 550 nm after 15 minutes. A reagent blank containing (1.0 ml) of phosphate buffer was processed as blank.

Estimation of phospholipids

Phospholipid (PL) levels were estimated by the method of Zilversmit and Davis (1950). An aliquot of 0.5 ml of the lipid extract was pipetted out into a Kjeldahl flask and evaporated to dryness. To the extract/0.2 ml of plasma, 1 ml of 5 NH_2SO_4 was added and digested in a digestion rack till the appearance of light brown color. Two to three drops of concentrated nitric acid was added and the digestion continued till it became colorless. The Kjeldahl flask was cooled and 1.0 ml of distilled water was added and heated in a boiling water bath for about 5 minutes. Then, 1.0 ml of 2.5% ammonium molybdate and 0.1 ml of 1-amino-2-napthol-4-sulfonic acid were added. The volume was then made upto 5.0 ml with distilled water and the absorbance was measured at 660 nm within 10 minutes.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using statistical package for the social science (SPSS) software version 20.0. Values were expressed as mean ± S.D. for six rats in each group. Values were considered significant when $P<0.05$.

3. Results

Blood pressure measurement

Fig. 1 shows the effect of TX at three different doses (25, 50 and 100 mg/kg) on diastolic blood pressure in L-NAME induced hypertensive rats. The L-NAME rats showed significantly increased diastolic blood pressure while treatment with TX significantly reduced the diastolic blood pressure.

Body weight

Fig. 2 shows the effect of TX at three different doses (25, 50 and 100 mg/kg) on body weight in L-NAME induced hypertensive rats. The L-NAME rats showed significantly decreased body weight while treatment with TX significantly elevated the body weight. The 100 mg/kg dose showed better effect in reducing diastolic blood pressure and enhancing body weight than other two doses (25 and 50 mg/kg), so we have chosen 100 mg/kg dosage for further evaluation.

Lipid peroxidation products

In L-NAME hypertensive rats, the levels of TBARS and LOOH were significantly increased in plasma when compared with control rats. TX supplementation (100 mg/kg) during the entire period of study significantly decreased the levels of TBARS and LOOH in plasma of L-NAME rats (**Table 1**).

Non-enzymatic antioxidants

Table 1 illustrates the levels of non-enzymatic antioxidants such as vitamin C, vitamin E and glutathione in plasma of control and L-NAME hypertensive rats. The levels of non-enzymatic antioxidants were significantly decreased in L-NAME hypertensive rats. Oral administration of TX significantly improved these parameters toward normalcy.

Plasma lipid level

Fig. 3 portray the levels of lipids (TC, TG, FFA and PL) in plasma of control and L-NAME hypertensive rats. The concentrations of plasma lipids (TC, TG, FFA and PL) were significantly increased in hypertensive rats as compared with control. TX supplementation significantly declined the levels of plasma lipids in L-NAME rats toward normal.

4. Discussion

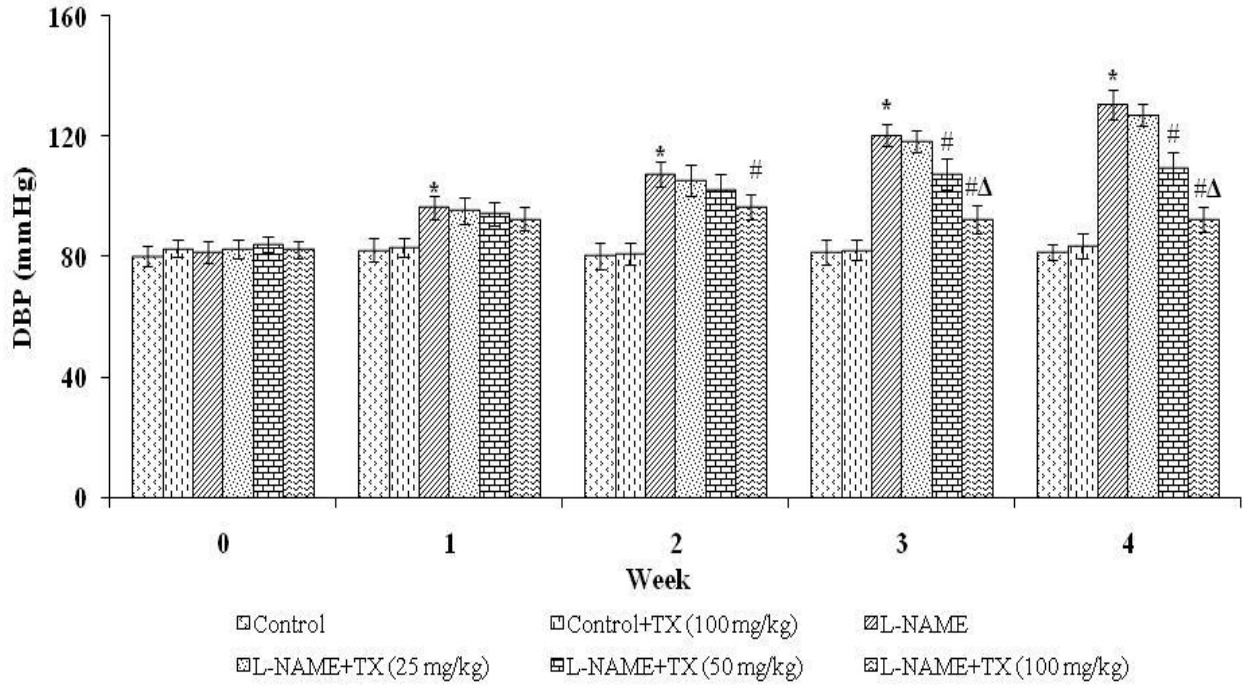
Nitric oxide (NO) is a vital regulator of vascular endothelial function and blood pressure (Silva-Herdade and Saldanha, 2011). Chronic inhibition of NO produces volume-dependent elevation of blood pressure, and its physiological and pathological characteristics resemble essential hypertension (Attia *et al*., 2001). Chronic NO inhibition with L-NAME can increase regional vascular resistance, raise the blood pressure, oxidative stress, and renal damage in both *in vitro* and *in vivo* models (Harrison, 1997). In the present study, there is a significant increase in diastolic blood pressure of L-NAME induced hypertensive rats. Studies reported that antioxidants such as vitamins and superoxide dismutase normalize the endothelial dysfunction and improve vascular remodeling in experimental hypertension (Akpaffiong and Taylor, 1998). TX has already been pharmacologically evaluated for its potent antioxidant efficacy (Fan *et al*., 2009). Our finding shows oral supplementation of TX resulted in a significant reduction in diastolic blood pressure. L-NAME rats showed significantly decreased body weight. After treatment with TX, the weight loss improved which might be as a result of its ability to reduce the loss or degradation of structural proteins (Varshavsky, 1997).

The increased levels of reactive oxygen species such as superoxide anion, hydrogen peroxide and lipid peroxides are reported in hypertensive patient (Touyz, 2000). Lipid peroxidation is an important pathogenic event in hypertension and accumulation of LOOH reflects the various stages of this disease and its complications (Hamberg *et al*., 1974). Our results showed that the lipid peroxidation end products, measured as TBARS and LOOH were increased in plasma of L-NAME-induced hypertensive rats.

Table 1. Effect of troxerutin on lipid peroxidation, non-enzymatic antioxidants in plasma of control and L-NAME induced hypertensive rats.

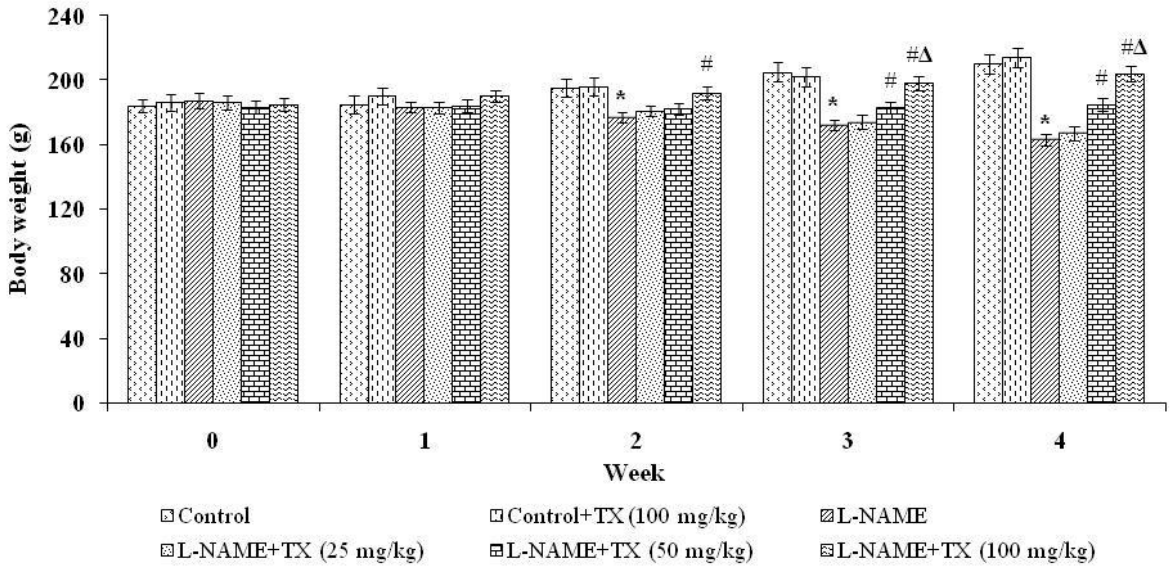
Parameter		Control	Control+TX	L-NAME	L-NAME+TX
TBARS	Plasma (mmol/dL)	$0.18 \pm 0.01^{*}$	$0.16 \pm 0.01^{*}$	$0.46 \pm 0.03^{\#}$	$0.27 \pm 0.01^{\Delta}$
LOOH		$10.23 \pm 0.62^{*}$	$10.45 \pm 0.54^{*}$	$24.31 \pm 1.84^{\#}$	$15.14 \pm 0.72^{\Delta}$
GSH		$38.42 \pm 2.11^{*}$	$37.33 \pm 2.23^{*}$	$25.42 \pm 1.67^{\#}$	$32.24 \pm 2.12^{\Delta}$
Vitamin-C	Plasma (mg/dL)	$3.15 \pm 0.21^{*}$	$2.94 \pm 0.18^{*}$	$1.18 \pm 0.05^{\#}$	$2.29 \pm 0.18^{\Delta}$
Vitamin-E		$2.87 \pm 0.19^{*}$	$2.59 \pm 0.16^{*}$	$1.1 \pm 0.05^{\#}$	$1.95 \pm 0.06^{\Delta}$

Values are mean ± S.D. for six rats in each group. Values not sharing a common symbol differ significantly at P<0.05 (DMRT).



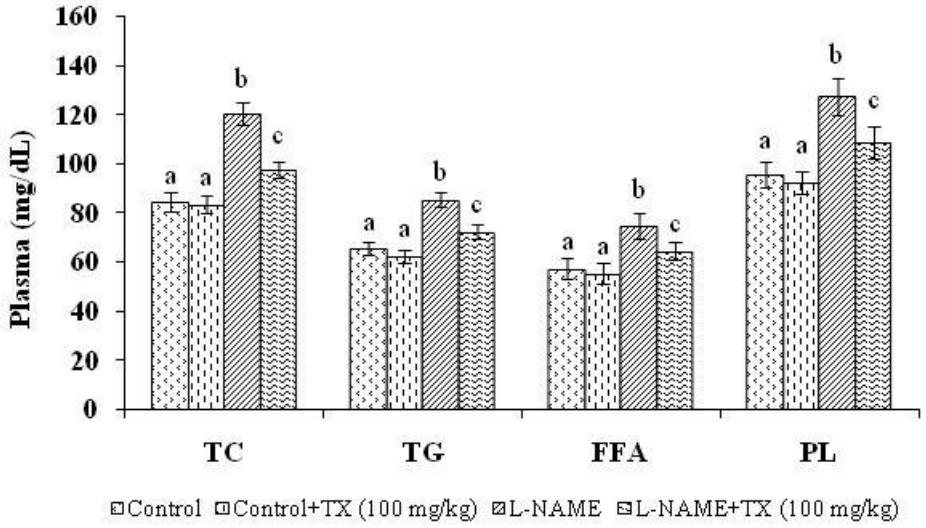
Values are mean ± S.D. for six rats in each group. * differs significantly at P<0.05 compared with control. # differs significantly at P<0.05 compared with L-NAME rats. Δ differs significantly at P<0.05 compared with 50 mg/kg troxerutin treated rats (DMRT).

Fig. 1. Effect of troxerutin on diastolic blood pressure in control and L-NAME induced hypertensive rats.



Values are mean ± S.D. for six rats in each group. * differs significantly at P<0.05 compared with control. # differs significantly at P<0.05 compared with L-NAME rats. Δ differs significantly at P<0.05 compared with 50 mg/kg troxerutin treated rats (DMRT).

Fig. 2. Effect of troxerutin on body weight in control and L-NAME induced hypertensive rats.



Values are mean ± S.D. for six rats in each group. Values not sharing a common letter differ significantly at P<0.05 (DMRT).

Fig. 3. Effect of troxerutin on lipid profile in plasma of control and L-NAME induced hypertensive rats.

A possible explanation for the enhancement of lipid peroxidation products might be due to increased free radical production and decreased antioxidant system. Treatment with TX decreased the levels of lipid peroxidation products in L-NAME hypertensive rats. Thus, TX inhibits lipid peroxidation may be due to scavenging of free radicals and is attributed to its antioxidant property (Fan *et al*., 2009).

The increase in lipid peroxidation products in L-NAME induced hypertensive rats might be a reflection of the decrease in enzymatic and non-enzymatic antioxidants defense system (Yu, 1994). Intracellular defense against active oxygen species is performed by antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic antioxidants such as GSH, vitamin C and vitamin E (Romero and Roche, 1996). The non-enzymatic antioxidants scavenge the residual free radicals escaping from decomposition enzymes (Roy *et al*., 1994). The major antioxidant of the aqueous phase is vitamin C, which acts as the first line of defense during oxidative stress. Vitamin E appears to be the most effective lipid soluble antioxidant in the biological system (Kitts *et al*., 1998). GSH, a tripeptide, is a powerful cellular antioxidant, which is directly involved in the removal of superoxide radicals, peroxyl radicals and singlet oxygen (Abidi *et al*., 1999). The lowered concentrations of vitamin C, vitamin E, and GSH observed in L-NAME-induced hypertensive rats might be due to neutralizing the production of free radicals. Treatment with TX enhanced the levels of non-enzymatic antioxidants in L-NAME-treated rats.

The presence of high blood pressure and hyperlipidemia is so common in hypertension that many have argued that the high blood pressure itself may play a role in altering lipid metabolism, resulting in abnormalities (Friedwald *et al*., 1972). High levels of circulating cholesterol and its accumulation in tissues are well associated with cardiovascular damage (Salter and White, 1996). In our study, we observed increased levels of TC in plasma of hypertensive rats. TX supplementation decreased the levels of TC in hypertensive rats.

Hypertriglyceridemia is independent risk factors that can accelerate the development of coronary artery disease and progression of atherosclerotic lesions (McKenney, 2001). In this study, we observed a higher concentration of TG in L-NAME hypertensive rats. TX supplementation lowered the levels of TG in plasma of hypertensive rats. This beneficial action might be due to the effective quenching of free radicals by TX. The oxidative tissue damage can release the membrane lipids such as FFA and PL into blood (Ohara *et al*., 1993). TX supplementation rescues the tissues from lipid peroxidation by mopping up free radicals and diminished the levels of FFA and PL in plasma of hypertensive rats.

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