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Full Length Research Paper

Ameliorative effects of rutin and ascorbic acid combination on hypercholesterolemia-induced hepatotoxicity in female rats

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It has been shown that female rats are more responsive to dietary cholesterol challenge than male and also, hypercholesterolemia induction is easier in female rats. The present study was designed to investigate the effects of rutin (RT) and ascorbic acid (AA) combination on high cholesterol diet (HCD)-induced hepatic damage in female Wistar rats. Rats were randomly divided into four groups and fed by respective diets for 6 consecutive weeks. Hepatic enzymes activity and lipid profile were estimated in plasma samples. Nucleic acids, total proteins, malondialdehyde (MDA), glutathione (GSH), total cholesterol (TC) and triglycerides (TG) levels were measured in liver. Histopathological changes were observed in hepatic tissue. Enzymatic activity and lipid profile increased significantly in plasma of HCD fed rats, which was normalized in HCD+RT+AA group. In hepatic cells, total protein, nucleic acids and GSH levels were significantly decreased, while MDA, TC and TG levels were increased by HCD. These changes were significantly corrected in HCD+RT+AA group. The apparent protection was further confirmed by the histopathological screening. In conclusion, the study provides the presence of oxidative stress in hypercholesterolemic female rats and suggests beneficial effects of RT and AA combinations in combating the oxidative process in nonalcoholic fatty liver disease.

Key words: Rutin, ascorbic acid, hypercholesterolemia, oxidative stress.

INTRODUCTION

Hypercholesterolemia is considered as one of the most familiar metabolic disorders and it is closely associated with obesity, diabetes mellitus, and several other metabolic syndromes (Farrell et al., 2008; Postic and Girard, 2008; Trauner et al., 2010). Hypercholesterolemia can eventually lead to nonalcoholic fatty liver disease (NAFLD) by depositing the lipids and triglycerides in liver which is usually progress to cirrhosis or even hepato cellular carcinoma (Kim et al., 2012; Lee et al., 2007). Experimentally induced hypercholesterolemia can impairs lipid metabolism leading to elevation of both blood and tissue lipid profile (Vasu et al., 2005). Moreover, studies demonstrated that even short exposure to high cholesterol diet (HCD) is capable of inducing hypercholesterolemia and is significantly associated with oxidative stress (Tomofuji et al., 2006).

Oxidative stress and generation of reactive oxygen

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species (ROS) are deemed to play a vital role in hepatocyte apoptosis and in the pathogenesis of NAFLD (Kojima et al., 2007; Trauner et al., 2010). Hypercholesterolemia was shown to impair oxidative stress biomarkers such as malondialdehyde (MDA) and superoxide dismutase (SOD) and also known to boost ROS production via different mechanisms and hence increase lipid peroxidation. Studies demonstrated that oxidative stress associated with hypercholesterolemia have harmful effect on different organs particularly heart, liver, and kidney. Moreover, generation of ROS have been implicated in the pathophysiology of various disease including; heart failure (Prasad et al., 1996), ischemic heart disease (Ferrari et al., 1998), hepatic injury (Jarrar et al., 2000), and chronic renal damage and failure (Baker et al., 1985; Galle, 2001). Therefore, it is necessary to search for effective approaches to control hypercholesterolemia and the associated fatty liver complication. Non pharmacological approaches for hypercholesterolemia include increased physical activity and weight reduction through lifestyle modification as well as dietary changes (Kim et al., 2012). Antioxidant supplementations may effectively suppress oxidative stress, which seems to be a useful therapy (Yang et al., 2012).

Rutin (RT), a quercetin-3-rutinosid or vitamin-P, is a well known flavonoidal glycoside and set as an affective phenolic compound. It is an antioxidant, which comprised of the flavonolquercetin and the disaccharide rutinose (Ihme et al., 1996; Lindahl and Tagesson, 1997). Phenolic compounds are mainly found in onions, apples, tea and red wine (Hertog et al., 1993; Khan et al., 2012). Various pharmacological properties were reported for rutin including antibacterial, antitumor, anti-inflammatory, anti-diarrheal, antiulcer, anti-mutagenic, vasodilator and immunomodulator (Janbaz et al., 2002). Moreover, rutin has inhibitory effects against membrane lipid peroxidation and generation of ROS like in other plant materials were reported (Lopez-Revuelta et al., 2006; Wang, 2012; Yin et al., 2012) and can suppress adipocyte differentiation from pre-adipocytes (Choi et al., 2006). In addition, rutin can also decrease the level of TBARS and increase the SOD activity suggesting a possible protective role in oxidative stress-mediated diseases (Park et al., 2002). On the other hand, ascorbic acid (AA; as a reduced form of vitamin C) is a famous effective antioxidant. Ascorbic acid is the most predominant form of vitamin C in the human body and is involved in tissue growth and repair. It is a water-soluble enzyme cofactor, abundantly present in different plants and animals. AA has a powerful antioxidant activity, which made it well known to protect tissues from oxidative injury via efficiently quenching the damaging free radicals produced by different biological processes (Heaney et al., 2008; Verrax and Calderon, 2008).

When multiple antioxidants are used in combination, they protect against vulnerability to other agents and synergistically potentiate their antioxidant properties. These synergistically potentiated antioxidant effects of agents contribute to the improvement of cognitive function. Thus the present study was designed to investigate the additive hepatoprotective effects of RT and AA combination against hypercholesterolemia induced oxidative injury following HCD supplementation to female Wistar rats.

MATERIALS AND METHODS

Animals

Twenty four young female Wistar albino rats, roughly the same age of 7 weeks, weighing 80 to 100 g were supplied from the Experimental Animal Care Center (King Saud University, Riyadh, Saudi Arabia). The animals were acclimatized to laboratory conditions before the tests for ten days. They were fed on Purina rat chow diet (Manufactured by Grain Silos and Flour Mills Organization, Riyadh, Saudi Arabia) and water *ad libitum* and were maintained under standard conditions of temperature (22±1ºC), humidity (50 to 55%), and light (12 h light/dark cycles). All methods including euthanasia procedure were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research, National Institute of Health (NIH Publications No. 80-23; 1996) and it was approved by the Ethics committee of Experimental Animal Care Center, College of Pharmacy, King Saud University (Riyadh, Saudi Arabia).

Dietary protocol

Experimental diets were prepared in pellet form by adding 0.1% RT + 0.2% AA (RT+AA), 1% cholesterol + 0.5% cholic acid (HCD) or 0.1% RT + 0.2% AA (RT+AA) + 1% cholesterol + 0.5% cholic acid (HCD+RT+AA) in rat chow powder. The diets were prepared weekly and shade dried. Animals were randomly divided into four groups (six in each group); (1) Control (rat cow), (2) RT+AA, (3) HCD and (4) HCD+RT+AA. All animals were kept on free access to food and water during the whole experimental period for six weeks. At the end of the experiment, animals were sacrificed by decapitation and the trunk blood was collected in heparinized tubes. Liver tissues were dissected, weighed and immediately dipped in liquid nitrogen for one min and then preserved at -75ºC (Ultra-low freezer, Environmental Equipment, Cincinnati, Ohio, USA) till analysis. Plasma samples were collected after centrifugation at 4000 rpm for 15 min and stored in freezer at -20ºC till analysis.

Blood chemistry

Plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), TG, TC, high density lipoprotein-cholesterol (HCD-C) and low density lipoprotein–cholesterol (LDL-C) were estimated by using commercially available diagnostic kits (Human, Wiesbaden, Germany).

Estimation of nucleic acids and total protein levels in liver

Nucleic acids (DNA and RNA) were estimated in liver by the method described by Bregman (1983). In brief, hepatic tissues were homogenized in ice-cold distilled water, and then the homogenates

Table 1. Effects of rutin (RT) and ascorbic acid (AA) combination on plasma level of AST, ALT, ALP, TG, TC, HDL-C and LDL-C, in high-cholesterol diet (HCD) fed rats for six consecutive weeks.

Parameter	Control	RT + AA	HCD	HCD + RT + AA
AST (U/L)	39.96 ± 3.36	41.04 ± 7.20	49.59 ± 4.41 **	39.39 ± 4.56 #
ALT (U/L)	20.65 ± 1.59	21.85 ± 2.37	24.69 ± 3.29 *	21.29 ± 1.54 #
ALP (U/L)	270.65 ± 34.85	281.21 ± 30.55	408.04 ± 48.70 ***	330.84 ± 18.54 ##
TG (mg/dl)	65.62 ± 4.86	64.72 ± 9.62	156.80 ± 24.59 ***	120.93 ± 7.89 ###
TC (mg/dl)	70.06 ± 22.08	65.94 ± 8.54	139.39 ± 24.61 ***	99.88 ± 12.04 ##
HDL-C (mg/dl)	27.43±4.95	28.36±2.6	20.35±1.89 **	27.51±2.69 ##
LDL-C (mg/dl)	48.32±4.24	46.04±5.03	86.62±6.88 ***	56.65±4.58 ###

Data were expressed as mean±S.D and analyzed using one-way ANOVA followed by Student Newman-Keuls method as post hoc test. Six rats were used in each group. *P<0.05, **P<0.01 and ***P<0.001 HCD and RT+AA vs. Control; #P<0.05, ##P<0.01 and ###P<0.001 HCD+RT+AA vs. HCD.

were suspended in 10% ice-cold trichloroacetic acid (TCA). Pellets were extracted twice with 95% ethanol. For quantification of DNA levels, the nucleic acids extract was treated with diphenylamine reagent and the intensity of blue color was measured at 600 nm. RNA levels were estimated by treating the nucleic acids extract with orcinol reagent and the green color was recorded at 660 nm on spectrophotometer (LKB-Pharmacia, Mark II, Ireland). The modified Lowry method by Schacterle and Pollack (1973) was used to estimate liver levels of total protein. Bovine plasma albumin was used as standard.

Estimation of MDA in liver

The method described by Ohkawa et al. (1979) was used to determine MDA concentrations in liver. Briefly, 200 mg of liver samples were homogenized in aqueous 0.15 M KCl solution to give 10% homogenate. 1 ml of homogenate was then mixed with one ml of 10% TCA and centrifuged at 3,000 rpm for 15 min. 1 ml of supernatant was suspended into 1 ml of 0.67% 2-thiobarbutaric acid. Sample tubes were then placed into a boiling water bath and kept for 15 min. Samples were allowed to cool down at room temperature followed by centrifugation at 3000 rpm for 15 min. The optical density of the clear pink supernatants was measured at 532 nm.

Estimation of GSH level in liver

The concentration of GSH was determined as described by Sedlak and Lindsay (1968). Briefly, 200 mg from liver samples were dissected out and homogenized in ice-cold 0.02 M ethylenediaminetetraacetic acid (EDTA). An aliquots of 0.5 mL of tissue homogenate was mixed with 0.2 M Tris buffer, pH 8.2 and 0.1 mL of 0.01 M Ellman's reagent, [5,5'-dithiobis-(2-nitro-benzoic acid)] (DTNB). Each sample tube was centrifuged at 3000 g at room temperature for 15 min. The absorbance of the clear supernatants was measured using spectrophotometer at 412 nm in one centimeter quarts cells.

Estimation of lipid contents in liver

Total cholesterol and triglycerides levels in liver tissues were estimated by using Folch et al. (1957) method. In brief, liver tissues were homogenized in 0.15 mol/L of ice-cold KCl (10% w/w) and lipids were extracted with chloroform:methanol (2:1). After the extraction and evaporation, tissue lipids were re-dissolved in isopropanol, and liver cholesterol and triglyceride levels were estimated enzymatically by commercially available kits (Human, Wiesbaden, Germany).

Histopathological evaluation

Randomly two rats from each group: control, HCD, and HCD+RT+AA were taken for histopathological examination. The cross-section from each liver was fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 3 µm, stained with Hematoxylin and Eosin (H & E) stain and placed in slides for light microscopic examination. Slides were evaluated by a histopathologist who was blinded to the treatment groups to avoid any kind of bias.

Statistical analysis

All data were expressed as mean ± Standard Deviation (SD) and statistically analyzed using one-way ANOVA followed by Student-Newman-Keuls multiple comparisons test. The differences were considered statistically significant at P<0.05. Graph Pad prism program (version 5) was used as analyzing software.

RESULTS

In HCD fed rats, mean liver weights were significantly increased as compared to control animals. Combined supplementation of RT and AA along with high cholesterol caused significant attenuation in liver weights compared to HCD group (Figure 1).

Plasma enzymatic activities of AST, ALT and ALP were significantly increased in HCD fed rats compared to control group increased in HCD fed rats compared to control group. These activities were significantly inhibited in HCD+RT+AA group when compared to HCD fed animals (Table 1). Plasma lipid levels including TC, TG and LDL-C were significantly increased in HCD fed rats as compared to control group of animals. In contrast,

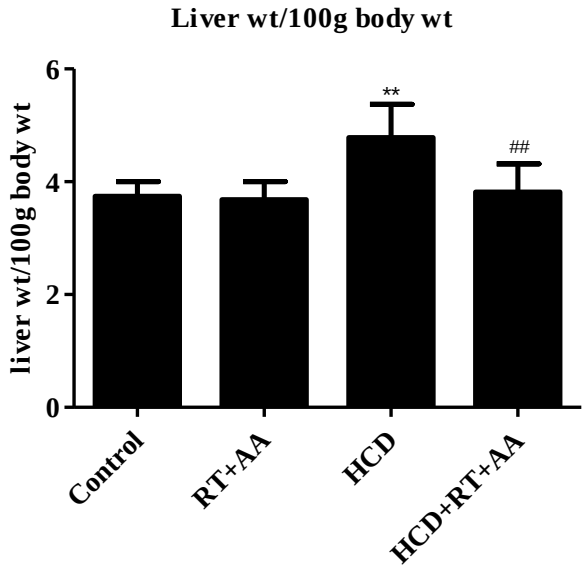

Figure 1. Effects of rutin (RT) and ascorbic acid (AA) combination on liver weight per 100 gram body weight in high-cholesterol diet (HCD) fed rats following 6 weeks of supplementation. Data were expressed as Mean±S.D and analyzed using one-way ANOVA followed by Student Newman-Keuls method as post hoc test. Six rats were used in each group. **P<0.01 HCD and RT+AA vs. Control group; ##P<0.01 HCD+RT+AA vs. HCD group.

plasma HDL-C levels were significantly decreased in HCD fed animals compared to controls. In HCD+RT+AA group, the mean levels of TC, TG and LDL-C were significantly decreased as compared to HCD fed rats respectively. This combined therapy of vitamins to HCD fed rats also significantly elevated the HCD-cholesterol levels compared to HCD group (Table 1).

In hepatic cells, DNA and RNA levels significantly decreased from 182.10±8.91 to 157.53±7.42 µg/100 mg and 612.88±26.53 to 539.13±26.45 µg/100 mg in HCD fed rats respectively. Similarly total protein levels also decreased from 15.26±0.35 to 14.46±0.31 mg/100 mg and it was found to be statistically significant. Feeding of animals with RT and AA in combination showed significant enhancement in the reduced levels of DNA, RNA and total protein when compared to HCD group respectively (Figure 2).

Oxidative markers showed significant changes in heaptic cells of HCD fed rats such as MDA levels increased and GSH levels decreased significantly while compared to control animals. The combined supplementation of RT and AA along with high cholesterol significantly brings back the MDA and GSH levels near to normal values (Figure 3).

Hepatic lipid compounds including TC and TG (mg/g tissue) levels were significantly increased in HCD fed rats compared to control animals. As similar to plasma lipid

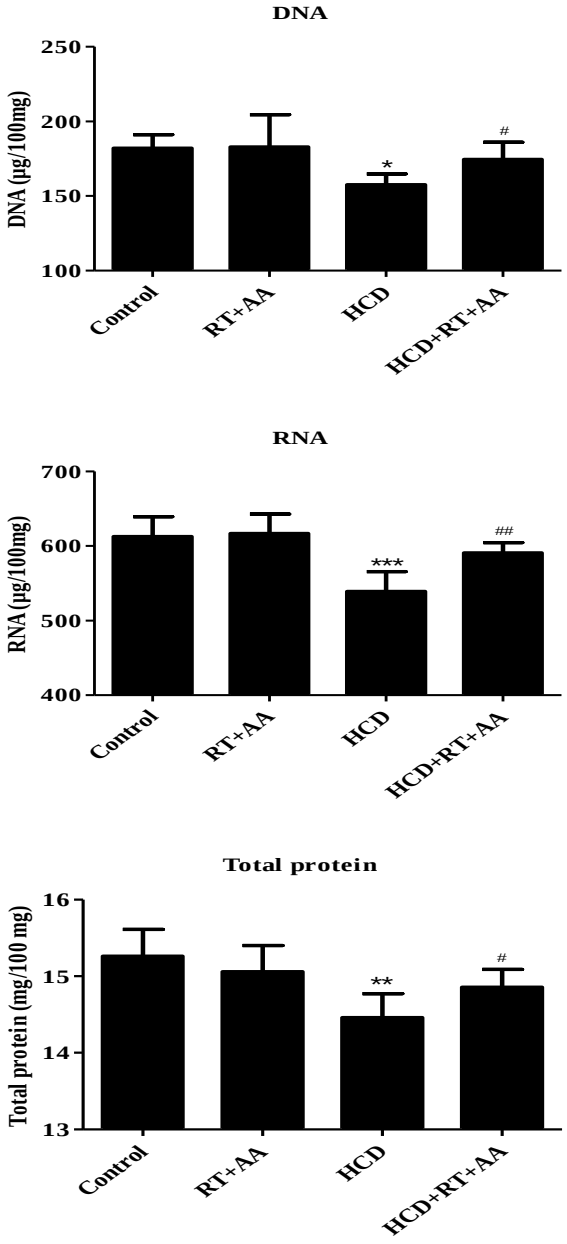

Figure 2. Effects of rutin (RT) and ascorbic acid (AA) combination on hepatic nucleic acids and total protein level in high-cholesterol diet (HCD) fed rats following 6 weeks of supplementation. Data were expressed as Mean±S.D and analyzed using one-way ANOVA followed by Student Newman-Keuls method as post hoc test. Six rats were used in each group. *P<0.05, **P<0.01 and ***P<0.001 HCD and RT+AA vs. Control group; #P<0.05 and ##P<0.01 HCD+RT+AA vs. HCD group.

levels, RT and AA combined supplementation to HCD fed rats significantly protected the elevated levels of these lipids in hepatic cells as compared to HCD group (Figure 4).

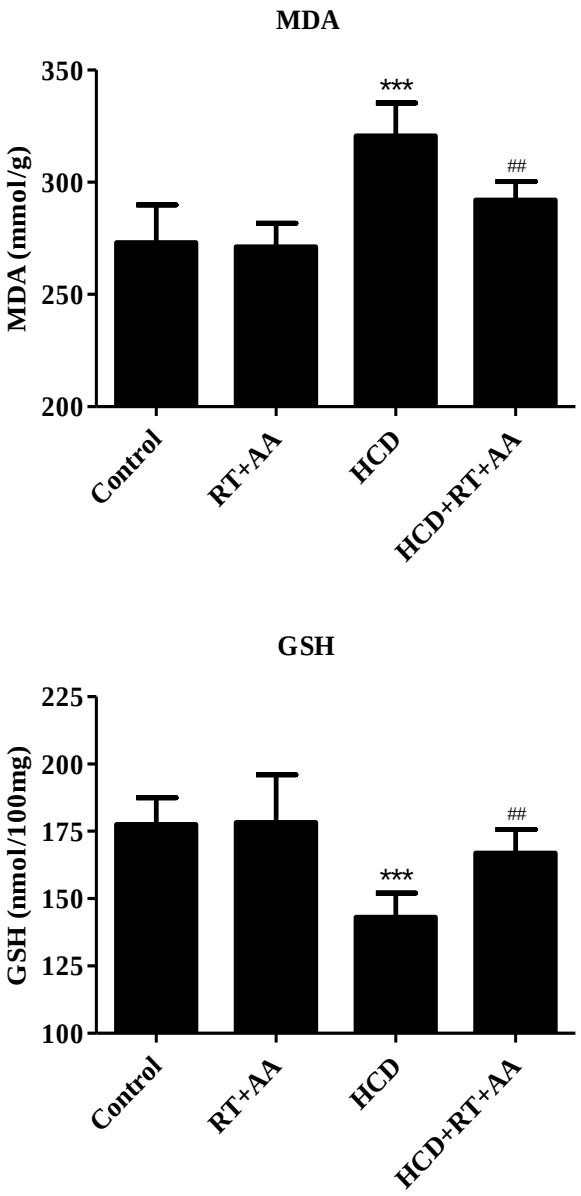

Figure 3. Effects of rutin (RT) and ascorbic acid (AA) combination on hepatic MDA and GSH level in high-cholesterol diet (HCD) fed rats following 6 weeks of supplementation. Data were expressed as Mean±S.D and analyzed using one-way ANOVA followed by Student Newman-Keuls method as post hoc test. Six rats were used in each group. ***P<0.001 HCD and RT+AA vs. Control group; $^{\#\#}$P<0.01 HCD+RT+AA vs. HCD group.

Histopathological changes in liver cross sections were presented in Figure 5. In control group, hepatocytes are looking benign and normal. Liver section from HCD group revealed scattered foci of steatohepatosis of liver, hepatocytes with swollen, epithelial cells associated with scattered foci of periportal to lobular inflammatory cell infiltrates. In conclusion, histopathological diagnosis showed moderate degree of hepatotoxicity in HCD fed rats. Liver sections from HCD+RT+AA group showed benign looking hepatocytes separated by congested central veins. Lobular lymphocytic infiltrate were noticed in few areas with no regenerating nodules or fibrosis. Finally, the histopathological diagnosis was mild degree of hepatotoxicity in RT and AA supplemented along with HCD fed rats.

DISCUSSION

In this study, combined protective effects of RT and AA on hypercholesrolemia induced hepatotoxicity were investigated in female Wistar albino rats. Hypercholesterolemia was induced by following HCD supplementation for six consecutive weeks and that has conformed through the biochemical and histopathological changes. Numerous studies reported that HCD has the ability to induce hepatotoxicity and contribute in causing fatty liver (Hirako et al., 2011; Park et al., 2002; Wang et al., 2011). Furthermore, hypercholesterolemic diet supplementation causes oxidative damage in liver by increasing enzymes and lipid profile (Choe et al., 2001; Katsube et al., 2006). The major hypothetical view of this study is to project the efficiency of antioxidant vitamins combined therapy against hypercholesrolemia-induced hepatotoxicity in female rats.

The present data of liver weights are in agreement with these reports as weights were significantly increased by HCD supplementation compared to controls. Histopathological screening also revealed the changes induced in liver of HCD supplemented rats by showing fat accumulation and inflammatory infiltrates. Park and his colleagues (2002) demonstrated that, HCD induces hypercholesterolemia which may contribute to cause oxidative stress and ROS generation. In another study, Balkan et al. (2002) reported that HCD-induced elevation in hepatic and plasma levels of lipids as well as oxidative enzymes. Our present results justified these studies when the plasma liver enzymes (ALT and AST) and lipid profile (TC, TG and LDL-C) levels significantly increased in HCD fed animals. The significant reduction in the HCD induced elevation in liver enzymes by RT and AA combination indicated that the combined antioxidant therapy is effective against oxidative process. These findings are in agreement with earlier experimental studies, where rutin and ascorbic acid individually were found to have protective effects against the oxidative stress (Abhilash et al., 2012; Banerjee et al., 2009; Janbaz et al., 2002). In the present study, lipid peroxidation markers including MDA and GSH revealed hepatic oxidative damage in HCD fed rats by significantly increasing MDA levels and decreasing the GSH levels while compared to control animals. Such alterations in lipid peroxidation products were also observed in brain, kidney and erythrocytes

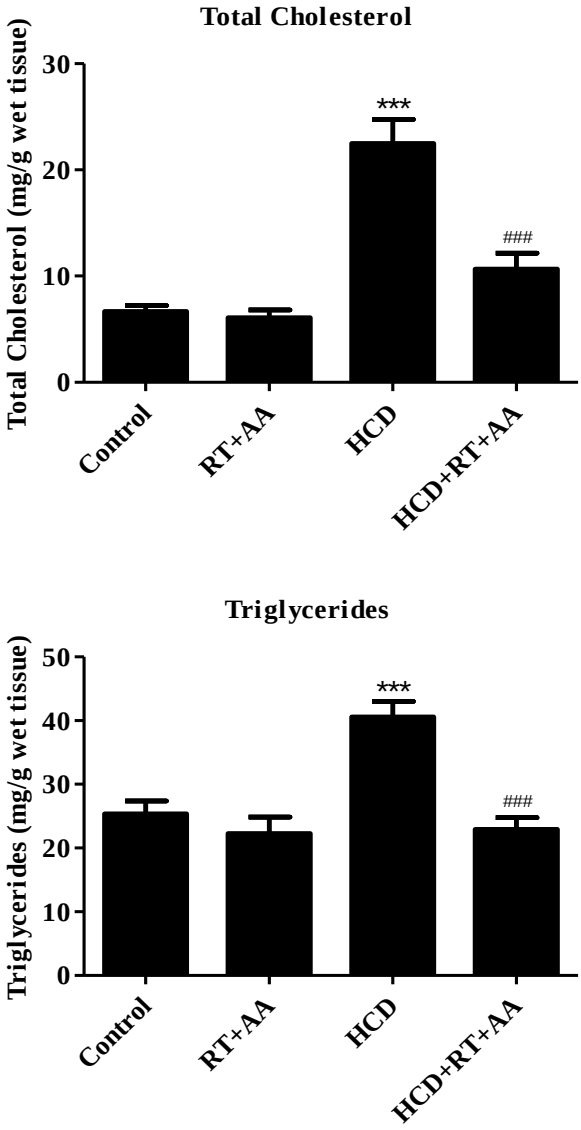

Figure 4. Effects of rutin (RT) and ascorbic acid (AA) combination on hepatic total cholesterol and triglycerides level in high-cholesterol diet (HCD) fed rats following 6 weeks of supplementation. Data were expressed as Mean±S.D and analyzed using one-way ANOVA followed by Student Newman-Keuls method as post hoc test. Six rats were used in each group. ***P<0.001 all HCD and RT+AA vs. Control group; $^{###}$P<0.001 HCD+RT+AA vs. HCD group.

of animals fed on HCD (Montilla et al., 2006). Moreover, HCD is reported to alter lipid composition in cell membranes and hence, the extracellular matrix to be more prone to free radical generation (Scheuer et al., 2000). Similar changes were observed in the present study, with significant increase at TC and TG levels of hepatic cells in HCD fed rats as compared to control animals. Furthermore, the present results showed the reduction in hepatic nucleic acids and total protein levels in HCD in HCD fed animals providing an evidenced for intracellular cytotoxicity.

Rutin is one of the flavonoids glycoside known as vitamin-P, which is widely accepted as physiologic antioxidants. Flavonoids are now believed to have a strong potential to protect against the many degenerative diseases linked to free radical-related tissue damage due to their capacity to protect critical macromolecules, such as chromosomal DNA, structural proteins and enzymes and membrane lipids (Dreosti, 2000; Rice-Evans et al., 1996). Rutin has been reported to exhibit multiple pharmacological activities including anti-inflammatory, vasoactive and membrane lipid peroxidation inhibitory properties (Ihme et al., 1996; Lindahl and Tagesson, 1997; Lopez-Revuelta et al., 2006; Park et al., 2002). Vitamin C (ascorbic acid) is recognized for its effective ability to prevent and control various diseases including allergic rhinitis (Thornhill and Kelly, 2000), diabetes (Anderson et al., 2006), heart disease (Ling et al., 2002) and cancer (Enwonwu and Meeks, 1995). In the present study, combined supplementation of RT and AA significantly prevented hypercholesterolemia induced liver injury. We believe that these effects are through the additive hepatoprotective effects of both vitamins. These findings are in accordance with other investigations, where both RT and AA were found to prevent hepatotoxicity and hepatic injury in different animal models (Abhilash et al., 2012; Banerjee et al., 2009; Janbaz et al., 2002; Rana et al., 2010; Shenbagam and Nalini, 2011). Both RT and AA are well recognized to protect against free radicals induced tissue damage through several biological processes in many extracellular and intracellular reactions (Mahmoud, 2011; Ozkaya et al., 2011). Measurements of hepatic DNA and RNA levels showed that RT and AA can attenuate HCD induced cytotoxic damage in liver tissues of the animals. ROS induced-cytotoxicity harmfully affects unsaturated fatty acids, which has been implicated in the pathogenesis of various diseases (Mahmoud, 2011). The cytoprotective effects of RT, as one of the phenolic flavonoids, and AA are also well established (Negre-Salvayre et al., 1995, Passoni and Coelho, 2008). Thus their combined cytoprotective effects are deemed to be through their ability to reduce free radicals production, which is expected to powerfully protect cellular membranes and components. Similar protective effects of this combination have been seen against hypercholesterolemia-induced oxidative stress as they efficiently reduced HCD-induced elevation in hepatic level of lipid peroxidation marker, MDA and significantly enhanced the reduced GSH levels. The elevated levels of total cholesterol and triglycerides in hepatic cells were also protected significantly by the combined supplementation of RT and AA.

The influence of the flavonoids on the endogenous regulation of cholesterol biosynthesis has been discussed in previous studies (Attaway and Buslig, 1998; Borradaile

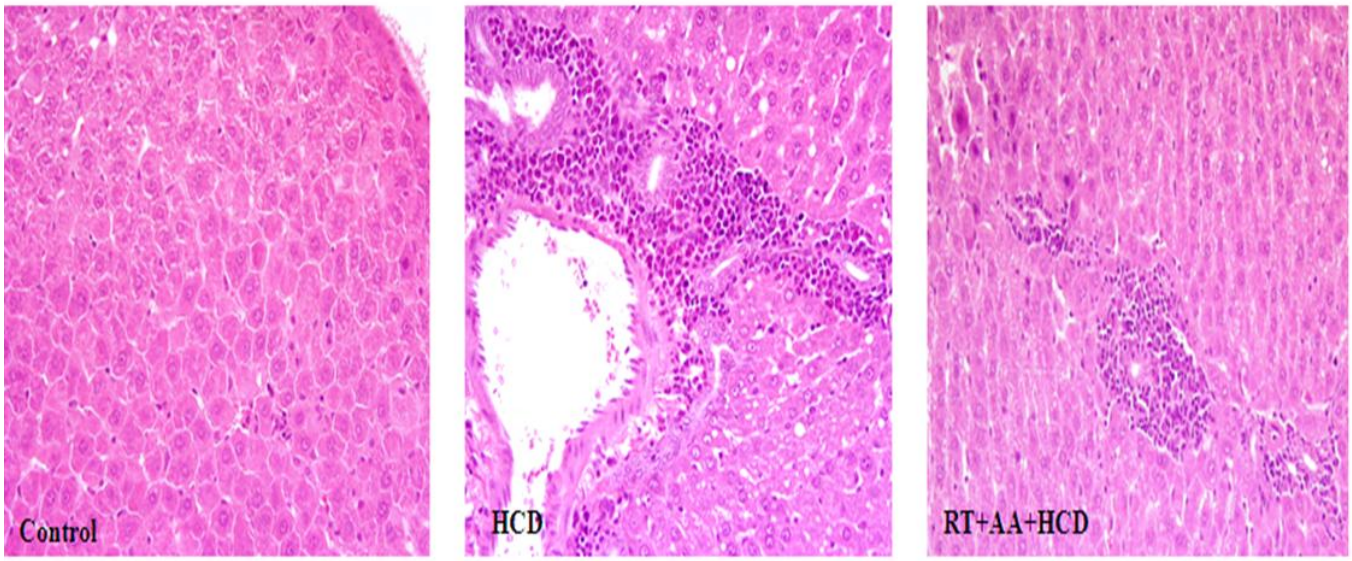

Figure 5. Histopathological sections from rats' liver showing: normal looking hepatocytes without steatosis or inflammatory cell infiltrates in control group; scattered foci of steatohepatosis of liver hepatocytes with swollen epithelial cells associated with scattered foci of periportal to lobular inflammatory cell infiltrates as well as few regenerative changes in HCD group; benign looking hepatocytes separated by congested central veins along with few inflammatory cell infiltrates in HCD+RT+AA group.

et al., 1999; Havsteen, 2002). Studies also suggested the potential improvement in the protective properties of dietary supplements and vitamins after their combination (Khan et al., 2012; Rozanowska et al., 2012). Qureshi et al. (2012), found that combining several dietary supplements can reduce cardiovascular risk factors in humans. Moreover, vitamin E was found to enhance protective effects of ascorbate on light-induced toxicity to retinal pigment epithelial cells (Rozanowska et al., 2012). According to the histopathological findings in the current study, supplementation of HCD and RT with AA significantly ameliorated hepatocelular ballooning and steatohepatosis.

In conclusion, our study demonstrated that oral co-administration of rutin and ascorbic acid attenuates hypercholesterolemia-induced hepatic toxicity in female rats by decreasing liver enzymatic and lipid peroxidative markers as well as increasing the antioxidative cascade.

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