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Trihoney reduces lipid peroxidation and enhances antioxidant enzyme activities in hypercholesterolaemic atherosclerotic rabbits

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Abstract

Oxidative stress and reactive oxygen species (ROS) constitute a major pathogenic mechanism for the development of atherosclerosis. In the present work, the antioxidant potential of Trihoney was investigated in hypercholesterolaemic rabbits. Thirty-six male New Zealand white (NZW) rabbits were grouped into: normal diet (C), normal diet with 0.6 g/kg/day of Trihoney (C+H), 1% cholesterol diet (HCD), 1% cholesterol diet with 0.3 g/kg/day of Trihoney (HCD+H_1), 1% cholesterol diet with 0.6 g/kg/day of Trihoney (HCD+H_2), and 1% cholesterol diet with 2 mg/kg/day of atorvastatin (HCD+At.). Animals were sacrificed following 12 weeks of treatment, and their serum was analysed for oxidised-low density lipoprotein (Ox-LDL). Serum and aortic tissue homogenate were assayed for superoxide dismutase (SOD), glutathione peroxidase (GPx), and malondialdehyde (MDA). Hypercholesterolemia caused a significant ($p < 0.05$) elevation in serum Ox-LDL and a significant ($p < 0.05$) reduction of antioxidant enzyme activities in serum of the HCD group. Trihoney induced a significant ($p < 0.05$) increase in antioxidant enzyme activities in serum as compared to the HCD group. The high cholesterol diet suppressed both antioxidant enzymes in aortic homogenate. Trihoney significantly ($p < 0.05$) enhanced both antioxidant enzymes in aortic homogenate. Hypercholesterolemia induced a significant ($p < 0.05$) elevation of serum lipid peroxidation in the HCD group. Trihoney caused a significant ($p < 0.05$) reduction of lipid peroxidation in aortic homogenate. These results demonstrated that Trihoney has the potential to ameliorate oxidative stress systemically, as well as locally in the atherosclerotic aorta.

Introduction

Atherosclerosis is a chronic inflammatory disease, and oxidative stress is one of its underlying pathogenic mechanisms (Morrow, 2003; Breitenbach and Eckl, 2015). It has been reported that oxidised lipids derived from low density lipoprotein (LDL) are major contributors to the pathogenic process of atherosclerotic plaque development and progression (Sakakura *et al.,* 2013). Oxidised-LDL induces foam cell (the pathological hallmark of atherosclerotic plaque) formation by reactive oxygen species (ROS)-induced inflammatory mechanisms (Liu *et al.,* 2014). An accumulation of ROS due to the disturbance of balance between their production and removal by antioxidant enzymes will result in a pathologic condition known as oxidative stress (Bhattacharya, 2015). Enzymatic antioxidants such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) play a very important role in the defence against the harmful effects of ROS (Bhattacharya, 2015). It has been established that honey functions as an antioxidant by inhibiting oxidative stress and through its radical scavenging ability (Vallianou *et al.,* 2014). Honey also has cardioprotective characteristics based on its antioxidative properties (Rakha *et al.,* 2008), its protective function against lipid peroxidation (Abd Jalil *et al.,* 2017), and its ability to lower malondialdehyde (MDA) concentration (Erejuwa *et al.,* 2010; Sahhugi *et al.,* 2014). In the present work, Trihoney, which is a combination of Trigona, Mellifera, and Dorsata honey, was investigated for its antioxidant potential for protection against atherosclerosis through assessment of serum Ox-LDL, antioxidant enzyme activities, and through a lipid

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peroxidation assay systemically, as well as locally in the atherosclerotic plaques.

Materials and methods

Chemicals, reagents, and assay kits

Chemicals, reagents, and assay kits used in the present work are as follows: pure cholesterol powder (Nacalai-Tesque, Japan); cholesterol-free extra virgin coconut oil (Philippines); OxiSelect™ superoxide dismutase activity assay kit (Cell Biolabs, USA); glutathione peroxidase assay kit (Abnova, Taiwan); OxiSelect MDA adduct competitive ELISA kit (Cell Biolabs, USA); MDA ELISA kit Elabscience (China); and rabbit oxidised low density-lipoprotein, Ox-LDL ELISA Kit (Cusabio, China). Other reagents and protein assay kit were supplied by Nacalai-Tesque (Japan) and Sigma Aldrich (USA).

Trihoney and atorvastatin

Trihoney was produced by the Department of Nutrition Sciences, Kulliyyah of Allied Health Sciences, International Islamic University Malaysia (IIUM), from three types of natural honey (Trigona, Mellifera, and Dorsata) at a ratio of 45:15:10. Trihoney was administered to the respective animal groups by oral route. Two doses were used (0.3 and 0.6 g/kg/day). Doses were calculated based on human and rabbit K_m factors, according to Reagan-Shaw *et al.* (2008). Atorvastatin, 40 mg film-coated tablets (Prague, Czech) were crushed into fine powder, reconstituted in 1 mL of distilled water, and given by oral gavage using a clean syringe (Jorge *et al.,* 2005; Du *et al.,* 2013; Song *et al.,* 2014) at a dose of 2 mg/kg body weight.

Preparation of 1% cholesterol diet

Preparation of 1% cholesterol diet was performed according to Jorge *et al.* (2005) with some modification as follows: 40 g of pure cholesterol powder was emulsified in 80 mL (= 80 g) of cholesterol-free extra virgin coconut oil. The cholesterol emulsion was evenly poured over 3,880 g of standard rabbit pellets (Perternakan Hong Lee Sdn. Bhd., Malaysia). The prepared food (1% cholesterol and 2% coconut oil rabbit pellet) was then packed in zipped bags and kept at 20 - 22°C prior to use.

Animal

Thirty-six male NZW rabbits were purchased from a certified experimental animal supplier (A Sapphire Enterprise, Seri Kembangan, Selangor, Malaysia). The animals' weight ranged from 2 to 2.5 kg, and the animals were 20 weeks of age. The animals were randomly housed in stainless-steel cages designed for rabbits, with a single rabbit per cage with free access to water and standard rabbits' pellet. The rabbits were maintained under standard animal care housing condition of 12 h dark/light cycle, at 15 - 21°C, and 45 - 65% humidity. The protocol of this experiment was approved by the Institutional Animal Care and Use Committee of International Islamic University Malaysia (IACUC-IIUM) with ID approval (IIUM/I-ACUC- Approval /2017 (19)).

Experimental study

The thirty-six male NZW rabbits were grouped into the following 6 groups: normal diet (C), normal diet with Trihoney dose of 0.6 g/kg/day (C+H), 1% cholesterol diet (HCD), 1% cholesterol diet with 0.3 g/kg/day of Trihoney (HCD+H_1), 1% cholesterol diet with 0.6 g/kg/day of Trihoney (HCD+H_2), and 1% cholesterol diet with 2 mg/kg/day of atorvastatin (HCD+At.). At the end of 12 weeks, animals were sacrificed at the animal surgical laboratory of Central Research and Animal Facility, International Islamic University Malaysia (CREAM, IIUM). General anaesthetic using a combination of ketamine and xylasine was given through intramuscular route (Torres *et al.,* 2014) at doses of 50 and 10 mg/kg, respectively (Bolayirli *et al.,* 2007). Blood was drawn from the central ear artery (Bolayirli *et al.,* 2007). Laparotomy and sternotomy were performed for full exposure of aorta (Fan *et al.,* 2001). The aortic arch was dissected 1 mm above the aortic root, immediately immersed in ice-cold PBS (Khalil *et al.,* 2015), then immediately stored at -80°C (Haier Ult Freezer, China) until homogenised (Otunola *et al.,* 2010).

Blood samples and serum preparation

Blood was collected from the animals into plain tubes, and allowed to clot at room temperature for 40 min (Brown, 2016), then centrifuged (Centrifuge Universal 320R Hettich, Germany) at 4°C and 3,500 rpm for 15 min (Chang *et al.,* 2012). The supernatant sera was immediately stored at -80°C (Haier Ult Freezer, China) prior to (Mikail *et al.,* 2016) Ox-LDL, antioxidant enzymes, and MDA analyses.

Aorta homogenate preparation

The aortic tissue was retrieved from -80°C freezer, defrosted, and then homogenised using bullet blender homogeniser at a ratio of 10% (w/v) (Ibrahim *et al.,* 2017). Homogenised tissue was centrifuged (ThermoFisher Scientific, Germany) for 15 min at 10,000 rpm at 4°C (Badalzadeh *et al.,* 2015). The supernatant was immediately stored at -80°C for later use (Soto *et al.,* 2014). The protein concentration in tissue homogenate was measured by Coomassie

(Bradford) protein assay method (Badalzadeh *et al.,* 2015), using Coomassie Brilliant Blue (CBB) solution (ready to use) (Nacalai Tesque, Japan).

Antioxidant study

Serum oxidized low-density lipoprotein (Ox-LDL)

The serum Ox-LDL was quantitatively determined using CUSABIO (Hubei Province-China) ELISA kit for rabbits. This assay employed a quantitative sandwich enzyme immunoassay technique, and was conducted following the manufacturer's protocol.

Lipid peroxidation assay in serum and aortic tissue homogenate

The concentration of MDA in serum and aortic tissue homogenate was quantitatively determined using a competitive ELISA kit.

Antioxidant enzyme assay in serum and aortic tissue homogenate

Superoxide dismutase enzyme activity was assayed using OxiSelect™ Superoxide Dismutase Activity Assay Kit. The enzyme activity as a function of optical density (OD) was expressed as units/μL, and expressed as percentage (%). The activity of GPx was assayed using GPx assay kit based on the decrease of NADPH (measured at 340 nm), which is proportionate to GPx activity. Activity of GPx in tissue homogenate was expressed in mU/mg tissue protein.

Statistical analysis

Statistical Package for Social Sciences (SPSS version 21, Chicago, Illinois, USA) software was used for data processing. Data were expressed as means ± standard deviations, and analysed by one-way analysis of variance (ANOVA). One-way ANOVA followed by *post hoc* test to determine significant differences between the means of two or more independent groups. $p < 0.05$ was considered statistically significant.

Results

Trihoney reduced serum Ox-LDL level

The high cholesterol diet group had significantly higher serum Ox-LDL level as compared to the control groups, C and C+H ($p < 0.05$). The Trihoney treated groups showed significantly elevated serum Ox-LDL levels as compared to the control groups. Despite the increase, the Trihoney treated groups had a slightly lower serum Ox-LDL mean as compared to the HCD group. Atorvastatin treated animals had comparable serum Ox-LDL levels to the control groups, but demonstrated a significant ($p < 0.05$) reduction of serum Ox-LDL as compared to the HCD group.

Table 1. Effects of Trihoney on serum oxidised low-density lipoprotein (Ox-LDL).

Group	Serum Ox-LDL (μmol/mL) week 12
C	0.36 ± 0.04^{a}
C+H	0.29 ± 0.02^{a}
HCD	0.52 ± 0.05^{b}
HCD+H_1	0.49 ± 0.04^{b}
HCD+H_2	0.49 ± 0.14^{b}
HCD+At.	0.37 ± 0.07^{a}

Values are means ± standard deviations (SD). The results of all experiment groups were analysed using one-way analysis of variance (ANOVA), followed by LSD *post hoc* test. Mean difference is considered significant at ($p < 0.05$). Means not sharing a common superscript letter within the same column differ significantly at $p < 0.05$.

Table 2. Correlation (r) between serum oxidised low-density lipoprotein (Ox-LDL) level and aortic lesion area percentage.

	Aortic lesion area percentage (%)
Serum Ox-LDL (μmol/mL)	$r = 0.693$**

Values are the Pearson correlation coefficient (r) between serum Ox-LDL level of all the experimental groups and aortic lesion percentage area at week 12. ** = $p < 0.01$ Sig. (2-tailed).

Atorvastatin treated group exhibited significantly ($p < 0.05$) lower serum Ox-LDL level than the Trihoney treated groups (Table 1). There was a positive ($p < 0.01$) correlation between the serum Ox-LDL levels with the percentage of aortic lesions (Table 2).

Trihoney ameliorated lipid peroxidation in the serum and in the atherosclerotic aorta

The group fed with 1% cholesterol diet alone for 12 weeks showed a significant ($p < 0.05$) increase in serum MDA level as compared to both control groups, C and C+H. All atherogenic groups which received treatment, whether Trihoney or atorvastatin, exhibited elevated serum MDA level in comparison to both control groups (Table 3). Although Trihoney treated groups showed a lower serum MDA level as compared to the HCD group, the levels were still

Table 3. Effect of Trihoney and 1% cholesterol diet on malondialdehyde (MDA) concentration in serum and aortic homogenate.

Group	Serum MDA (ng/mL) week 12	Aortic homogenate MDA week 12 (µg/mg protein)
C	0.00 ± 0.00^{a}	0.03 ± 0.03^{a}
(C+H)	0.00 ± 0.00^{a}	0.08 ± 0.07^{ad}
(HCD)	38.68 ± 15.32^{b}	1.48 ± 0.25^{b}
(HCD+H_1)	36.97 ± 28.81^{b}	0.32 ± 0.09^{c}
(HCD+H_2)	37.43 ± 25.72^{b}	0.31 ± 0.16^{c}
(HCD+At.)	25.34 ± 23.12^{b}	0.25 ± 0.08^{cd}

Values are means ± standard deviations (SD). The results of all experiment groups were analysed using one-way analysis of variance (ANOVA), followed by LSD *post hoc* test. Mean difference is considered significant at ($p < 0.05$). Means not sharing a common superscript letter within the same column differ significantly at $p < 0.05$.

Table 4. Effects of Trihoney on activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the serum.

Group	Serum SOD activity (%) week 12	Serum GPx activity (mU/mL) week 12
C	85.90 ± 1.67^{a}	108.03 ± 62.82^{ab}
C+H	86.09 ± 0.91^{a}	249.12 ± 93.67^{ac}
HCD	65.11 ± 4.95^{b}	88.03 ± 64.50^{b}
HCD+H_1	71.32 ± 2.47^{c}	218.94 ± 28.32^{cd}
HCD+H_2	72.00 ± 4.77^{c}	177.09 ± 41.62^{ac}
HCD+At.	74.38 ± 7.04^{c}	146.48 ± 32.34^{bc}

Values are means ± standard deviations (SD). The results of all experiment groups were analysed using one-way analysis of variance (ANOVA), followed by LSD *post hoc* test. Mean difference is considered significant at ($p < 0.05$). Means not sharing a common superscript letter within the same column differ significantly at $p < 0.05$.

Table 5. Effects of Trihoney on activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in aortic homogenate.

Group	SOD activity (%) week 12	GPx activity (mU/mg protein) week 12
C	25.12 ± 5.92^{a}	12.25 ± 7.05^{ab}
C+H	24.82 ± 6.14^{a}	15.72 ± 1.85^{ac}
HCD	17.12 ± 5.89^{a}	3.14 ± 3.92^{b}
HCD+H_1	19.70 ± 10.79^{a}	22.60 ± 3.31^{c}
HCD+H_2	34.68 ± 1.41^{b}	19.07 ± 8.84^{ac}
HCD+At.	24.97 ± 6.78^{a}	49.30 ± 13.45^{d}

Values are means ± standard deviations (SD). The results of all experiment groups were analysed using one-way analysis of variance (ANOVA), followed by LSD *post hoc* test. Mean difference is considered significant at ($p < 0.05$). Means not sharing a common superscript letter within the same column differ significantly at $p < 0.05$.

significantly ($p < 0.05$) higher than the control groups. HCD+At. group had a lower MDA level as compared to HCD group, but no significant difference was observed. On the other hand, lipid peroxidation in aorta homogenate was significantly ($p < 0.05$) higher in HCD group as compared to the control groups. Trihoney treated groups, as well as the atorvastatin treated group showed significantly ($p < 0.05$) lower lipid peroxidation as compared to the HCD group. All treated atherogenic groups showed a significant ($p < 0.05$) increase in lipid peroxidation as compared to the control groups.

Trihoney enhanced antioxidant enzyme activities in the serum and in the atherosclerotic aorta

The HCD group had very significant reduction ($p < 0.05$) in serum SOD and GPx activities as compared to the control groups, C and C+H (Table 4). All treated atherogenic groups showed a significant ($p < 0.05$) decrease in serum SOD activity as compared to the control groups, but exhibited higher serum SOD activity in comparison to the high cholesterol group, HCD. Trihoney treated groups were comparable to the atorvastatin treated group. Trihoney treated groups exhibited a significant ($p < 0.05$) increase in serum GPx activity as compared to the HCD group. Atorvastatin treated group showed a significant ($p < 0.05$) reduction in serum GPx activity in comparison to the C+H group. However, the serum GPx activity in this group was comparable to Trihoney treated groups. The results of SOD and GPx activities in homogenate of the aorta are displayed in Table 5. In the atherosclerotic aorta of HCD group, activities of both SOD and GPx were markedly reduced. The HCD+H_2 group had the highest SOD activity in aortic homogenate in comparison to all experimental groups, including the control groups. The atherogenic groups, HCD+H_1, and HCD+At. showed SOD activity comparable to each other and to the control groups, but both groups had significantly ($p < 0.05$) reduced SOD activity in comparison to the atherogenic group which received a higher dose of Trihoney HCD+H_2. Activity of GPx in aorta homogenate of the HCD group was markedly ($p < 0.05$) reduced in comparison to the control groups. Trihoney treated groups showed a significant ($p < 0.05$) increase in GPx activity in aorta homogenate as compared to the HCD group. The atorvastatin treated group had the highest GPx activity in aorta homogenate in comparison to all experimental groups.

Discussion

In the present work, we demonstrated that feeding NZW rabbits with a 1% cholesterol diet caused a significant elevation of serum Ox-LDL. Oxidised-LDL which is generated due to oxidation of LDL in association with oxidative stress, has been found to play a crucial role in the pathogenesis of atherosclerosis (Sangle and Shen, 2010). Elevated serum Ox-LDL is considered to be the main contributor to the induction of foam cells in the vascular intima following massive uptake of Ox-LDL by macrophages (Lara-Guzmán *et al.,* 2018). Our investigation showed an elevation of serum Ox-LDL in all experimental groups which received the 1% cholesterol diet. The treatment groups that received Trihoney exhibited a decrease in serum Ox-LDL as compared to the high cholesterol diet group. While the decrease itself was not significant, the implication of this diet may be clinically important. Coincidently, the group that received atorvastatin also showed a significant reduction in serum Ox-LDL as compared to the high cholesterol diet. Taken together, these findings may indicate that Trihoney has the potential to reduce serum Ox-LDL level, although not to the potency of atorvastatin. Reduction of serum Ox-LDL protects againstatherogenesis (El-Hady and Shaker, 2013). Moreover, trapping Ox-LDL within the vascular walls not only triggers more inflammation, but promotes atherosclerotic plaque formation, and furthers vessel narrowing (Nwachuku *et al.,* 2017).

In the present work, we found a strong positive correlation between serum Ox-LDL level and the percentage of aortic lesions. Consequently,-this suggests that Trihoney has the potential to reduce the percentage of aortic lesions by reducing serum Ox-LDL. In agreement with our results, Skopelitis *et al.* (2014) reported a positive correlation between serum Ox-LDL and carotid artery stenosis. In line with our findings, in an *in vitro* study, Hegazi and El-Hady (2009) highlighted the ability of four types of natural honey to reduce lipid peroxidation and to inhibit oxidation of LDL. In the present work, we used atorvastatin as a positive control to compare Trihoney with a known hypercholesterolaemic drug. Atorvastatin supplementation with 1% cholesterol diet resulted in a significant reduction of serum Ox-LDL. This is in line with a clinical trial conducted by Tavridou *et al.* (2010) who showed that statins significantly reduced circulating Ox-LDL in subjects with combined hyperlipidaemia, and in subjects with isolated hypercholesterolemia. In the present work, lipid peroxidation was assayed in the serum, and in the homogenate of aorta by quantifying serum MDA, a lipid peroxide which forms in oxidative stress situations (Mikail *et al.,* 2016). Feeding NZW rabbits with

a 1% cholesterol diet for 12 weeks resulted in a systemic, and aortic wall oxidative stresses, which is evident by our reported MDA results.

In the present work, the high cholesterol diet group had a significant increase of MDA concentration in serum, as well as in aortic wall homogenate, indicating a causal relationship between hypercholesterolemia and induction of oxidative stress. In this investigation, supplementation of Trihoney with the 1% cholesterol diet led to a reduction in oxidative stress, systemically, as well as locally in the aorta. The effectiveness of Trihoney against oxidative stress in reducing MDA concentration was comparable to atorvastatin. The impact of Trihoney on lipid peroxidation and oxidative stress was very pronounced and more significant in aortic homogenate than in serum. This led us to postulate that Trihoney has significant protective effects against lipid peroxidation and ongoing oxidative stress in atherosclerotic aortic wall, in a state of concomitant hypercholesterolemia. It is crucial to point out that the mechanisms by which honey affects oxidative stress has not been fully addressed. Xiao *et al.* (2016) who used a NASH rat model, concluded that the effect of honey against oxidative stress in NASH may be via inhibition of overexpression of thioredoxin-interacting protein (TXNIP) and suppression of Nod-like receptor protein 3 (NLRP3). Suppression of the TXNIP-NLRP3 inflammasome pathway was found to reduce cellular damage caused by oxidative stress. Mohamed *et al.* (2011) who showed protective effect of honey against cigarette smoking-induced oxidative stress in a rat model, suggested the probable mechanism was through nutritional constituents of honey, in addition to a synergistic effect between honey and antioxidant enzymes, SOD and GPx, in the biological system. The mechanisms by which statins modulate lipid peroxidation are also still controversial. van Tits *et al.* (2006) suggested that the antioxidant function of atorvastatin is related to its hydroxyl metabolites, which protect against LDL-c peroxidation.

Antioxidant enzymes constitute a very important defence mechanism against the deleterious effects of ROS, and are capable of deactivating them before they attack cellular components (Krishnamurthy and Wadhwani, 2012). In our investigation, feeding NZW rabbits with 1% cholesterol diet for 12 weeks resulted in a very significant drop in SOD and GPx activities, systemically, and locally, in the atherosclerotic aorta. This observation is supported by Mikail *et al.* (2016). In the present work, we reported an enhanced activity of SOD and GPx in the Trihoney treated groups. Although serum SOD was lower than that of the control groups, it was higher than the HCD group, which may indicate the potential of Trihoney to induce SOD activity in hypercholesterolaemic conditions. The reported serum SOD activity of Trihoney treated groups was comparable to atorvastatin treated group. Coincidentally, there was increased activity of serum GPx in Trihoney treated groups, with a significant difference from the HCD group. Serum SOD and GPx results were supported by findings in the homogenate of the atherosclerotic aorta. The SOD activity in homogenate of aorta of Trihoney treated groups was higher as compared to other treated and untreated groups. On the other hand, GPx activity in aorta homogenate was enhanced in Trihoney treated groups, as well as in atorvastatin treated group. Atorvastatin treated group showed the highest GPx activity among treated and untreated groups.

Interestingly, we noticed that within the Trihoney treated groups, whenever aorta homogenate GPx activity decreased, the SOD activity increased. This may be due to the negative impact of H_2O_2 on GPx, since GPx activity has been reported to be suppressed under chronic exposure to H_2O_2 (Ryan *et al.,* 2008). In agreement with our study, Malaysian Tualang honey has been shown to induce elevation in SOD and CAT activities in diabetic rats (Erejuwa *et al.,* 2010). Mohamed *et al.* (2011) also showed significant improvement of SOD and GPx activities with honey supplementation following cigarette smoking-induced oxidative stress in rat model.

In the present work, treatment with atorvastatin resulted in increased SOD and GPx activities in serum, as well as in aorta homogenate, as compared to HCD group. Moreover, HCD+At. group exhibited significant GPx activity in aorta homogenate. Mikail *et al.* (2016) reported similar findings to ours. This is the first study to investigate the effect of Trihoney on antioxidant enzymes in hypercholesterolaemic rabbit model. The antioxidant effects of Trihoney were comparable to that of atorvastatin. From results, we conclude that Trihoney was able to protect against Ox-LDL elevation and lipid peroxidation, and exhibited marked enhancement of SOD and GPx activities, owing to its natural antioxidant constituents, in addition to other possible mechanisms that necessitate future studies.

Conclusion

The present work demonstrated that Trihoney had potential protective effects against Ox-LDL and lipid peroxidation, in addition to a marked beneficial ability to ameliorate oxidative

stress through enhancement of antioxidant enzymes activities. Trihoney, through its health beneficial functions, may be suggested as an adjuvant remedy to statins for management of atherosclerosis.

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