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Protective Effects of Chitosan, Ascorbic Acid and *Gymnema Sylvestre* Against Hypercholesterolemia in Male Rats

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Abstract: This study was designed to test chitosan and the mixture of chitosan, ascorbic acid and *Gymnema sylvestre* (10:2:1) as lipid-lowering and the antioxidative activities on hypercholesterolemic rats. The plasma lipid levels, transaminases (ALT and AST), lactate dehydrogenase (LDH) activities, glucose, malondialdehyde (MDA) and whole blood reduced glutathione (GSH), the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) in erythrocytes and plasma glutathione reductase (GR), glutathione-S-transferase (GST) and catalase (CAT) were examined. The hypercholesterolemia-induced diet was manifested in the elevation of total lipids (TL), total cholesterol (TC), triglycerides (TG), LDL-C levels, ALT, AST, LDH activities and MDA content and depletion of GSH and enzymic antioxidants. Supplementation of hypercholesterolemia-induced diet with three levels of chitosan and three levels of the mixture significantly lowered the plasma levels of TL, TC, TG and LDL-C. ALT, AST and LDH activities slightly decreased in treated groups compared with hypercholesterolemic group (HC). Furthermore, the content of GSH significantly increased while MDA significantly decreased in treated groups compared with HC group. In addition, chitosan and mixture groups improved enzymic antioxidants compared with HC group. In general, the results indicated that, the mixture supplements are better than chitosan. These results suggested that the hypocholesterolemic effect of chitosan and the mixture supplements might be due to their abilities to lower plasma cholesterol level as well as to slow down the lipid peroxidation process and to enhance the antioxidant enzyme activity.

Key words: Chitosan, ascorbic acid, *Gymnema sylvestre*, oxidative stress, hypercholesterolemia, rats

INTRODUCTION

Hypercholesterolemia (HC) is a major risk factor for atherosclerosis and coronary heart disease. It is characterized by coronary endothelial dysfunction, the hallmark of which is an altered vasodilation to endothelial dependent vasodilators (Luscher *et al.*, 1992). It may also promote ischemic tissue damage by enhancing the vulnerability of the microcirculation to the deleterious effects of ischemia and other inflammatory stimuli (Stokes *et al.*, 2002). This leads to increase in the incidence of myocardial ischemia and cardiac events (Sudhahar *et al.*, 2007). In recent years, many reports have focused on how to decrease plasma lipid concentrations and the absorption of fat in the intestinal tract to reduce diet-related chronic disease. Dietary fiber such as pectin and psyllium show some potent hypolipidemic effect (Vergara-Jimenez *et al.*, 1998 and Zhang *et al.*, 2008).

The use of dietary fiber has been attractive because of the reduction in the energy density of the diet (Beereboom, 1979), an improvement in bowel habits (Cummings *et al.*, 1976) and the prevention of colon cancer (Burkitt, 1971). Chitosan, a polyglucosamine derived from chitin, is a cellulose-like polymer located mainly in the exoskeletons of arthropods, such as crabs, shrimps, lobsters and insects (Razdan and Pettersson, 1994). It can be defined both chemically and physiologically as a dietary fibre since it is a polysaccharide, which cannot be digested by digestive enzymes of humans (Razdan and Pettersson, 1996). Moreover, it is the only abundant polysaccharide derived from animals, and its cationic characteristics are different from other dietary fibers (Muzzarelli, 1996). It is natural and nontoxic, and growing evidence indicate that it exhibits a marked hypolipidemic activity that would reduce the risk of cardiovascular diseases (Zhou *et al.*, 2006). It has exhibited a potent hypocholesterolemic activity in rats (Simunek and Bartonova, 2005 and Liu *et al.*, 2008)

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and humans (Guerciolini *et al.*, 2001; Maezaki *et al.*, 1993). Previous study have reported that chitosan reduced the concentration of plasma cholesterol in animals (Chiang *et al.*, 2000; Yao and Chiang, 2002, 2006a,b) and type II diabetes patients in combination with hypercholesterolemia (Tai *et al.*, 2000 and Yao *et al.*, 2008). Increased fecal cholesterol accompanied with or without bile acid excretion by interfering intestinal micelle formation was proposed to be the mechanisms responsible for the hypocholesterolemic properties (Gallaher *et al.*, 2000; Yao and Chiang, 2006a,b). Maezaki *et al.* (1993) was the first to report the hypocholesterolemic effect of chitosan in humans and found that chitosan effectively decreased plasma lipid level and had no side effect.

Prospective studies have demonstrated reduced risk of coronary artery disease in subjects with a greater intake of vitamin E (Rimm *et al.*, 1993) or ascorbic acid (Enstrom *et al.*, 1992). Because these antioxidant vitamins inhibit oxidation of low density lipoprotein (LDL), a critical event in the pathogenesis of atherosclerosis. It has recently become apparent that antioxidants may favorably influence coronary artery disease through alternative mechanisms, including improvement of endothelial function, inhibition of platelet aggregability and a decrease in the risk of plaque rupture (Vita *et al.*, 1998).

Recently, it has also been reported that the addition of ascorbic acid to chitosan causes a larger increase in fecal fat excretion without affecting protein digestibility (Kanauchi *et al.*, 1994 and Tsujikawa *et al.*, 2003).

Gymnema sylvestre leaves contain gymnemic acids, which are known to suppress transport of glucose from the intestine into the blood stream and a small protein, gurmar, that can interact with receptors on the tongue to decrease the sensation of sweetness in many foods. This dual action has been shown to reduce blood sugar and cholesterol levels in diabetic animals and humans and may provide some benefits in terms of regulating appetite control and food cravings (Daisy *et al.*, 2009).

There are many reports in the literature concerning the biological activities of chitosan, ascorbic acid and *Gymnema sylvestre* individually, and few studies on the biological activities of the mixture of chitosan and ascorbic acid. However, no studies have focused on the effect of the mixture of chitosan, ascorbic acid and *Gymnema sylvestre* on oxidative stress and hypercholesterolemia.

The present study was designed to compare the effect of chitosan and the mixture of chitosan, ascorbic acid and *Gymnema sylvestre* in protecting experimental animals fed hypercholesterolemia-induced diet of oxidative stress and hypercholesterolemia.

MATERIALS AND METHODS

Materials:

High molecular weight (MW) chitosan and the mixture of high MW chitosan, ascorbic acid and *Gymnema sylvestre* (10:2:1) donated from Aldebeiky Pharma Co., Egypt.

Animals and Diets:

Forty eight male Sprague-Dawley rats weighing 100 ±10 g were purchased from animal house of Helwan Station for Experimental Animals, Helwan, Egypt. They were raised in the animal house of Biochemistry Department, Faculty of Agriculture, Cairo University, Giza, Egypt. The animals were housed in polyethylene cages in groups of six rats per cage in a controlled environment (25±2 ºC, 50-60% relative humidity and 12-hour light-dark cycle). The animals were fed *ad libitum* with a basal diet and water for two weeks, and were then randomly assigned to 8 groups (6 rats each): normal control group (NC), receiving basal diet consisting of corn starch 65%, casein 10%, corn oil 10%, salt mixture 4%, vitamins mixture 1% and cellulose 10% (AOAC,2000), high-cholesterol control group (HC) receiving hypercholesterolemia-induced diet which prepared as basal diet preparation, except that the 10% corn oil portion was replaced with 10% sheep perineal fat and it was supplemented with 1% cholesterol and 0.25% cholic acid (Fukushima *et al.*, 1997). Three chitosan groups (chitosan I, chitosan II, chitosan III) receiving hypercholesterolemia-induced diets supplemented with 1.2, 2.4 and 3.6 g chitosan/kg diet, respectively. Three mixtures (chitosan, ascorbic acid and *Gymnema sylvestre*) groups (Mix I, Mix II and Mix III) receiving hypercholesterolemia-induced diets supplemented with 1.56, 3.12 and 4.68 g mixture/kg diet, respectively.

Experimental Design:

During the experimental period (4 weeks), water and diets were available *ad libitum*. At the end of the experiment, all the animals were scarified by cervical decapitation. Blood samples were collected in three heparinized tubes. The first one (0.1 ml blood) was used for the determination of reduced glutathione (GSH),

the 2nd heparinized tube (0.5 ml blood) was used to extract the erythrocytes lysate according to the procedure of Quist (1980) to study antioxidants enzymes. The 3rd heparinized tube was centrifuged at 2500 rpm at 37 ºC for 15 min to separate the plasma.

Biochemical Analysis:

Lipid Analysis:

Plasma total lipids (TL), triglycerides (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) were determined according to Knight *et al.* (1972), Fossati and Prencipe (1982), Allain *et al.* (1974), Levy (1981) and Burstein (1970), respectively. Atherogenic Index (AI) was calculated according to Lee and Niemann (1996) using following equation:

$$\text{Atherogenic Index (AI)} = \frac{\text{Total cholesterol} - \text{HDL-C}}{\text{HDL-C}}$$

Determination of LDH, AST and ALT activities:

Lactate dehydrogenase (LDH) activity in plasma was determined according to the method of Young (2001). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured colourimetricaly in plasma according to the method described by Reitman and Frankel (1957).

Determination of Glucose:

Plasma glucose level was determined according to Trinder (1969).

Determination of Lipid Peroxidation:

Plasma lipid peroxidation was estimated by measuring the thiobarbituric acid reactive substances (TBARS) and was expressed in terms of malondialdehyde (MDA) content, according to the method of Uchiyama and Mihara (1978). The MDA values were estimated using 1,1,3,3-tetraethoxy propane as the standard.

Determination of Non-enzymic Antioxidant (GSH):

Reduced glutathione (GSH) in whole blood was determined by the method of Beutler *et al.* (1963). This method was based on the reaction of GSH with 5,5`dithiobis(2-nitrobenzoic acid) to give a yellow compound that absorbs at 412 nm.

Determination of Enzymic Antioxidant Activities:

Superoxide dismutase (SOD) and glutathione peroxidase (GPx) in erythrocytes were assayed by the methods of Nishikimi *et al.* (1972) and Paglia and Valentine (1970), respectively.

Plasma glutathione reductase (GR) activity was assayed by the method of Goldberg and Spooner (1983). The enzyme activity was quantified by measuring the disappearance of NADPH at 340 nm. Plasma glutathione-S-transferase (GST) activity was determined using the procedure of Habig *et al.* (1974) by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione. The conjugation is accompanied by an increase in absorbance at 340 nm. The rate of increase is directly proportional to the GST activity. Plasma catalase (CAT) activity was determined according to the method of Aebi (1984).

Statistical Analysis:

Statistical analysis (standard deviation ‘‘SD’’ and standard error ‘‘SE’’) was carried out according to Fisher (1970). LSD (Least significant difference) test was used to compare the significant differences between means of treatment (Waller and Duncan, 1969). The statistical package for social science S.P.S.S. (1999) program version was used for all analysis.

RESULTS AND DISCUSSION

In the present study, hypercholesterolemia-induced diet feeding for four weeks was chosen as experimental model of early phase atherogenesis. Cholic acid addition enhances the hypercholesterolemic effect of cholesterol feeding (Shinnick *et al.*, 1990). The role of chitosan and the mixture of chitosan, vitamin C and *Gymnema sylvestre* in countering the lipidemic-oxidative aberrations accompanying diet-induced hypercholesterolemia have been investigated here.

Rats fed on hypercholesterolemia-induced diet (HC) developed hypercholesterolemia mark by significant ($P < 0.05$) increase in plasma total lipids (TL), triglycerides (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) and atherogenic Index (AI) compared with normal control rats (NC). Supplementation with chitosan and the mixture showed significant ($P < 0.05$) falls in total lipids, triglycerides, total cholesterol, LDL-C and AI and insignificant alteration in HDL-C compared with hypercholesterolemic group (HC) as shown in Tables (1 and 2). It must be noticed that, the mixture supplements is more effective than chitosan for lowering lipids profile. The best reduction in lipids profile was recorded by Mix III supplement, the levels of total lipids, total cholesterol, triglycerides, LDL-C and AI were decreased by 35.34%, 43.89%, 35.87%, 54.00% and 41.47%, respectively. No significant change in HDL-C level was observed. Results showed that mixture supplemented diets are more effective against hypercholesterolemia than chitosan. Yao *et al.* (2008) demonstrated that high MW chitosan had plasma cholesterol-lowering effect in diabetic rats. Liu *et al.* (2008) and Ormrod *et al.* (1998) found that rats fed hypercholesterolemia-induced diets containing chitosan significantly lowering plasma cholesterol and LDL-C. The strong positive charge carried by the chitosan molecule (amino groups) causes it to bind negatively charged substrates such as lipids. Chitosan binds fat in the intestine, blocking absorption, and has been shown to lower blood cholesterol in animals and humans (Ormrod *et al.*, 1998 and Gallaher *et al.*, 2000). Reduction of fatty acid and bile acid will lead to less absorption of fat from the diets (Gallaher *et al.*, 2000), and the reduction of endogenetic cholesterol because of the interruption of enterohepatic bile acid circulation (Razdan and Pettersson, 1996), will influence cholesterol metabolism. Chitosan is soluble in the acidic conditions of the stomach and forms a gel when the molecular weight is high. When fat and chitosan in the diets are eaten together, the viscous chitosan will entrap the fat droplet in the stomach. In the small intestine, which is at neutral pH, chitosan forms a precipitate and prevents the digestion of fat (Zhou *et al.*, 2006). Tsujikawa *et al.* (2003) and Kanauchi *et al.* (1994) speculated that gastric acid–soluble chitosan mixes with dietary fat in the stomach, with the emulsifying process effectively mediated by ascorbic acid. Vitamin C supplementation provided a significant reduction in both LDL cholesterol and triglycerides (McRae, 2007 and 2008 and Knekt *et al.*, 2004). Administration of *Gymnema sylvestre* to the STZ-induced diabetic rats significantly ($P < 0.05$) improved lipids profile. The observed hypolipidaemic effect may be due to of decreased cholesterogenesis and fatty acid synthesis. Significant lowering of total cholesterol, triglycerides, LDL-cholesterol and raise in HDL-cholesterol is a very desirable biochemical state for prevention of atherosclerosis and ischaemic conditions (Jouad *et al.*, 2003 and Daisy *et al.*, 2009). These studies confirmed the present study which indicated that, the mixture of chitosan, vitamin C and *Gymnema sylvestre* has more lowering effect of lipid profile than chitosan alone. In fact the mixture (especially Mix III) may prevent of the increase in the factors causing coronary heart diseases (CHD) and cardiovascular diseases (CVD), so it may prevent of atherosclerosis.

Table (3) presents the results of plasma AST, ALT and LDH activities in the controls and experimental groups. There were significant increases ($P < 0.05$) in the plasma AST, ALT and LDH activities of hypercholesterolemic rats (HC) as compared to normal control rats (NC). The present finding are in agreement with those obtained by Ahmed *et al.* (1987) who found that hypercholesterolemia state significantly stimulate ALT and AST activity in the plasma. Plasma ALT activity slightly decreased in rats fed hypercholesterolemia-induced diets containing different levels of chitosan, mixture I and II compared with hypercholesterolemic control (HC). Significant decrease ($P < 0.05$) in plasma ALT activity by 19.27% of rats fed hypercholesterolemia-induced diet containing mixture III (Mix III, 4.68 g/Kg) that containing chitosan, ascorbic acid, *Gymnema sylvestre* (10:2:1). Moreover, plasma AST activity significantly decreased in all treated groups as compared to hypercholesterolemic control (HC). The highest decrease in plasma AST activity was recorded by Mix III group. No changes in LDH activity was observed by chitosan I, chitosan II and Mix I groups when compared with hypercholesterolemic control group (HC). However, there was significant decrease in the plasma LDH activity of chitosan III, Mix II and Mix III groups as compare to hypercholesterolemic control. The best supplement was Mix III which significantly decreased the LDH activity near to that of normal control (NC). It must be noticed that no significant difference in plasma LDH activity between normal control group and Mix III group.

The actions of chitosan include interference of lymphatic absorption of cholesterol and fat (Zhang *et al.*, 2008), increased fecal excretion of neutral steroids and fat, and improvement of liver function (LeHoux and Grondin, 1993 and Zhang *et al.*, 2008). Moreover, *Gymnema sylvestre* caused reduction in the activity of AST, ALT, ALP and ACP enzymes in plasma of diabetic rats (Daisy *et al.*, 2009).

The liver is a central organ for many physiological and biochemical process necessary for maintenance of life (Souba and Wilmore, 1983). Morphological alterations that occur in the liver affect many metabolic processes in the organism. Peroxide formation induced by hypercholesterolemia (Sudhahar *et al.*, 2007) result

in the release of some enzymes by interacting with cellular structure and function. Thus, the serum activities of cellular enzymes such as transaminases, alkaline phosphatase, and lactate dehydrogenase do increase. With the increase in cellular membrane permeability, intracellular fluid transfers onto intercellular space, resulting in muscle and liver cell degeneration.

In our study, it was observed that as a result of hypercholesterolemia, enzymes such as AST, ALT and LDH were released into blood. Their increase in the plasma activities of these enzymes was directly proportional to the degree of cellular damage. These values decreased by chitosan and mixture supplements.

Rats fed hypercholesterolemia-induced diet showed significant (P<0.05) increase and decrease in plasma malondialdehyde (MDA) level and blood reduced glutathione (GSH) content respectively compared to normal control rats (Table 4). MDA significantly increased (P<0.05) by 225.74% and GSH significantly depleted (P<0.05) by 39.88% in hypercholesterolemic rats (HC). These results are related to the results of Kempaiah and Srinivasan (2004 and 2005). Supplementation of hypercholesterolemia-induced diets with different levels of chitosan or mixtures showed significant (P<0.05) decrease and increase in MDA level and GSH content respectively compared with hypercholesterolemic rats (Table 4). The best results of MDA and GSH were recorded by Mix III supplement.

MDA level is the most important factor indicating increased peroxidative level, while glutathione is substance with an important role in cell detoxification and protection from hazardous compounds. Glutathione is synthesized in the erythrocytes and is found in living cells. It has been reported that cellular glutathione has an important function against chemical agents by protecting the cell membrane integrity. A decrease in the amount of glutathione and increase in the amount of MDA may result in the destruction of membrane integrity (Kempaiah and Srinivasan, 2005 and Tauseef *et al.*, 2007). In this study, the decrease in the reduced glutathione and the increase in malondialdehyde levels of hypercholesterolemic group indicate that hypercholesterolemia damaged the integrity of the erythrocyte membrane. On the contrary, the observed increase in the amount of GSH and the decrease in MDA in the chitosan and mixture groups indicate that chitosan, ascorbic acid and *Gymnema sylvestre* effectively protect membrane integrity.

Chitosan, ascorbic acid and *Gymnema sylvestre* have hypolipidemic effect which may prevent the increase in lipid peroxidatin and depleting GSH in hypercholesterolemia.

In regard to mechanism of action, it has been shown that vitamin C is able to intercept reactive oxygen species in the aqueous phase of plasma, thereby significantly reducing plasma lipid peroxide levels and thus inhibiting oxidative modification of LDLs (Polidori *et al.*, 2004). Vitamin C's antioxidant protection of very low-density lipoprotein may therefore facilitate its uptake by the liver and hence promote its removal from the plasma. It has also been shown that vitamin C stimulates fatty acid utilization in hepatocytes by enhancing carnitine synthesis. Carnitine is synthesized from the amino acids lysine and methionine, and vitamin C is required as a cofactor in 2 hydroxylation reactions in the pathway of carnitine biosynthesis. If increased hepatic carnitine concentration results in further hepatic fatty acid β-oxidation, then as a result, there will be a reduction in the plasma triglyceride concentration (McRae, 2008). Furthermore, vitamin C improved circulating antioxidant level, i.e., GSH:GSSG ratio, in periodontitis-induced rats. The chemical and biological properties of vitamin C suggest that vitamin C can act as an antioxidant *in vivo*. Lipid peroxidation is implicated in development of atherosclerosis, and vitamin C protects against oxidation of isolated LDL primarily by scavenging reactive oxygen species in the aqueous milieu. Vitamin C may contribute to improve atherosclerosis with decreasing lipid peroxidation and increasing antioxidant level (Ekuni *et al.*, 2009).

Plasma glucose level of hypercholesterolemic rats (HC) was significantly (P<0.05) increased compared to normal control (NC). However, slight decrease in plasma glucose level was observed in all treated groups compared with hypercholesterolemic group (HC). It was noticed that the effect of mixture supplements are better than the effect of chitosan on glucose level, while no significant difference between NC and Mix III group was found.

Gymnema sylvestre is reported to increase glucose uptake and utilization and improve the function of pancreatic beta cells. *Gymnema sylvestre* may also decrease glucose absorption in the gastrointestinal tract (Shanmugasundaram *et al.*, 1990 and Daisy *et al.*, 2009). In this study, the decrease in the glucose level in mixture supplements groups may be due to the mixture supplements containing *Gymnema sylvestre*.

Table (5) displays the activities of antioxidant enzymes in erythrocytes and plasma. The erythrocytes superoxide dismutase (SOD) and plasma glutathione reductase (GR) were significantly (P<0.05) depressed (23.95% and 17.64%, respectively) in hypercholesterolemic rats (HC). Slight decrease in the activities of erythrocytes glutathione peroxidase (GPx), plasma catalase (CAT) and plasma glutathione-S-transferase (GST) were observed in the hypercholesterolemic control group (HC) compared to the normal control group (NC) by 7.37%, 12.86% and 4.54%, respectively. Fed hypercholesterolemia-induced diets supplemented with chitosan

and the mixture of chitosan, ascorbic acid and *Gymnema sylvestre* improved the activities of these enzymes. The effect of mixture in this context was even better since the levels of enzymes were brought almost to nearly that of normal control (NC). At Mix III feeding a stimulation of SOD, GPx, CAT and GR activities by 14.59%, 15.72%, 11.39% and 19.62%, respectively. There was an increase in the levels of these antioxidant enzymes and GSH in the treated groups, thereby indicating that treatment with chitosan or mixture supplements protects against oxidative stress induced by the depletion of enzymic and non-enzymic antioxidants.

Table 1: Plasma total lipids, total cholesterol and triglycerides (mg/dl) in rats fed hypercholesterolemia-induced diets supplemented with different levels of chitosan or mixture of chitosan, ascorbic acid and *Gymnema sylvestre*

Groups	Total lipids	Total cholesterol	Triglycerides
NC	331.19 ±27.11 ^{e}	95.05 ± 3.35 ^{d}	89.16 ± 5.64 ^{d}
HC	1237.34 ± 56.30 ^{a}	456.82 ± 32.18 ^{a}	269.30 ± 16.40 ^{a}
Chitosan I	1022.87 ± 31.88 ^{b}	341.31 ± 16.39 ^{b}	252.64 ± 15.41 ^{ab}
Chitosan II	974.36 ± 54.08 ^{bc}	304.24 ± 18.85 ^{bc}	233.91 ± 14.79 ^{ab}
Chitosan III	834.76 ± 37.02 ^{cd}	291.30 ± 7.74 ^{bc}	212.82 ± 14.05 ^{bc}
Mix I	905.98 ± 69.35 ^{bcd}	304.85 ± 28.76 ^{bc}	221.15 ± 10.51 ^{b}
Mix II	899.57 ± 48.04 ^{bcd}	287.04 ± 24.60 ^{bc}	211.54 ± 13.72 ^{bc}
Mix III	800.21 ± 32.76 ^{d}	256.34 ± 13.36 ^{c}	172.69 ± 16.39 ^{c}
LSD	136.15	59.65	40.26

Values are expressed as means ± SE (n = 6).
Values with different superscript letters within the same column are significantly different ($P < 0.05$).

Table 2: Plasma HDL-cholesterol, LDL-cholesterol (mg/dl) and AI in rats fed hypercholesterolemia-induced diets supplemented with different levels of chitosan or mixture of chitosan, ascorbic acid and *Gymnema sylvestre*

Groups	HDL-cholesterol	LDL-cholesterol	AI
NC	36.16 ± 2.04 ^{b}	44.91 ± 5.80 ^{f}	1.63
HC	49.92 ± 3.66 ^{a}	360.18 ± 22.09 ^{a}	8.15
Chitosan I	51.99 ± 2.84 ^{a}	249.61 ± 10.41 ^{b}	5.56
Chitosan II	37.58 ± 2.53 ^{b}	219.88 ± 12.05 ^{bc}	7.10
Chitosan III	37.20 ± 3.21 ^{b}	211.53 ± 7.94 ^{bc}	6.83
Mix I	52.74 ± 2.93 ^{a}	199.68 ± 13.80 ^{cd}	4.78
Mix II	46.61 ± 3.03 ^{a}	197.28 ± 15.13 ^{cd}	5.16
Mix III	44.46 ± 1.66 ^{ab}	165.67 ±14.32 ^{e}	4.77
LSD	8.19	38.18	

Values are expressed as means ± SE (n = 6).
Values with different superscript letters within the same column are significantly different ($P < 0.05$).

Table 3: Plasma ALT, AST and LDH activities (U/L) in rats fed hypercholesterolemia-induced diets supplemented with different levels of chitosan or mixture of chitosan, ascorbic acid and *Gymnema sylvestre*

Groups	ALT	AST	LDH
NC	22.63 ± 1.13 ^{c}	20.80 ± 1.17 ^{e}	542.6 ± 22.8 ^{d}
HC	39.85 ± 2.05 ^{a}	97.00 ± 5.04 ^{a}	1236.4 ± 52.4 ^{a}
Chitosan I	36.25 ± 2.01 ^{ab}	74.83 ± 3.90 ^{b}	1249.8 ± 97.5 ^{a}
Chitosan II	35.71 ± 1.42 ^{ab}	69.37 ± 2.70 ^{bc}	1141.7 ± 72.9 ^{ab}
Chitosan III	35.18 ± 1.60 ^{ab}	64.01 ± 3.08 ^{cd}	1023.3 ± 73.5 ^{b}
Mix I	35.33 ± 1.41 ^{ab}	70.59 ± 3.15 ^{bc}	1276.2 ± 46.5 ^{a}
Mix II	35.17 ± 0.88 ^{ab}	63.67 ± 2.75 ^{cd}	827.5 ± 50.3 ^{c}
Mix III	32.17 ± 1.20 ^{b}	58.99 ± 2.12 ^{d}	698.8 ± 23.1 ^{cd}
LSD	4.53	9.51	172.9

Values are expressed as means ± SE (n = 6).
Values with different superscript letters within the same column are significantly different ($P < 0.05$).

Table 4: Whole blood GSH (mg/dl) and plasma MDA (nmol/L) and glucose (mg/dl) in rats fed hypercholesterolemia-induced diets supplemented with different levels of chitosan or mixture of chitosan, ascorbic acid and *Gymnema sylvestre*

Groups	GSH	MDA	Glucose
NC	39.97 ± 1.68 ^{a}	27.19 ± 0.90 ^{d}	81.10 ± 3.35 ^{d}
HC	24.03 ± 0.94 ^{d}	88.57 ± 2.78 ^{a}	112.62 ± 2.70 ^{a}
Chitosan I	25.20 ± 0.71 ^{d}	84.19 ± 2.61 ^{a}	105.45 ± 3.76 ^{ab}
Chitosan II	29.86 ± 0.91 ^{c}	74.47 ± 3.90 ^{b}	96.00 ± 4.69 ^{bc}
Chitosan III	30.68 ± 1.19 ^{c}	74.89 ± 3.25 ^{b}	99.60 ± 1.96 ^{bc}
Mix I	30.55 ± 1.35 ^{c}	71.78 ± 3.19 ^{b}	101.20 ± 3.22 ^{b}
Mix II	31.15 ± 1.42 ^{bc}	74.46 ± 2.72 ^{b}	96.60 ± 4.07 ^{bc}
Mix III	34.44 ± 1.05 ^{b}	57.07 ± 3.29 ^{c}	90.40 ± 2.08 ^{cd}
LSD	3.48	8.60	9.78

Values are expressed as means ± SE (n = 6).
Values with different superscript letters within the same column are significantly different ($P < 0.05$).

Table 5: Erythrocytes SOD (U/ml) and GPx (U/L) and plasma CAT, GR and GST (U/L) activities in rats fed hypercholesterolemia-induced diets supplemented with different levels of chitosan or mixture of chitosan, ascorbic acid and *Gymnema sylvestre*

Groups	SOD	GPx	CAT	GR	GST
NC	173.74± 2.49 ^{a}	324.53 ± 15.02^{ab}	426.23 ± 19.06^{a}	40.99 ± 2.58^{a}	42.08 ± 2.28^{a}
HC	132.12± 4.26 ^{cd}	300.62 ± 15.13^{ab}	371.41 ± 14.60^{a}	29.66 ± 0.47^{c}	40.17 ± 2.33^{a}
Chitosan I	131.73± 6.45 ^{cd}	301.20 ± 9.41 ^{ab}	364.46 ± 13.85^{a}	29.47 ± 0.90^{c}	44.00 ± 1.53^{a}
Chitosan II	133.21± 7.96 ^{cd}	305.44 ± 12.32^{ab}	372.82 ± 20.29^{a}	30.66 ± 0.87^{c}	41.02 ± 2.10^{a}
Chitosan III	133.19± 4.09 ^{cd}	322.71 ± 14.53^{ab}	395.28 ± 17.67^{a}	33.37 ± 0.70^{bc}	39.13 ± 2.72^{a}
Mix I	129.46± 4.18 ^{d}	295.42 ± 8.43 ^{b}	366.40 ± 20.05^{a}	30.53 ± 1.06^{c}	42.93 ± 1.68^{a}
Mix II	146.83± 4.35 ^{bc}	336.95 ± 23.69^{ab}	378.53 ± 19.84^{a}	31.77 ± 1.63^{bc}	39.23 ± 1.00^{a}
Mix III	151.40± 6.07 ^{b}	347.87 ± 9.27 ^{a}	413.72 ± 22.67^{a}	35.48 ± 0.39^{b}	41.78 ± 0.45^{a}
LSD	15.62	8.60	56.04	3.79	5.68

Values are expressed as means ± SE (n = 6).

Values with different superscript letters within the same column are significantly different ($P < 0.05$).

A hypercholesterolemia-induced diet brings about remarkable modification in the antioxidant defence mechanisms. Studies have shown that hypercholesterolemia diminishes the antioxidant defence system and decreases the activities of SOD, CAT, GPx and GST, elevating the lipid peroxide content (Fki *et al.*, 2005 and Kempaiah and Srinivasan, 2005).

Oxidative stress occurred as a consequence of imbalance between production of reactive oxygen species and the antioxidative process in favor of radical production (Dringen, 2000). In the current study, the decrease in the antioxidant enzymes proved the failure of antioxidant defence system to overcome the influx of reactive oxygen species generated by hypercholesterolemic diet. However, the inhibition of enzymes involved in free radical removal led to the accumulation of H_2O_2, which promoted lipid peroxidation and modulation of DNA, altered gene expression and cell death (Halliwell and Gutteridge, 1999). Glutathione (GSH) was the major compound in the intracellular redox status regulation and it was an important substrate and cofactor in many drug's metabolism. The decrease in the activities of GPx and GST could result directly from the decreased levels of GSH following hypercholesterolemia-induced diet feeding since both enzymes were independent on GSH for their activity. The positive correlation observed, in our study, between GSH, GPx and GST supported these findings. Many studies observed that vitamin C reducing plasma lipid peroxide levels, increasing GSH and enhance enzymic antioxidants (Ekuni *et al.*, 2009 and Polidori *et al.*, 2004).

In this study, the hypolipidemic and hypocholesterolemic effects of chitosan, ascorbic acid and *Gymnema sylvestre* improved antioxidant enzymes activities with increasing antioxidant GSH level and decreasing lipid peroxide level (MDA)

It could be summarized that both chitosan and mixture (chitosan, ascorbic acid and *Gymnema sylvestre*; 10:2:1) supplements at different levels protected and prevented of the increase in hypercholesterolemic agents compared with hypercholesterolemic group (HC); but the effects of the mixture supplements (Mix I, Mix II and Mix III) were more better than chitosan. On the other hand, mixture supplements (especially Mix III) significantly decreased plasma transaminases and LDH activities, while slight decrease in these enzymes was observed by chitosan supplements. Also, it can be said that chitosan and mixture supplements kept MDA less than that in hypercholesterolemic diet. Moreover, chitosan and mixture supplements kept GSH more than that in hypercholesterolemic diet. On the other hand, mixture supplements improve antioxidant enzymes better than chitosan as compared to hypercholesterolemic control.

In general, lipids profile, transaminases, LDH, MDA were still more than that in normal control group, while GSH and enzymic antioxidants were still less than that in normal control group. Also, it could be noticed that Mix III gives the best results.

Chitosan, ascorbic acid and *Gymnema sylvestre* are natural, normal, healthy and appropriate mixture to reduce oxidative stress, hyperlipidemic, hypertriglyceridemic and hypercholesterolemic factors. This stand in stark contrast to the use of hypercholesterolemic drugs that have life-threatening side effects like aching or weakness of skeletal muscles.

Finally the present results clearly refer to possibility using the mixture of chitosan, ascorbic acid and *Gymnema sylvestre* as hypocholesterolemic agents, but may need further studies using higher concentrations of the mixtures to normalize the rest of biochemical parameters. However, serious experiments must be carried out on human patients.

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