

Hypocholesterolemic Effects of Soybean and Sweet Lupine Tempeh in Hypercholesterolemic Rats

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Abstract

A study was designed to evaluate the hypocholesterolemic effects of soybean and sweet lupine tempeh fermented with *Rhizopus oligosporus* in hypercholesterolemic rats. Soybean and sweet lupine seeds and *Rhizopus oligosporus* were used in this study to produce tempeh using traditional method. Both types of tempeh were subjected to biological study. Thirty-six male albino rats of Sprague-Dawley strain were divided into 6 groups as follows: (1) control negative (C-); (2) hypercholesterolemic (control positive, C+); (3) hypercholesterolemic + 3.5% protein from soybean tempeh; (4) hypercholesterolemic + 7% protein from soybean tempeh; (5) hypercholesterolemic + 3.5% protein from sweet lupine tempeh; (6) hypercholesterolemic + 7% protein from sweet lupine tempeh. All animal groups received the selected experimental diets for 4 weeks. Blood samples were drawn after the end of the experimental period. The evaluation of histopathological liver tissue sections and serum biochemical markers (aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, triglycerides, lipoprotein fractions, urea nitrogen and uric acid) were conducted. The animal groups fed a hypercholesterolemic diet + 3.5% or 7% protein either from soybean or sweet lupine tempeh significantly ($P < 0.05$) reduced the levels of total cholesterol (-37.41% to -48.43%), LDL-C (-57.51% to -75.35%), VLDL-C (-25.87% to -42.55%), triglycerides (-25.77% to -42.1%), AST (-10.19% to -37.15%), ALT (-23% to -45.86%), urea nitrogen (-9.38% to -19.04%) and uric acid (-9.04 to -14.27%), while HDL-C levels were increased (+57.67% to +76.09%) compared to that of the positive control group. The ratios of serum TC/HDL-C, LDL-C/HDL-C and AI were also decreased. On the other hand, the histopathological examination of the liver tissue sections of hypercholesterolemic rats fed a diet containing tempeh

produced from soybean or sweet lupine revealed that the two types of tempeh reduced the degenerative changes. However, the higher levels of soybean and sweet lupine tempeh (7%) induced a better effect than the lower levels (3.5%), and were capable of recovering the damaged hepatocytes to almost their normal structure. These results indicated that tempeh obtained from soybean or sweet lupine, in particular, the high level (7%) significantly improved the levels of lipid profiles, activity of liver enzymes, concentrations of uric acid and urea nitrogen as well as induced a better protective effect in the hepatocytes, consequently, the two types of tempeh exhibited hypocholesterolemic, anti-atherogenic and hepatoprotective effects. Therefore, incorporating soybean or sweet lupine tempeh into the diet can be a possible coadjutor in the treatment and prevention of hypercholesterolemia.

Keywords: Soybean, Sweet lupine, Tempeh, Hypercholesterolemic rats, Biochemical markers, Histopathological evaluation

Hypercholesterolemia and its implications in cardiovascular diseases is a major problem in human health, and much attention has been paid to dietary intervention as a tool for its prevention and treatment (Kerckhoffs *et al.*, 2002). It is estimated that fermented foods and beverages incorporate about one-third of the human diet (Campbell-Platt, 1994). Fermented foods containing probiotics and prebiotics can be important diet components, due to their nutritional characteristics and ability to reduce the risk of chronic diseases (Rossi *et al.*, 2000 and Monzani *et al.*, 2008).

Fermented soy products include, but are not limited to, miso, soy paste, soy sauces (shoyu), douchi, fermented tofu, natto, and tempeh. Tempeh is a compact, sliceable cake of mold fermented soybean cotyledons. Tempeh is an indigenous fermented food that originated from the Javanese people in Indonesia, where it is most popular (Aderibigbe and Kolade, 2003). It is normally consumed as fried, boiled, steamed or roasted. Fermentation process of tempeh completely transforms the soybeans to produce a new flavor, aroma, texture and also increases the nutritional values of some nutrients, development of vitamins, phytochemicals and antioxidative constituents (Berghofer *et al.*, 1998; Astuti *et al.*, 2000 and Chen-Tien *et al.*, 2009). Studies have reported that isoflavones levels determined in tempeh are relatively high compared to other soybean products such as tofu and soy beverages (Hutabarat *et al.*, 2001).

Fermentation process of tempeh decreases the phytic acid and enhances the bioavailability of minerals such as calcium, zinc and iron (Astuti *et al.*, 2000). Whereas soybeans are the main ingredient of tempeh and the health effects of soybeans can also be associated with tempeh, tempeh is associated with certain health effects.

Gamma-aminobutyric acid (GABA), an important non-protein constituent amino acid, is also produced during tempeh fermentation and this compound has been proven to have pharmaceutical effects on the human body (Aoki *et al.*, 2003 and Oh and Oh, 2004). Previous studies showed that feeding tempeh containing GABA

at 0.1% level had significant antihypertensive effect in spontaneously hypertensive rats (Aoki *et al.*, 2003), antioxidant activity, and anticancer (Watanabe *et al.*, 2007; Babu *et al.*, 2009 and McCue and Shetty, 2004). Dietary GABA from tempeh has also been reported to protect the filtration function of the kidneys from damage induced by high blood pressure (Nakamura *et al.*, 2000). Some reports also stated that GABA is a strong secretagogue of insulin from pancreas; therefore, effectively preventing diabetic conditions (Adeghate and Ponery, 2002).

In the fermentation, β -glucosidase enzyme hydrolyze the β -glucosidic isoflavones forms to their corresponding aglycone forms which are readily available to human organism and has high biological activity (Liggins *et al.*, 2000). Isoflavones, mainly aglycones, have been studied due to their ability to reduce cardiovascular disease risk (Zhuo *et al.*, 2004), inhibit some types of cancer cell growth (Lund *et al.*, 2004), reduce the risks of diseases, including osteoporosis (Liggins *et al.*, 2000) and relieve symptoms of menopause (Messina and Hughes, 2003). Furthermore, several studies demonstrated the hypolipidemic and antidiarrheal effect of tempeh (Astuti *et al.*, 2000; Karyadi and Lukito, 1996; Karyadi and Lukito, 2000 and Kiers *et al.*, 2003). Soybean tempeh can also have beneficial physiological effects in case of malfunction of the gastrointestinal digestive system (Kiers *et al.*, 2000).

In Indonesia, the traditional and original material for producing tempeh is soybean (Shurtleff and Aoyagi, 2001). However, possible use of other types of legumes as a raw material for fermented foods should be explored its potential, because they are not only a source of protein but also can be used as a functional food (Cornelia *et al.*, 2012). Lupine is an economically and agriculturally valuable plant (Sujak *et al.*, 2006). Its seeds are employed as a protein source for animal and human nutrition in various parts of the world.

The lupine seed is high in protein, high in dietary fiber, low in oil content, and contains minimal starch (Martinez-Villaluenga *et al.*, 2006). Lupines contain phytochemicals with antioxidant capacity, such as polyphenols, mainly tannins and flavonoids (Oomah *et al.*, 2006). Therefore, many researches have paid more attention towards the possibility of using lupines as a human food (Pettersson *et al.*, 1997), and their potential health benefits. Due to low glycemic index of lupine seeds, it was found that lupine kernel fibers have appetite suppression (Archer *et al.*, 2004) and cholesterol lowering properties (Hall *et al.*, 2005b), that they lower blood glucose and insulin levels (Hall *et al.*, 2005a), and aid bowel health as a fecal bulking agent, because it reduces transit time, lower the colon pH (anticancer) and acts as a “prebiotic” to improve bowel functions (Johnson *et al.*, 2006).

The present study was designed to develop soybean and sweet lupine tempeh, fermented with *Rhizopus oligosporus* and to evaluate the hypocholesterolemic effects of the two types of tempeh in hypercholesterolemic rats.

Materials and Methods

Materials: Soybean (*Glycine max*) variety Giza 111 and sweet lupine (*Lupinus termis*) variety Giza 1 were obtained from Agriculture Research Center, Ministry of Agriculture, Giza, Egypt.

Mold strain: *Rhizopus oligosporus* strain ATCC 22959 was obtained from the Egyptian Microbial Culture Center (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University, Egypt.

Chemicals and Kits: Corn starch and corn oil were purchased from local market. Cholesterol (as a pure white crystalline powder), casein, choline chloride, cellulose, bile salts (as a pure yellow powder), vitamins and minerals were obtained from EL-Gomhoria Company, Cairo, Egypt. All kits were purchased from Gamma Trade Company, El-Mohandessen, Cairo, Egypt.

Preparation of inoculum and tempeh: The method of Aderibigbe and Kolade (2003) was used to prepare the inoculum (spore's suspension). The mold was grown on potato dextrose agar. The spore's count was determined by serial dilution and plating and expressed as cfu/ml. Tempeh was prepared using one of the Indonesian methods as described by Aderibigbe and Adebayo (2002), as shown in Figure 1.

Animals: Thirty six male albino rats of Sprague-Dawley strain weighing (110±10g), 6 weeks old, were obtained from the laboratory of animal colony, Ministry of Health and Population, Helwan, Cairo, Egypt. The rats were housed in clean polypropylene cages and kept in the animal room of the Faculty of Home Economics, Helwan University under a controlled environment (temperature 22±1°C, relative humidity 55±5%), with a 12h light and a 12h dark cycle. The rats were fed on basal diet and water ad libitum for one week before starting the experiment for acclimatization. Animal were cared according to the guidelines and protocol in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). After that, the rats were divided into two main groups. The first group (n=6 rats) was fed on the basal diet as a control negative (C-). The second group (n=30 rats) was fed on a high cholesterol diet (1% cholesterol + 0.2% bile salts) for two weeks, to induce hypercholesterolemia (Ashraf *et al.*, 1999). After this period the second group (hypercholesterolemic rats) was divided into 5 subgroups having six rats in each group as follows:

G1. Normal control group, rats fed on basal diet (negative control group).

The other groups comprised rats with hypercholesterolemia (G2 to G6).

G2. Rats fed on a high cholesterol diet (positive control group).

G3. Rats fed on a high cholesterol diet + 3.5% protein from soy tempeh + 10.5% protein from casein.

- G4.** Rats fed on a high cholesterol diet + 7% protein from soy tempeh + 7% protein from casein.
- G5.** Rats fed on a high cholesterol diet + 3.5% protein from sweet lupine tempeh + 10.5% protein from casein.
- G6.** Rats fed on a high cholesterol diet +7% protein from sweet lupine tempeh + 7% protein from casein.

The experiment lasted for 4 weeks. During the experimental period, the food intake was recorded every day and body weight gain percentage (BWG%) was calculated by the following formula:

$$\text{BWG\%} = [\text{Final weight (g)} - \text{Initial weight (g)} / \text{Initial weight}] \times 100$$

Blood Sampling and Organs Preparation: At the end of the experimental period, the rats were fasted overnight, anesthetized with ether and blood samples were collected from the portal vein in clean and dry tubes, then the blood was allowed to clot and serum was separated by centrifugation at 3000 rpm for 15 min for measurements of some serum biochemical parameters. Organs (Liver, kidneys, spleen and heart) were rapidly excised and immediately placed in ice-cold physiological saline to wash off the excess blood and cool off the tissue. The tissues were blotted dry and weighed.

Biochemical Analysis: Serum total cholesterol was assayed by the method of Allain *et al.*, (1974), serum triglycerides were determined according to the method described by Fossati and Prencipe (1982), serum HDL-cholesterol was determined according to the method described by Richmond (1973), serum LDL-cholesterol and VLDL-cholesterol were assayed according to Friedwald *et al.*, (1972).

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured according to Reitman and Frankel (1975), serum urea nitrogen was determined according to Patton and Crouch (1977), while serum uric acid was estimated by the method of Fossati *et al.*, (1980).

Histopathological Examination: The animals were sacrificed and liver was excised immediately and thoroughly washed with ice-cold physiological saline then specimens from liver tissue were fixed immediately in 10% neutral buffered formalin, dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin wax. Sections of 5 μm in thickness were prepared and stained with haematoxylin and eosin and examined microscopically (Bancroft *et al.*, 1996).

Statistical Analysis: All data were expressed as means values \pm SD for six rats in each group. Statistical analysis was performed using one way analysis of variance (ANOVA). Differences among means were compared using Duncan's Multiple

Range test with a level of significance of $P < 0.05$. Statistical analysis was conducted with the Statistical Analysis System (SAS, 1996).

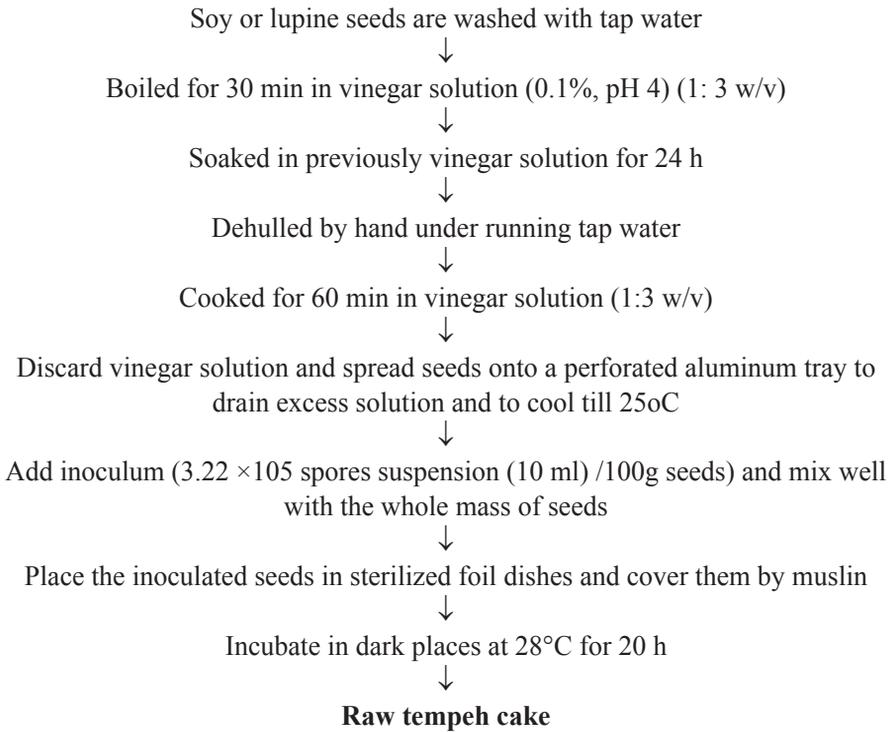


Fig. 1. Process of soybean and sweet lupine tempeh production according to the method of Aderibigbe and Adebayo (2002)

Results and Discussion

Food intake, body weight gain (%) and the relative organs weights/body weights

Food intake was reduced in the group of rats fed on a high cholesterol diet and in the groups of hypercholesterolemic rats fed on the both types of tempeh at higher or lower levels (Table 1). There were significant differences in the body weight gain (%) pattern of the animals fed on a high cholesterol diet compared to those fed on the control diet as well as fed on 3.5% or 7% protein either from soybean or sweet lupine tempeh. While the group of rats fed on the control diet (control

Table 1. Food intake, body weight gain (%) and the relative organs weights/body weights (%) of hypercholesterolemic rats fed on different experimental diets for 4 weeks.

Groups	Food intake g/day	*BWG (%)	Liver	Kidney	Spleen	Heart
G1 Control (-ve) (14 % protein from casein)	15.297±2.521 ^a	37.800±3.355 ^a	2.431±0.162 ^d	0.643±0.089 ^c	0.207±0.025 ^c	0.264±0.026 ^{cd}
G2 Control (+ve) (14% protein from casein)	13.875±3.455 ^b	18.685±5.731 ^d	3.694±0.192 ^a	1.034±0.108 ^a	0.307±0.045 ^a	0.360±0.032 ^a
G3 (3.5 % soy tempeh protein + 10.5% protein from casein)	14.285±3.136 ^b	30.167±3.067 ^c	2.998±0.277 ^b	0.786±0.058 ^b	0.259±0.033 ^b	0.298±0.028 ^{bc}
G4 (7 % soy tempeh protein + 7 % protein from casein)	14.881±3.102 ^a	30.893±2.155 ^b	2.55±0.185 ^{cd}	0.681±0.028 ^c	0.203±0.045 ^c	0.253±0.015 ^d
G5 (3.5 % lupine tempeh protein + 10.5 % protein from casein)	13.095±2.355 ^c	14.152±4.209 ^e	2.912±0.218 ^b	0.787±0.062 ^b	0.257±0.039 ^b	0.328±0.051 ^{ab}
G6 (7 % lupine tempeh protein + 7 % protein from casein)	12.916±2.302 ^c	11.801±3.103 ^f	2.764±0.157 ^{bb}	0.646±0.055 ^c	0.198±0.033 ^c	0.268±0.022 ^{cd}

Values are the mean±Standard deviation (n=6).

Means within each column followed by different superscript letters are significantly different at P<0.05.

*BWG (%): Body weight gain (%).

negative) showed the highest weight gain (%) when compared with the other experimental groups. The high cholesterol diet was not efficient in raising body weight gain (%) of rats in the positive control group (hypercholesterolemic rats). These results are in accordance with those obtained by Otunola *et al.*, (2010) and De Abreu *et al.*, (2014).

Matos *et al.*, (2005) and Hartvigsen *et al.*, (2007) reported similar weight loss as observed in this study with diet-enriched hypercholesterolemic rat models. While Harnafi *et al.*, (2009) reported a linear weight increase in all the animal fed with both experimental and control diets. Another study showed no significant changes in weight gain among the control and hypercholesterolemic animals (Ramachandran *et al.*, 2003 and Rossi *et al.*, 2008). The body weight gain (%) of hypercholesterolemic rats fed on 3.5% and 7% protein from soybean tempeh was significantly ($P<0.05$) increased as compared to positive control group. However, the body weight gain (%) in hypercholesterolemic rats treated with soy tempeh at lower or higher level was lower than the normal control rats (control negative).

The body weight gain (%) of hypercholesterolemic rats fed on 3.5% and 7% protein from sweet lupine tempeh was significantly less than the other experimental groups (Table 1). These results are in agreement with those of Yee *et al.*, (2001) who reported that consumption of soy protein contributes to weight loss by decreasing the amount of fat stores in body while increasing lean muscle mass. In addition, Maier *et al.*, (2000) found that inclusion of 30% white lupine to the diet of broilers and laying hens reduced growth of body and Bunger *et al.*, (2000) found that fiber content of sweet lupine significantly reduced body weight.

Matsumoto *et al.*, (2007) noticed significantly lower body weight in mice fed with 20% and 40% okara supplemented to their basal diet for 10 weeks, indicating a preventive effect in the obesity. Préstamo *et al.*, (2007) and Jiménez-Escrig *et al.*, (2008) observed a decrease in body weight gain from the second week in rats fed okara, compared to the control animals.

A recent study has found that enrichment of bread with lupine-kernel flour can reduce appetite acutely. Lupine-enriched bread significantly reduced within meal food intake by approximately 30%, food intake at a subsequent meal by approximately 15%, and self-reported hunger and fullness for 3h following a fixed energy meal (Lee *et al.*, 2006). In addition, lupine-enriched bread compared to white bread at breakfast significantly altered the 3h post meal plasma ghrelin response. The changes in plasma ghrelin were consistent with the observed acute effects on satiety and energy intake (Lee *et al.*, 2006). Furthermore, incorporation of lupine kernel fiber into processed foods was found to result in higher post meal satiety up to 4.5h, and lower energy intake by approximately 15% over the test day (Archer *et al.*, 2004).

Furthermore, the protein and fiber content in the diet may be an important determinant of ghrelin (an appetite-regulating hormone) secretion (Erdmann *et al.*, 2004), thereby influencing post meal satiety and subsequent energy intake.

The group of rats fed on a high cholesterol diet showed highest relative weights for liver, kidneys, spleen and heart when compared with the other experimental groups (Table 1). In the present study, feeding a high cholesterol diet produced a significant increase in the relative organs weights of the hypercholesterolemic rats. This could be as a result of high levels of lipids such as cholesterol, tend to become deposited in liver and on the arterial walls of blood vessels forming hard plaques (Jiunrong *et al.*, 2002), leading to increased resistance to blood flow. Thus the heart in an effort to pump through the narrow arteries becomes enlarged and hence the increased weight, the same trend was also observed for other organs of hypercholesterolemic rats.

The high cholesterol diet caused lipid accumulation in the organs of hypercholesterolemic rats. Treatment with the two types of tempeh at higher or lower levels (Groups 3, 4, 5 and 6) significantly ($p < 0.05$) reduced the cholesterol-induced enlargement of the different organs. However, the best effect was obtained by the higher level (7%) of both soybean and sweet lupine tempeh as could be seen in Table 1. These results are in line with those obtained by Cavallini *et al.*, (2009); Jaekel and Rodrigues (2011); Kobayashi *et al.*, (2012) and Fontanari *et al.*, (2012).

Treatment the hypercholesterolemic rats with 7% protein either from soybean or sweet lupine tempeh reversed the increase in the relative organs weights. The improving effect of the two types of tempeh on the enlargement that induced in different organs of hypercholesterolemic rats could be explained by the hypocholesterolemic effect of tempeh, prevented the cholesterol-induced enlargement of the different organs, and also indicating that the regular intake of tempeh produced either from soybean or sweet lupine could protect against heart defects and failures.

Fontanari *et al.*, (2012) investigated the whole lupine seeds and lupine protein isolate in hypercholesterolemic hamsters and reported that the lowest relative weight of the liver was for the group of animals that consumed whole lupine seeds in comparison to the other groups. Similar results were also obtained by Frota *et al.*, (2008) who found that significant reduction in the relative liver weight for the group of animals that consumed whole cowpea bean in relation to the other groups and the authors also observed that in this same group there was a greater excretion of total sterols in the faeces, this being a possible mechanism which could explain the hypocholesterolemic effect of whole legumes.

Alkhamees (2013) found that feeding of hypercholesterolemic diet to female albino rats significantly elevated liver weights (27.8%, $P < 0.01$) compared to rats fed on

normal diet and fed on hypercholesterolemic diet supplemented with vitamin-P (a quercetin-3-rutinosid or rutin) or vitamin C significantly ($P<0.01$) attenuated liver weights elevation (13.59% and 18.41%, respectively) as compared with hypercholesterolemic group.

Serum lipid profile

Serum lipid profile concentrations in normal control group, hypercholesterolemic group and hypercholesterolemic groups fed on both types of tempeh for 4 weeks are presented in Table 2. Rats fed the hypercholesterolemia-induced diet developed hypercholesterolemia marked by a significant ($P<0.05$) increase in levels of TC, TG, LDL-C and VLDL-C (124.01%, 75.52%, 642.52% and 75.88%, respectively), while HDL-C level and HTR% value were reduced (47.77% and 76.66%, $P<0.05$, respectively) compared to the normal control rats (Figure 2 and Table 2).

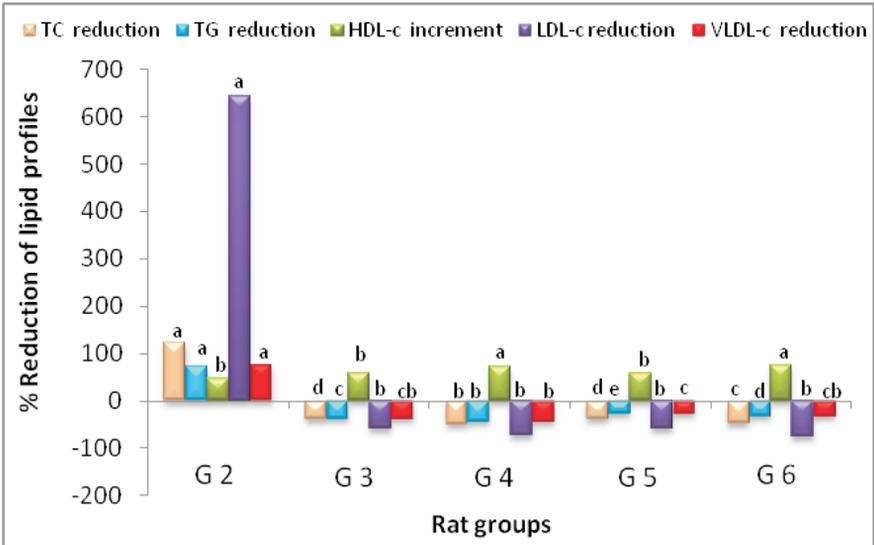


Fig. 2. Effect of soybean and sweet lupine tempeh on the reduction of serum lipid profiles in hypercholesterolemic rats. Means within each bar with different letters are significantly different at $P<0.05$.

Rats fed a high cholesterol diets containing 3.5% and 7% protein either from soybean or sweet lupine tempeh showed a significant decrease in serum levels of TC (-37.91%, -48.43%, -37.41% and -46.01%, $P<0.05$, respectively), TG (-36.35%, -42.1%, -25.77% and -30.74%, $P<0.05$, respectively), LDL-C (-57.51%, -73.34%, -58.2% and -75.35%, $P<0.05$, respectively) and VLDL-C (-36.69%, -42.55%, -25.87% and -31.12%, $P<0.05$, respectively) as compared to hypercholesterolemic rats (Figure 2). In contrast, HDL-C serum levels and HTR% values were

Table 2. Effect of feeding soybean and sweet lupine tempeh on serum lipid profile in hypercholesterolemic rats.

Groups	Parameters (mg/dl)					*HTR%
	Cholesterol	Triglycerides	HDL-C	LDL-C	VLDL-C	
G1 Control (-ve) (14% protein from casein)	66.75±5.484 ^d	45.15±3.686 ^d	42.51±3.007 ^a	15.20±2.886 ^d	9.03±0.737 ^d	63.67±1.253 ^a
G2 Control (+ve) (14% protein from casein)	149.50±5.263 ^a	78.30±4.151 ^a	22.24±2.339 ^d	111.70±6.894 ^a	15.66±0.830 ^a	14.86±0.945 ^d
G3 (3.5% soy tempeh protein + 10.5 % protein from casein)	92.83±5.776 ^b	49.85±5.506 ^{cd}	34.96±0.826 ^e	47.47±4.826 ^b	9.96±1.101 ^{cd}	37.65±0.487 ^c
G4 (7% soy tempeh protein + 7% protein from casein)	77.12±4.921 ^c	45.35±3.654 ^d	38.25±2.294 ^{bc}	29.79±6.155 ^e	9.07±0.730 ^d	49.58±0.639 ^b
G5 (3.5 % lupine tempeh protein + 10.5% protein from casein)	93.58±8.293 ^b	58.13±3.646 ^b	35.25±3.523 ^c	46.70±6.763 ^b	11.65 ±0.729 ^b	37.65±0.773 ^c
G6 (7% lupine tempeh protein + 7 % protein from casein)	80.73±4.626 ^c	54.24±1.432 ^{bc}	39.06±3.105 ^b	27.55±6.771 ^c	10.84±0.308 ^{cb}	48.37±0.546 ^b

Values are the mean±Standard deviation (n=6).

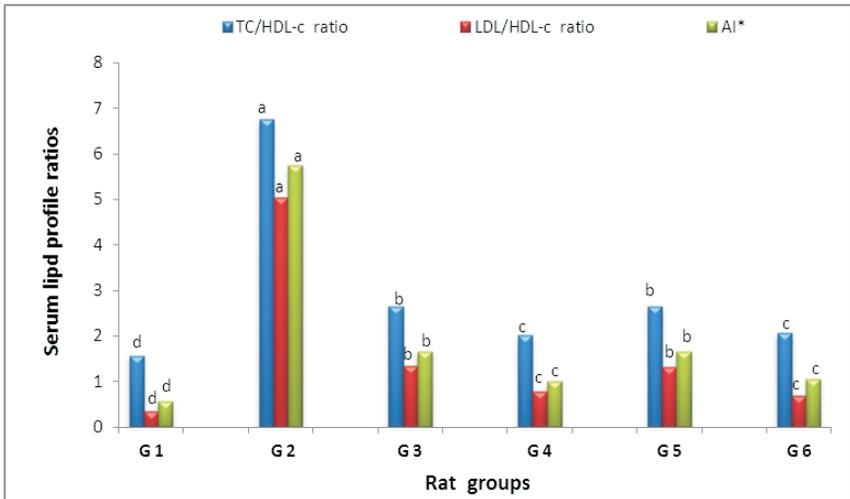
Means within each column followed by different superscript letters are significantly different at P<0.05.

*HTR% = (HDL-C / TC) X 100.

significantly increased (57.67%, 72.43%, 58.88% and 76.09%, $P < 0.05$, respectively) and (153.35%, 233.65%, 153.35% and 225.48%, $P < 0.05$, respectively), following 4 weeks of 3.5% and 7% protein from soybean and sweet lupine tempeh consumption to hypercholesterolemic fed rats, respectively, (Figure 2 and Table 2).

The ratios of serum TC/HDL-C, LDL-C/HDL-C and AI are illustrated in Figure 3. In hypercholesterolemic rats these ratios were significantly ($P < 0.05$) increased (329.79%, 1313.45% and 907.36%, respectively), whereas a significant ($P < 0.05$) decrease in the ratios of TC/HDL-C (-60.65%, -70.13%, -60.65% and -69.37%, respectively), LDL-C/HDL-C (-73.09%, -84.56%, -73.72% and -86.03%, respectively) and AI (-71.19%, -82.32%, -71.19% and -81.43%, respectively) in hypercholesterolemic rats fed on soybean and sweet lupine tempeh at 3.5% and 7% protein, respectively, compared with the hypercholesterolemic rats (Figure 3).

The higher levels (7%) of soybean and sweet lupine tempeh supplemented diets were more effective against hypercholesterolemia than the lower levels (3.5%) of the two types of tempeh. These results indicated that the two types of tempeh exhibited hypocholesterolemic and anti-atherogenic effects. These results are in agreement with the observations of Osman *et al.*, (2011) and Fontanari *et al.*, (2012).



AI* = (TC-HDL-C)/HDL-C

Fig. 3. Effect of soybean and sweet lupine tempeh on serum lipid profile ratios in hypercholesterolemic rats. Means within each bar with different letters are significantly different at $P < 0.05$.

In addition, in randomized crossover study in humans revealed that a modest amount comprising 25 g/d of additionally consumed lupine protein isolate was capable of lowering total (-5%) and LDL cholesterol concentrations (-12%) as well as the LDL:HDL cholesterol ratio (-16%) from baseline to 4 weeks, primarily in subjects with higher hypercholesterolemia (>6.6 mmol/L). According to Anderson and Konz (2001) a 1% increase in either total or LDL cholesterol increases the risk for coronary heart disease by 2% to 3% and 1%, respectively. Thus, after 4 weeks of lupine protein isolate intervention the risk for cardiovascular events such as coronary heart diseases would be reduced by 10% to 15% in subjects with higher hypercholesterolemia (Bähr *et al.*, 2013).

The effects of protein and fiber rich lupine kernel flour on blood lipids remain uncertain. However, results of several animal studies suggested cholesterol-lowering effects of lupine. Whole lupine grain fed to chickens (Viveros *et al.*, 2007) and pigs (Martins *et al.*, 2005) has been shown to lower cholesterol. Furthermore, protein and fiber isolates from lupine may also improve lipids. In rats, lupine protein isolates significantly reduced plasma total cholesterol concentrations by 21% (Sirtori *et al.*, 2004). In humans, an additional 17 to 30 g/d of dietary fiber from lupine fiber isolate reduced total and LDL cholesterol concentrations by approximately 5% (Hall *et al.*, 2005b).

Moreover, Radtke *et al.*, (2014) showed that the reduction circulating plasma cholesterol concentrations in response to lupine protein isolate administration was associated with a significant increase of fecal cholesterol output. In addition, the continued drain of bile acids and lipids by sequestration and increased elimination as fecal acidic and neutral sterols may ultimately influence the absorption of lipids and cholesterol in the intestine. The theory that viscous non-starch polysaccharides from lupine seeds lowers cholesterol by enhanced bile excretion has been explored as follows: cholesterol lost from the body in the form of bile shifts the liver to toward providing more cholesterol for increased bile acid synthesis and increases LDL receptor activity. Thus the end result is increased LDL removal from the blood (Costa *et al.*, 1994).

The hypocholesterolemic possible mechanisms of lupine seeds that have studies in animals and humans include stimulation of LDL receptors and thus an increase in LDL cholesterol degradation, reduction in the intestinal absorption of cholesterol and increased bile acid excretion (Sirtori *et al.*, 2004; Martins *et al.*, 2005; Hall *et al.*, 2005b; Viveros *et al.*, 2007 and Bähr *et al.*, 2013).

On the other hand, recent study reported that soybean protein containing isoflavones significantly reduced serum total cholesterol, LDL cholesterol and triacylglycerol and significantly increased HDL cholesterol, but the changes were related to the level and duration of intake, and gender and initial serum lipid concentrations of persons (Zhan and Ho, 2005). Soy protein (20% of diet) with

isoflavones also inhibits formation of atherosclerotic lesions in primates (Anthony *et al.*, 1997). Isoflavones also act as antioxidants and can inhibit LDL oxidation, since LDL oxidation has a central role in the pathogenesis of atherosclerosis, thus, soy isoflavones may have protective effects against atherogenesis in humans. In vitro studies suggested that genistein and daidzein inhibit LDL oxidation in subendothelium of vessels in a similar manner to that of vitamin E (Hodgson *et al.*, 1996).

In addition, consumption of fermented soy milk was positive in lowering total cholesterol and triglycerides accumulation in the liver and under oxidative stress (Lin *et al.*, 2005). Okara tempeh as well as soybean protein and fiber, reduce plasma cholesterol levels in rats significantly compared with casein (Matsuo and Hitomi, 1993). Moreover, Okara tempeh achieved reduced liver cholesterol and increase of cholesterol excretion in feces, most probably as a result of its increased water-soluble dietary fiber levels.

Yusof *et al.*, (2013) demonstrated that 1000 mg/kg body weight of nutrient enriched soybean tempeh could significantly reduce the levels of cholesterol and triglycerides in the liver under alcohol-induced oxidative stress in mice. Previous studies conducted by Shivashankara *et al.*, (2012) and Yang *et al.*, (2011) showed that soybean and soybean-based products have revealed to have hypocholesterolemic effect. Nagaoka *et al.*, (2010) observed that soybean protein derived Val-Ala-Trp-Trp-Met-Tyr peptide, designated soystatin inhibits cholesterol absorption by reducing the micellar solubility of cholesterol.

In a Number charges of clinical intervention trials, total cholesterol and low-density lipoprotein cholesterol were significantly reduced in subjects treated with tempeh (Sudarmadji *et al.*, 1997). Tempeh has also been found to lower blood cholesterol levels (Guermani *et al.*, 1993) and that it may thus be of benefit as a protective agent against cardiovascular disease.

Several studies revealed that the constituents of soybean responsible for lipid-lowering effects involved small peptide components, individual amino acid ratios, non-protein components such as isoflavones, saponins, fiber, or a combination of factors may alter lipoprotein metabolism (Sirtori *et al.*, 1997; Anthony *et al.*, 1996; lovati *et al.*, 1996 and Anderson *et al.*, 1995).

The hypocholesterolemic possible mechanisms of soy foods that have been studied in animals and humans include a decrease in the intestinal absorption of cholesterol and / or bile acids, enhancement of the bile acids excretion, increased plasma cholesterol clearance through enhancing hepatic LDL-receptor, reduced cholesterol metabolism, changes in hepatic biotransformation of cholesterol, increased thyroid hormones, and reduced insulin-glucagon ratios (Anderson *et al.*, 1995; Potter, 1995 and Sirtori *et al.*, 1995).

Liver enzymes

Serum aspartate and alanine amino transferases concentrations of rats fed on different experimental diets are shown in Table 3. Significant ($P<0.05$) increase of serum AST and ALT concentrations was found in hypercholesterolemic rats group compared to negative control group, indicating that lipid accumulation was harmful to the liver. This could be as a result of leakage of the enzymes into the serum as a result of damage to integrity of the liver. The present findings are in agreement with those obtained by Ahmed *et al.*, (1987) who found that hypercholesterolemia states significantly stimulates ALT and AST activity in the plasma. According to Pincus and Schaffner (1996), AST and ALT are released into serum when there is severe hepatic cellular injury. In addition, Otunola *et al.*, (2010) showed hepatic injury and cardiovascular distress in the rats fed with hypercholesterolemic diet.

Table 3. Effect of feeding soybean and sweet lupine tempeh on serum AST and ALT activities (U/L) in hypercholesterolemic rats.

Groups	Liver parameters (U/L)	
	AST	ALT
G1 Control (-ve) (14 % protein from casein)	60.117±2.416e	13.117±1.742c
G2 Control (+ve) (14 % protein from casein)	105.263±5.664a	25.960±1.872a
G3 (3.5 % soy tempeh protein + 10.5 % protein from casein)	94.540±2.420b	20.000±2.280b
G4 (7 % soy tempeh protein + 7 % protein from casein)	72.767±5.256c	18.333±3.669b
G5 (3.5 % lupine tempeh protein + 10.5 % protein from casein)	66.167±2.316d	14.640±1.248c
G6 (7 % lupine tempeh protein+ 7 % protein from casein)	66.167±1.471d	14.100±1.337c

Values are the mean±Standard deviation (n=6).

Means within each column followed by different superscript letters are significantly different at $P<0.05$.

Positive control group recorded a significant increase in serum levels of AST (75.15%, $P<0.05$) and ALT (99.19%, $P<0.05$) compared to the normal control group (Figure 4).

Soybean tempeh at level of 3.5% and 7% protein was able to reduce (- 10.19% and - 30.88%, respectively) the elevated AST level significantly ($P < 0.05$) compared to the hypercholesterolemic rats. As well as, serum level of ALT was reduced significantly ($P < 0.05$) after both levels of soybean tempeh feeding (-23% and 29.46%, respectively) compared to the hypercholesterolemic rats. The same trend was also observed for the two levels (3.5% and 7% protein) of sweet lupine tempeh feeding, whereas the reduction in the levels of serum AST and ALT were 37.15%, 37.11%, 43.77% and 45.86%, $P < 0.05$, respectively) as could be seen in Figure 4. These decreases were considered as indicators of the improvement in the functional states of liver cells that may be due to the antioxidants which are found in tempeh such as tocopherol, isoflavones and the antioxidant enzyme superoxide dismutase (Astuti *et al.*, 2000), which act as power antioxidants against lipid oxidation.

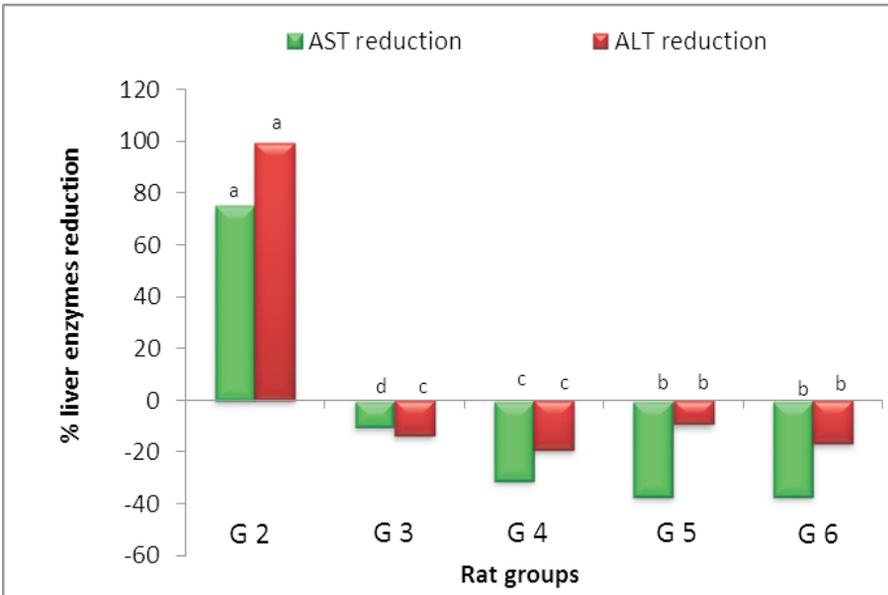


Fig. 4. Effect of soybean and sweet lupine tempeh on the reduction of serum AST and ALT activities in hypercholesterolemic rats. Means within each bar with different letters are significantly different at $P < 0.05$.

These results showed that sweet lupine tempeh was more effective on the activities of both serum AST and ALT, when compared to soybean tempeh. The present results are in accordance with those obtained by Sugiyama *et al.*, (2002) who reported that plasma ALT and AST activities in rats fed on 25% soybean protein diet were suppressed to about 0.25 and 0.2 of the values in rats fed on 25% casein diet, respectively. Le Blanc *et al.*, (2003) revealed that, the activity of some hepatic enzymes was enhanced by soybean lecithin. Moreover, bile acid and lipid output were significantly increased by fed on diet enriched with soybean lecithin for 14

days. Kuba *et al.*, (2004) and Ali *et al.*, (2005) reported that, the fermented soy product (Tofuyo) had a significant lowering effect on ALT and AST.

Osman *et al.*, (2011) observed that a hypercholesterolemia-induced diet was manifested by the elevation of AST, ALT and LDH activities and the supplementation of a hypercholesterolemia- induced diet with bitter and sweet lupine seeds significantly lowered the plasma levels of AST, ALT and LDH activities.

Mansour *et al.*, (2002) found that a treatment of alloxan-induced diabetic rats with lupinus albus for 28 consecutive days could restore the activities of AST, ALT and LDH to their normal levels. A possible explanation for the effect of lupine seeds on the activities of AST, ALT and LDH in plasma and liver is that these seeds may inhibit the liver damage induced by hypercholesterolemia.

The liver is a central organ for many physiological and biochemical processes necessary for the maintenance of the life. Morphological alterations that occur in the liver affect many metabolic processes in the organism. Peroxide formation induced by hypercholesterolemia (Sudhahar *et al.*, 2007) results 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 (Noori *et al.*, 2009). With the increase in cellular membrane permeability, intracellular fluid transfers into intercellular space, resulting in muscle and liver cell degeneration.

Yusof *et al.*, (2013) reported that nutrient enriched soybean tempeh at 1000 mg/kg body weight was the most effective concentration that restrained the levels of AST and ALT activity compared to the ethanol control group.

In the present study, it was observed that as a result of hypercholesterolemia, enzymes such as AST and ALT were released into the blood. Their increase activities in the serum of these enzymes were directly proportional to the degree of cellular damage. These values significantly decreased with soybean and sweet lupine tempeh supplements which may prevent oxidative damage by detoxifying reactive oxygen species; thus reducing hypercholesterolemia.

Kidney functions

As shown in Table (4), significant increases were observed in serum uric acid and urea nitrogen levels of hypercholesterolemic rats (21.94% and 58.64%, $P < 0.05$, respectively) compared to the normal control group (Figure 5). Studies in a variety of animal models have shown that hypercholesterolemia accelerate the rate of progression of kidney disease (Abrass, 2004). A high-fat diet causes macrophage infiltration and foam cell formation in rats, leading to glomerulosclerosis (Hattori *et al.*, 1999). In humans more than a decade ago, a relationship between serum

cholesterol levels and glomerular filtration rate (GFR) decline was shown in 31 patients with type1 diabetes and established overt nephropathy (Mulec *et al.*, 1990). In those with a total cholesterol level >7 mmol/L, the rate of decline in GFR was at least three times higher than in those with a level <7 mmol/L.

However, feeding on a high cholesterol diets containing tempeh either from soybean tempeh or sweet lupine at lower or higher levels (3.5% or 7%) normalized values of serum uric acid compared to the positive control group, whereas no significant difference was found for serum uric acid among the normal control rats and soybean or sweet lupine tempeh-fed hypercholesterolemic rats (Table 4). Both levels of soybean tempeh were able to reduce (- 9.04% and - 13.51%, respectively) the elevated uric acid levels significantly ($P<0.05$) as could be seen in Figure (5).

On the other hand, feeding a high cholesterol diets containing 3.5% or 7% protein either from soybean or sweet lupine tempeh for 4 weeks resulted in a significant reduction in serum levels of urea nitrogen (-13.57%, -19.04%, -9.38% and

Table 4. Effect of feeding soybean and sweet lupine tempeh on the concentrations of serum uric acid and urea nitrogen (mg/dl) in hypercholesterolemic rats.

Groups	Kidney parameters (mg/dl)	
	Uric acid	Urea nitrogen
G1 Control (-ve) (14% protein from casein)	1.724±0.119 ^b	24.655±3.245 ^c
G2 Control (+ve) (14% protein from casein)	2.102±0.293 ^a	38.988±3.889 ^a
G3 (3.5% soy tempeh protein+ 10.5% protein from casein)	1.912±0.097 ^b	33.717±2.924 ^b
G4 (7% soy tempeh protein + 7% protein from casein)	1.818±0.111 ^b	31.588±4.269 ^b
G5 (3.5% lupine tempeh protein+ 10.5% protein from casein)	1.861±0.151 ^b	35.354±2.530 ^{ab}
G6 (7% lupine tempeh protein+ 7% protein from casein)	1.802±0.083 ^b	32.516±2.432 ^b

Values are the mean±Standard deviation (n=6).

Means within each column followed by different superscript letters are significantly different at $P<0.05$.

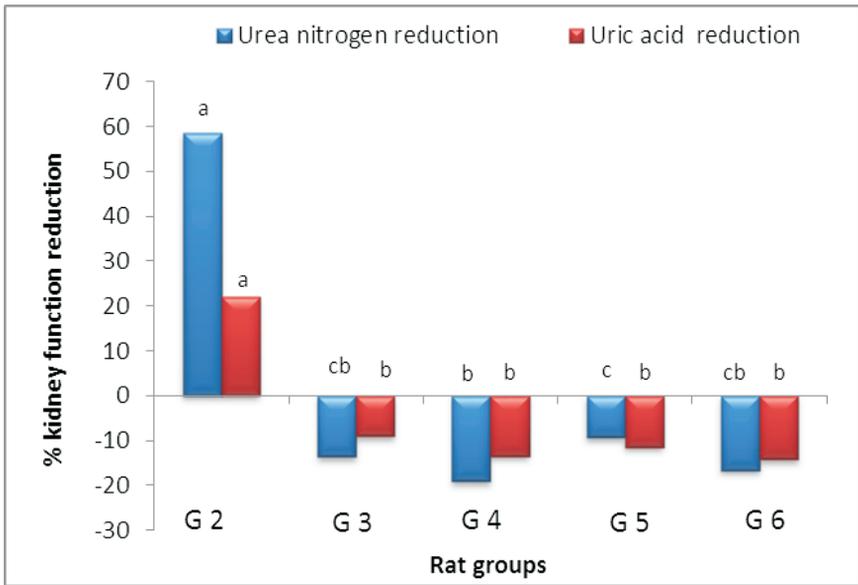


Fig. 5. Effect of soybean and sweet lupine tempeh on the reduction of serum uric acid and urea nitrogen in hypercholesterolemic rats. Means within each bar with different letters are significantly different at $P < 0.05$.

-16.61%, $P < 0.05$, respectively) compared to the positive control group, as could be seen in Figure (5). These results are in harmony with those of Astuti *et al.*, (2000) who found that feeding tempeh formula for hypercholesterolemic subjects decreasing uric acid concentrations for about 3.39-5.5%. It was found that dietary Gamma-aminobutyric acid from tempeh has been able to protect the filtration function of the kidneys from damage induced by high blood pressure (Nakamura *et al.*, 2000 and Aoki *et al.*, 2003). Furthermore, in healthy humans, substituting soy protein for animal protein decreases renal blood flow and glomerular filtration rate (Bosch *et al.*, 1983 and Viberti *et al.*, 1987). In humans with diabetic kidney disease (nephropathy), replacing animal protein with soy protein improves renal function and decreases protein loss (proteinuria) (Stephenson *et al.*, 2005 and Azadbakht *et al.*, 2003), thus substitution of soy protein for animal protein in diabetic individuals results in less hyperfiltration and glomerular hypertension resulting in protection from diabetic nephropathy (Anderson *et al.*, 1998).

Most soy protein products provide these components: soy protein with its unique amino acid profile, soy peptides, and isoflavones. Each of these components appears to have specific and unique effects on renal function. The amino acid profile of soy protein, differing from that of most animal proteins, may specifically affect renal blood flow and glomerular filtration rates. Soy peptides, with sizes ranging from four to 20 amino acids, have very important effects on vascular reactivity, blood

pressure (Imura *et al.*, 1993) and blood lipid values (Hori *et al.*, 2001). Thus, soy peptides may affect renal function in many different ways and may be the most active component of the soy protein package. However, soy isoflavones also have a wide range of activities that probably act synergistically with the soy peptides to mediate favorable effects on renal function (Stephenson *et al.*, 2005).

Histopathological examination

Histopathological observation was based on the liver tissue sections from different experimental groups which had been stained with haematoxylin and eosin staining and observed via a light microscope (Figure 6, A-F). Normal hepatocytes were observed in normal control group (control negative) (Figure 6 A). Histopathological data of the liver tissue sections of hypercholesterolemic rats showed marked structural alterations in the hepatocytes as compared to normal rats. The major alterations were marked vacuolar degeneration of hepatocytes, atrophied hepatocytes with dilated hepatic sinusoid and collagen fibers deposition in the portal vein associated with activation of epithelial lining of the bile duct (Figure 6 B, I-III). These alterations could be attributed to lipid accumulation in the hepatocyte cell cytoplasm.

Hypercholesterolemic rats fed on a high cholesterol diet containing 3.5% protein either from soybean or sweet lupine tempeh showed a moderate recovery effect of hepatocytes with vacuolar degeneration in some of hepatocytes to massive Number charges of fat vacuolar in the cytoplasm of hepatocytes, respectively, (Figure 6 C and D). While hypercholesterolemic rats fed on a high cholesterol diet containing 7% protein either from soybean or sweet lupine tempeh showed a better effect than the lower level (3.5%), since, the higher level (7%) of soybean tempeh protein was capable of recovering the damaged hepatocytes to almost their normal structure (Figure 6 D). However, the high level (7%) of sweet lupine tempeh protein showed a well improving effect with some vacuolar degeneration of sporadic hepatocytes (Figure 6 F).

The diet-induced hypercholesterolemia has often been used to modify the normal lipid profile in order to study hypercholesterolemia-related injuries in different body organ. Feeding of the experimental rodents with high cholesterol diet is reported to cause hypercholesterolemia and deposit cholesterol in liver (Lee *et al.*, 2007). Liver plays a central role in the balance and metabolism of cholesterol, which is derived from endogenous biosynthesis, chylomicron remnants and lipoprotein fractions and so liver is the primary organ to be affect from ingested excessive cholesterol and subsequent complication (Kumar *et al.*, 2006 and Amin and Abd El-Twab, 2009).

Hypercholesterolemia is a lipoprotein metabolic disorder characterized by high serum levels of low density lipoprotein and blood cholesterol. Alteration in

cholesterol and triglycerides metabolism as a result of hypercholesterolemia has been shown to negatively affect oxidative stress biomarkers and promotes production of reactive oxygen species (ROS) by various mechanisms, that leads to increased lipid peroxidation (Abuhashish *et al.*, 2013). It has been reported that hypercholesterolemia increased generation of oxygen free radicals, which contributed to the deleterious effects on the organ tissues, inducing blood vessels, liver and kidney (Scheuer *et al.*, 2000 and Zou *et al.*, 2003).

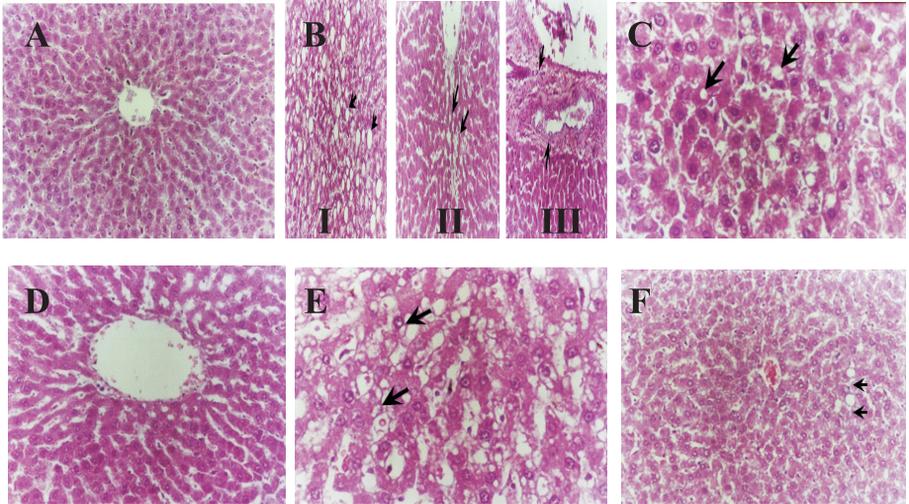


Fig. 6. Histopathological changes of liver tissue. Photomicrographs of liver tissue sections from normal group (A), hypercholesterolemic group (B, I-III) and hypercholesterolemic groups treated with 3.5% protein from soybean tempeh (C), 7% protein from soybean tempeh (D), 3.5% protein from sweet lupine tempeh (E) and 7% protein from sweet lupine tempeh (F), respectively. (Hx. and E. X 200).

Oxidative stress is derived from an imbalance between the generation of free radicals such as ROS and the endogenous antioxidant systems so that the latter is overwhelmed (Ahmed *et al.*, 2013). It has been reported to lead to increased lipid peroxidation, which in turn is involved in the aetiology of several chronic diseases (Kasdallah-Grissa *et al.*, 2008), 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 (Galle, 2001). Similarly, lipid peroxidation and oxidative stress are one of the most important pathological mechanisms that explain metabolic changes and hepatic injury following high cholesterol diet feeding (Kojima *et al.*, 2007). The rise of ROS could lead to the rise of serum biochemical markers such as AST, ALT, triglycerides and cholesterol.

Histopathological of liver tissue sections of hypercholesterolemic rats (Figure 6 B, I-III) showed a clear damage with association of fatty liver degeneration. While, groups of rats fed on a high cholesterol diet containing 7% protein either from

soybean or sweet lupine tempeh showed a well improving effect since, the two types of tempeh reduced the degenerative changes and were capable of recovering the damaged hepatocytes to almost their normal structure.

These results are in harmony with those obtained by Yusof *et al.*, (2013) who reported that nutrient enriched soybean tempeh was able to enhance hepatoprotective and antioxidant effects in vivo. The authors also found that treatment the mice with 1000 mg/kg of nutrient enriched soybean tempeh showed a recovery effect of hepatocytes from having a microvesicular steatosis. Some studies have shown that soybean has potential bioactive substances (i.e., isoflavones, folate, vitamin B12, dietary fiber, a beneficial fatty acid composition, soy protein and saponins) that exhibit hepatoprotective properties (Park *et al.*, 2004 and Shivashankara *et al.*, 2012).

Hodgson *et al.*, (1996) and Esaki *et al.*, (1996) revealed that soybean and the antioxidant compound extract from tempeh might inhibit the oxidative of lipoprotein in serum and may play an effective role in the protection of hepatic cells. Yang *et al.*, (2011) have recently reported that soy saponins-rich extract from soybean may able to improve on the acute alcohol-induced hepatotoxicity in mice. Another study was conducted by Fontanari *et al.*, (2012) have demonstrated a hepatoprotective effect of both lupine protein isolate and whole lupine seed in hypercholesterolemia, whereas, lupine protein isolate and whole lupine seed reduced the accumulation of fat in the hepatocytes of hamsters, even in the presence of hypercholesterolemic diet. This study revealed also that lupine protein isolate has potential bioactive substances (i.e., bioactive peptides) that exhibit hepatoprotective and hypocholesterolemic effects.

Matsuo (1997) reported that 3-hydroxyanthranilic acid (3-HAA), a byproduct of soy milk fermentation was found to be effective as α -tocopherol and was more effective than genistein in inhibiting lipid oxidation in vivo (Esaki *et al.*, 1996 and Thomas *et al.*, 1996). Other than (3-HAA), many components in soybean are antioxidative. Soy isoflavones, soy protein, and saponins all possess antioxidative abilities (Esaki *et al.*, 1998). Soybean tempeh was found to contain the highest amount of total isoflavones (daidzein and genestein) compared to other soy products (Haron *et al.*, 2009). The antioxidant activity of soybean tempeh might be attributed by various groups, namely free amino acids, peptides and phenolic compounds. These compounds are known to pose a wide spectrum of biochemical activities such as antioxidant, antimutagenic and anticarcinogenic which play an important role in human health (Tapiero *et al.*, 2002; Nakamura *et al.*, 2003 and Watanabe *et al.*, 2007).

Markus *et al.*, (1997) suggested that the antioxidant activity of tempeh had a synergistic effect with tocopherols (presented in soybeans) and amino acids (liberated during the fermentation). Furthermore, the aqueous extracts of natto

and tempeh also have strong antioxidant potential in vivo (Iwai *et al.*, 2002 and Sheih *et al.*, 2000). In addition, soybean protein could inhibit lipid peroxidation in plasma (Carrol, 1991).

The results in the present study indicated that the protective role of either soybean tempeh or sweet lupine tempeh against hypercholesterolemic-induced hepatic oxidative damage in rats might be due to the decrease of lipid peroxidation with their antioxidant properties.

Conclusion

Overall, feeding 7% protein either from soybean or sweet lupine tempeh to hyper-cholesterolemic rats demonstrated an effective amelioration against hypercholesterolemic-induced liver cell injury. These results also showed that the higher level (7% protein) from the two types of tempeh was more effective than the lower level (3.5%) in reverting the serum biochemical markers (AST and ALT), lipid profile (TC, TG, LDL-c, VLDL-c and HDL-c), kidney functions (uric acid and urea nitrogen), and histopathological conditions back to normal, consequently, the two types of tempeh exhibited hypocholesterolemic, anti-atherogenic and hepatoprotective effects. Thus, it could be concluded that a possible use of tempeh as dietary supplement might have greater significance in the treatment and prevention of hypercholesterolemia.

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