

Effect of Substituting Pumpkin Seed Protein Isolate for Casein on Serum Liver Enzymes, Lipid Profile and Antioxidant Enzymes in CCl₄-intoxicated Rats

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Abstract: The aim of the present study is to prepare pumpkin seed protein isolate (PSPI) by alkaline extraction and to study the effect of its substitution for casein on body weight, feed efficiency ratio, serum liver function enzymes, serum lipids profile and antioxidant enzymes in carbon tetrachloride (CCl₄) intoxicated rats. Forty two male rats were divided into 6 equal groups. One group of rats was fed on basal diet and kept as a negative control while the others were subcutaneously injected by CCl₄ twice at a dose of 1 ml /kg body weight for induction of acute liver toxicity. The first group of the intoxicated rats was left as a positive control while the others were fed on experimental diets in which casein was replaced with PSPI at 25, 50, 75 and 100% instead of casein. At the end of experimental period (4 weeks), the rats were sacrificed and blood samples were collected for biochemical analyses. Livers of the sacrificed rats were removed and prepared for histopathological examination. Results showed that substitution of PSPI for casein especially at 75 and 100% in CCl₄-intoxicated rats increased their feed intake and body weight gain. This substitution also decreased the levels of serum liver function enzymes, improved lipid profile and increased the activity levels of antioxidant enzymes in CCl₄-intoxicated rats. Histopathological examination revealed alleviation of hepatic lesions caused by CCl₄ by increasing the percentage of PSPI used. In conclusion, it was suggested that pumpkin seed protein isolate could protect the liver cells from CCl₄-induced liver damages perhaps, by its antioxidative effect on hepatocytes, hence eliminating the deleterious effects of toxic metabolites from CCl₄. So the present study recommended that the use of pumpkin seed protein isolate may be useful for patients suffering from liver diseases due to its hepatoprotective and hypolipidemic activities.

Key words: Pumpkin seeds • Protein isolate • Body weight • Liver enzymes • Lipid profile • Antioxidant enzymes • Histopathology • CCl₄ • Rats

INTRODUCTION

Pumpkin (*Cucurbita pepo* L) is a squash like fruit with usually orange or yellow color. Pumpkins vary in their uses for cooking, from the fleshy shell, to the seeds and it has been used for centuries in Latin America, Africa and India in traditional medicine. Pumpkin seed appear to be non-toxic and a good source of a range of nutrients [1, 2].

Pumpkin seed is a rich natural source of proteins and phytosterols [3,4]. Evaluation of the nutritional value of pumpkin seeds revealed that it contained 45.4% crude oil, 32.3% crude protein, 12.1% crude fiber and 4.65% ash while the defatted flour of the pumpkin seeds contained 55.4% crude protein. The seeds were also

found to have considerable amounts of essential minerals such as Zn, K, Ca, Mg, Fe, Cu and P [5]. On the other hand, on a dry weight basis pumpkin seeds contained 58.8% protein and it represents a useful source of many nutrients essential to humans especially in rural areas of Africa [6]. In addition fortification of bread with pumpkin seeds resulted in an increase of protein, lysine and mineral content compared to the control. Moreover, *in vitro* protein digestibility improved when the pumpkin seed protein isolate was added [7].

Pumpkin seed protein isolate has promising antioxidant properties and is effective in alleviating the detrimental effects associated with protein malnutrition and acetaminophen intoxication [8,9].

The present work aimed to prepare pumpkin seed protein isolate and to study the effects of substituting different percentages of pumpkin seed protein isolate with casein on some biological parameters, serum liver function enzymes, serum lipids profile and antioxidant enzymes in CCl₄-intoxicated rats.

MATERIALS AND METHODS

Materials

Pumpkin Seeds: was purchased from a local company for medicinal plants and herbs, Cairo, Egypt. The dry seeds were authenticated in the Botany Department, Agricultural Research Center, Giza, Egypt.

Chemicals: 10% liquid solution of Carbon tetrachloride (CCl₄), chemicals and other kits used in the experiment were obtained from El-Gomhuryia Company for Chemical Industries, Cairo, Egypt.

Experimental Animals: Forty two adult male albino rats of Sprague Dawley strain weighing (150-165 g) were obtained from Laboratory Animal Colony, Helwan, Egypt. Rats were placed in wire bottom cages under hygienic conditions and were allowed access to the experimental diets and water *ad libitum*. Rats were observed, fed daily and weighed twice a week.

Methods

Preparation of Pumpkin Seed Protein Isolate (PSPI): Pumpkin seeds protein isolate was prepared by alkaline extraction and subsequent isoelectric precipitation [10]. The grounded pumpkin seeds (without hulls) were defatted with hexane. Defatted pumpkin seed was dispersed in 5 volumes of 1 M NaCl solution, stirred for 15 min. at ambient temperature followed by adjusting to pH 9.5 using 1 M NaOH and stirring for 30 min. After extraction, the suspension was centrifuged and supernatant was filtered through glass wool to remove insoluble material, adjusted to pH 4.0 with 1 N HCl to precipitate the protein. The precipitate was dispersed in 50% ethyl alcohol (1:5), stirred for 30 min. and centrifuged for 20 min. at ambient temperature. The precipitate was washed several times with distilled water and was air dried. The dispersed product denoted as pumpkin seed protein isolate.

Experiment Design: Animals were kept for one week before starting of the experiment for acclimatization. Then animals were divided into six groups of seven rats in each.

The first group was fed on the basal diet [11] and served as a negative control (-Ve). The rest five groups were given carbon tetrachloride (CCl₄) for induction of acute liver damage. CCl₄ was diluted in an equal volume of paraffin oil as a vehicle and subcutaneously injected in the first and the second day of the experiment in a dose of 1 ml/kg body weight [12]. The first hepatotoxic group was fed basal diet and kept as a positive control (+ Ve) while the other hepatotoxic groups were fed on basal diets that substitute 25, 50, 75 and 100% PSPI for casein.

Feed intake (FI) and body weight were recorded daily and body weight gain (BWG) and feed efficiency ratio (FER) were calculated at the end of the experimental period according to the following equations:

$$\text{BWG (g)} = \text{final weight (g)} - \text{initial weight (g)}$$

$$\text{FER} = \text{body weight gain (g)} / \text{feed intake (g)}$$

At the end of experiment period (4 weeks), rats were anaesthetized by ether and blood samples were collected from the portal vein into dry centrifuge tubes and were centrifuged for 20 minutes at 3000 rpm. to separate the sera which were kept at -10°C till biochemical analysis. Livers of the sacrificed rats were removed for histopathological examination.

Biochemical Analyses: Total polyphenol content of pumpkin seed protein isolate (Gallic acid equivalent) was determined [13].

The collected serum samples were used for estimating aspartate amino transferases (AST), alanine amino transferases (ALT) [14], and alkaline phosphatase enzymes (ALP) [15].

Serum total cholesterol (TC) [16], triglycerides (TG) [17] and high density lipoprotein (HDL-c) [18] were determined calorimetrically. Low density lipoprotein cholesterol (LDL-c) and very low density lipoprotein cholesterol (VLDL-c) were calculated mathematically according to Friedwald's equations [19]

$$\text{LDL-c} = \text{TC} - [\text{HDL-c} + (\text{TG}/5)]$$

$$\text{VLDL-c} = \text{Triglycerides} / 5.$$

The activity of serum glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT) were determined [20-22] respectively.

Histopathological Studies of the Liver: Livers of the sacrificed rats were dissected, removed, washed with normal saline and put in 10% formalin solution. The fixed

specimens were then trimmed, washed and dehydrated in ascending grades of alcohol. The tissue specimens were cleared in xylene, embedded in paraffin, sectioned at 4-6 microns thickness, stained with Hematoxylen and Eosin (H and E) and then studied under an electronic microscope [23].

Statistical Analysis: Results were expressed as means±SD. Statistical analysis was carried out using computerized SPSS program (version 11.0, Chicago, IL, USA) with one way ANOVA. The differences among means were tested for significance using Duncan post hoc test at $P < 0.05$ [24].

RESULTS

The pumpkin seed protein isolate showed a total polyphenolic content of (2.49±0.049 mg/g). This value is means of triplicate analyses.

The effect of PSPI on FI, BWG and FER of CCl₄-intoxicated rats is shown in Table 1. Substitution of pumpkin seed protein isolate for casein at 75 and 100% in the diet after CCl₄ intoxication significantly increased FI in CCl₄-intoxicated rats compared to negative control group. The body weight gain indicated that the CCl₄-treated group had a lower weight gain as compared to the negative control group. The body weight gain observed in the 50, 75 and 100% PSPI fed groups, however, being more significantly pronounced than the CCl₄-treated control group. FER was not differing by the substitution of pumpkin seed protein isolate for casein.

Our results using the model of CCl₄-induced hepatotoxicity in rats demonstrated that substitution of pumpkin seed protein isolate for casein at 75 and 100% caused significant inhibition of ALT and AST levels in serum compared to the positive control group. In addition, substitution of pumpkin seed protein isolate for casein especially at all percentages used caused significant inhibition of ALP level as shown in Table 2.

It is clear from Table 3 that administration of CCl₄ caused significant elevation in serum lipids parameters compared to negative control group. CCl₄-intoxicated rats fed with pumpkin seed protein isolate which substitute 25, 50, 75 and 100% for casein showed significant decreases in serum levels of total cholesterol and triglycerides in comparison to positive control group. Substitution of pumpkin seed protein isolate for casein at 25, 50, 75 and 100% in the diet of CCl₄-intoxicated rats caused a significant decrease in the serum level of LDL-c, while there were no significant changes in levels of HDL-c in the serum, compared to the positive control group as recorded in Table 4.

Table 5 shows that CCl₄ injected rats had significantly lower levels of GPX, SOD and CAT antioxidant enzymes activity compared to negative control group. Substitution of pumpkin seed protein isolate for casein at 25, 50, 75 and 100% in the diet of CCl₄-intoxicated rats increased the activity levels of GPX, SOD and CAT antioxidant enzymes.

Histopathological examination of livers of the negative control rats fed on basal diet revealed normal histological picture of hepatic lobule which consists of

Table 1: Effect of substituting pumpkin seed protein isolate (PSPI) for casein on food intake (FI), body weight gain (BWG) and feed efficiency ratio (FER) in CCl₄-intoxicated rats.

Groups	FI(g/ day)	BWG(g)	FER
Control-ve	11.23±0.87 ^c	17.00±0.98 ^c	1.52±0.10 ^b
Control+ve	8.01±0.98 ^a	9.33±0.58 ^a	1.10±0.16 ^a
PSPI 25%	8.67±0.57 ^a	9.00±0.98 ^a	1.05±0.19 ^a
PSPI 50%	9.33±0.58 ^{ab}	11.01±0.98 ^b	1.18±0.13 ^a
PSPI 75%	10.50±0.71 ^{bc}	12.50±0.71 ^b	1.19±0.09 ^a
PSPI 100%	10.66±1.15 ^{bc}	11.75±0.25 ^b	1.11±0.10 ^a

Values are mean±SD. Values in the same column sharing the same superscript letters are not statistically significantly different.

Table 2: Effect of substituting pumpkin seed protein isolate (PSPI) for casein on liver function enzymes in CCl₄-intoxicated rats.

Groups	AST(U/L)	ALT(U/L)	ALP(U/L)
Control-ve	57.5±4.81 ^a	28.5±3.64 ^a	92.98±1.42 ^a
Control + ve	99.4±3.20 ^c	59.5±2.27 ^c	126.95±2.68 ^c
PSPI 25%	86.6±2.42 ^c	48.5±3.32 ^c	112.97±3.87 ^b
PSPI 50%	85.3±2.33 ^c	47.7±5.61 ^c	110.90±1.26 ^b
PSPI 75%	80.9±3.81 ^b	44.1±3.95 ^b	108.16±4.34 ^b
PSPI 100%	78.5±2.81 ^b	40.1±3.91 ^b	106.00±2.55 ^b

Values are mean±SD. Values in the same column sharing the same superscript letters are not statistically significantly different.

Table 3: Effect of substituting pumpkin seed protein isolate (PSPI) for casein on serum total cholesterol and triglycerides in CCl₄-intoxicated rats.

Groups	Total cholesterol (mg/dL)	Triglycerides (mg/dL)
Control-ve	90.98±2.49 ^a	53.33±2.56 ^b
Control + ve	109.96±2.61 ^d	62.62±1.99 ^c
25% PSPI	102.97±1.89 ^c	52.60±2.41 ^b
50% PSPI	101.90±3.23 ^c	50.50±1.29 ^b
75% PSPI	98.16±1.96 ^c	49.30±1.47 ^b
100% PSPI	96.00±2.58 ^b	48.00±1.38 ^a

Values are mean±SD. Values in the same column sharing the same superscript letters are not statistically significantly different.

Table 4: Effect of substituting pumpkin seed protein isolate (PSPI) for casein on lipoprotein fractions in CCl₄-intoxicated rats.

Groups	HDL-c (mg/dL)	LDL-c (mg/dL)	VLDL-c (mg/dL)
Control-ve	63.96±1.12 ^a	16.35±0.89 ^a	10.67±0.51 ^b
Control + ve	75.69±2.23 ^b	20.95±0.98 ^b	12.52±0.40 ^c
25% PSPI	74.75±1.31 ^b	17.70±0.10 ^b	10.52±0.48 ^b
50%PSPI	73.80±1.39 ^b	16.50±1.58 ^a	9.90±0.26 ^b
75%PSPI	70.70±1.10 ^b	16.80±0.57 ^a	10.66±0.29 ^b
100%PSPI	70.10±2.06 ^b	16.40±0.24 ^a	9.80±0.28 ^a

Values are mean±SD. Values in the same column sharing the same superscript letters are not statistically significantly different.

Table 5: Effect of substituting pumpkin seed protein isolate (PSPI) for casein of serum antioxidant enzymes activity in CCl₄-intoxicated rats.

	GPX(n mole)	SOD(U/ml)	CAT(n mole)
Control-ve	20.60±1.81 ^c	95.70±3.6 ^c	68.53±3.91 ^c
Control + ve	6.25±0.30 ^a	55.5±3.2 ^a	35.41±2.60 ^a
25% PSPI	8.80±0.86 ^b	63.72±5.3 ^b	42.70±3.81 ^b
50% PSPI	9.50±0.91 ^b	80.50±7.3 ^b	48.63±4.10 ^b
75% PSPI	10.00±0.73 ^b	83.60±6.2 ^b	50.52±2.52 ^b
100% PSPI	12.00±0.94 ^b	85.61±4.2 ^b	54.50±2.91 ^b

Values are mean±SD. Values in the same column sharing the same superscript letters are not statistically significantly different.

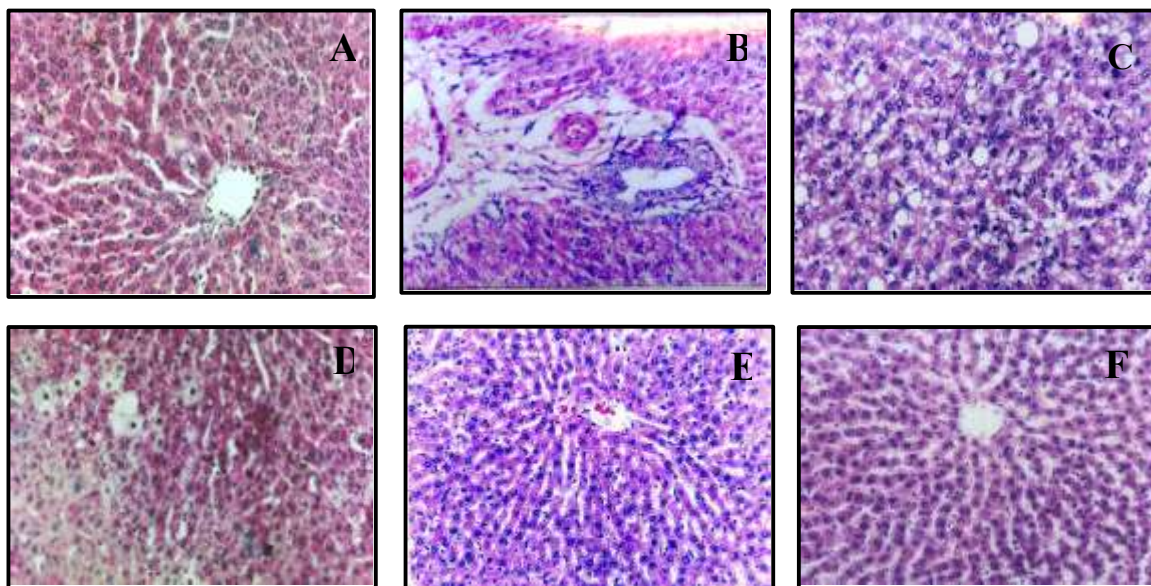


Fig. 1: Histopathological changes detected in the liver of (A) control-ve, (B) control + ve, (C) 25% PSPI, (D) 50% PSPI, (E) 75% PSPI and (F) 100% PSPI. (H and E X 100)

central vein surrounded by normal hepatocytes as shown in Fig. (1-A). Examination of liver of the CCl₄-intoxicated positive control rats showed severe fatty degeneration of hepatocytes and infiltration of leucocytes in hepatic sinusoid (Fig. 1-B). Livers of CCl₄-intoxicated rats fed on diet containing 25% pumpkin seed protein isolate showed little vacuolar degeneration of hepatocytes as shown in Fig. (1-C) while livers of CCl₄-intoxicated rats fed on diet containing either 50,75 or 100% pumpkin seed protein isolate showed almost normal histology of the hepatic lobule (Fig. 1-D,E,F). The higher the percentage of PSPI the higher the improvement in liver histopathology.

DISCUSSION

Recent interest in food phenolics has increased greatly, owing to their antioxidant capacity (free radical scavenging and metal chelating activities) and their possible beneficial implications in human health. The polyphenol content in PSPI measured in the present study may indicate antioxidative activity of PSPI and is confirmed by two studies. The first one stated that pumpkin seed cake contain (2.61 g/kg) phenolic compounds[25] while the second one found that pumpkin seed protein isolate exhibited a polyphenol content of (2.30 mg/g) [9].

The liver, the key organ involved in numerous metabolic functions and detoxification of hazardous substances, is a frequent target of a number of toxicants [26]. There is no doubt that reactive oxygen species play an important role in pathological changes in the liver, particularly in the cases of alcoholic and toxic liver diseases [27]. It is now generally accepted that the hepatotoxicity of CCl₄ is the result of reductive dehalogenation, which is catalyzed by P-450 enzyme system and which forms highly reactive trichloromethyl free radical. This readily interacts with molecular oxygen to form the trichloromethyl peroxy radical. Both trichloromethyl and its peroxy radical are capable of binding to proteins or lipids, or of abstracting a hydrogen atom from an unsaturated lipid, initiating lipid peroxidation and liver damage and by doing so playing a significant role in pathogenesis of diseases [28].

The body weight decrease as a result of CCl₄ injection was considered to be the result of direct toxicity of CCl₄ and/or indirect toxicity related to the liver damage. Changes in the body weight after CCl₄ dosing have been used as a valuable index of CCl₄-related organ damage [29,30]. on the other hand, no available literature could be found concerning the effect of PSPI on FI, BWG and FER.

Assessment of liver can be made by estimating the activities of serum ALT, AST and ALP which are enzymes originally present higher concentration in cytoplasm. When there is hepatopathy, these enzymes leak into the blood stream in conformity with the extent of liver damage [8]. The elevated level of these entire marker enzymes observed in the positive control group corresponded to the extensive liver damage induced by toxin. These results are in agreement with previous finding that the activity levels of serum ALT and AST were significantly elevated in rats after CC14 administration [8, 31,32]. The reduced concentrations of ALT and AST as a result of PSPI administration observed during the present study might probably be due in part to the presence of polyphenol. The tendency of these marker enzymes to return towards a near normalcy in PSPI fed groups point towards an early improvement in the secretory mechanism of the hepatic cell and is a clear manifestation of anti-hepatotoxic effect of PSPI. This effect was similar to that reported in another study that pumpkin seed protein isolate was observed to lower effectively the increased levels of ALT,AST and ALP in low protein fed CCl₄ intoxication rats in comparison with their counterparts [8].

In accordance with the present study, CCl₄-intoxicated rats exhibited significant higher levels of TC and TG [33]. This perhaps due to the presence of damage in the liver. On the other hand, no available literature could be found concerning the effect of pumpkin seed protein isolate on serum lipids but the observed improvement in the levels of TC, TG, LDL-C and VLDL-C is probably indicative of hepato-protective effect of PSPI in CC14 injected rats.

Biological systems protect themselves against the damaging effects of activated species by several means. These include free radical scavengers and chain reaction terminators; enzymes that found in blood and liver such as SOD, CAT and GPX system [34]. Inhibition of these protective mechanisms results in enhanced sensitivity to free radical induced cellular damage. The noticed decrease in the activity of these enzymes in positive control group is in agreement with other finding [35, 36]. The observed increase of SOD activity suggests that the substitution of PSPI in different percentages for casein had an efficient protective mechanism in response to reactive oxygen species derived from the peroxidative process in the liver of CCl₄ injected rats. The reported antioxidant activity of PSPI was in accord with that found in other study [36] that pumpkin seed protein isolate has promising antioxidant properties and is effective in alleviating the detrimental effects associated with protein malnutrition and acetaminophen intoxication.

From these results, it was suggested that pumpkin seed protein isolate could protect the liver cells from CCl₄-induced liver damages perhaps, by its antioxidative effect on hepatocytes, hence eliminating the deleterious effects of toxic metabolites from CCl₄. So the present study recommended that the use of pumpkin seed protein isolate may be useful for patients suffering from liver diseases due to its hepatoprotective and hypolipidemic activities. Further studies are required in this field.

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