

**EFFECT OF PUMPKIN SEED (*Cucurbita pepo*) PROTEIN
ISOLATE ON THE ANTIOXIDANT ENZYMES IN CCl₄-INDUCED
LIVER INJURY IN LOW-PROTEIN FED RATS.**

BY

Cynthia Zanele Nkosi

B.Sc. (Hons.) (University of Zululand)

Dissertation presented in fulfillment of the requirements for the degree

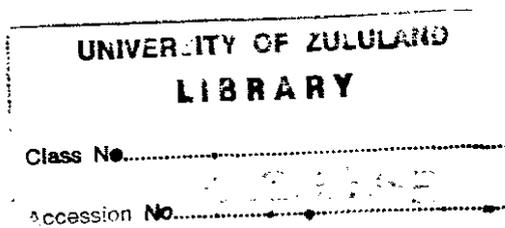
Magister Scientiae (Biochemistry)

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UNIVERSITY OF ZULULAND

Supervisor: Prof. A. R. Opoku

Co-Supervisor: Prof. S. E. Terblanche



(i)

Declaration

I the undersigned Cynthia Zanele Nkosi hereby declare that the work contained in this dissertation is my own original work and has not been previously submitted at any University for a degree.

Signature

Date

(ii)

Dedicated to

In Memory of my Father

Johannes Mandla Nkosi

(iii)

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Section 1

Effect of Pumpkin Seed (*Cucurbita pepo*) Protein Isolate on The Activity Levels of Certain Plasma Enzymes in CCl₄-Induced Liver Injury in Low-Protein Fed Rats

1.1 ABSTRACT

The effect of pumpkin seed (*Cucurbita pepo*) protein isolate on the activity levels of lactate dehydrogenase (LD), alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) against carbon tetrachloride (CCl₄)-induced acute liver injury in low-protein fed rats were investigated.

A group of male Sprague-Dawley rats which were maintained on a low-protein diet for five days were divided into three subgroups. Two subgroups were injected with carbon tetrachloride and the other group with an equivalent amount of olive oil. Two hours after CCl₄ intoxication one of the two subgroups was administered with pumpkin seed protein isolate. All three subgroups of rats were maintained on the low- protein diet for the duration of the investigation. Groups of rats from the different subgroups were sacrificed at 24, 48 and 72 hours after their respective treatments. After five days on the low-protein diet the activity levels of all four enzymes were significantly higher than their counterparts on a normal balanced diet. CCl₄ intoxication resulted in significant increases in the activity levels of all four enzymes investigated. The administration of pumpkin seed protein isolate after CCl₄ intoxication resulted in significantly reduced

activity levels of all four enzymes. It is concluded that pumpkin seed protein isolate administration was effective in alleviating the detrimental effects associated with protein malnutrition.

1.2 INTRODUCTION

Protein energy malnutrition (PEM) is defined as a wasting condition resulting from inadequate intake of energy. The causative factor is therefore nutritional, mainly protein deficiency. Children are more vulnerable to the detrimental effects of malnutrition because of their special food requirements. About 60% of deaths in children under the age of 5 in developing countries are thought to be related to malnutrition (UNICEF, 1989). In adults, PEM occurs as a result of partial or total starvation.

The causative factor of PEM has initially been attributed to nutritional deficiency (of protein) (Williams, 1935; Williams *et al.*, 1935), but recent advances in the understanding of the disease seem to suggest that PEM may be more complex than just unavailability of rich protein diet, and adaptation failure (Gopalan, 1993; Gopalan, 2001), genetic deficiency of transaminases (Phadke *et al.*, 1995) and the built up of free radicals (Golden and Ramdath, 1987) have been suggested.

If the built up of free radicals is indeed the cause of PEM then the addition of oil to the diet of PEM children in order to boost their energy level as recommended by UNICEF (1989) could be inappropriate.

In severely malnourished individuals a marked decrease in whole body protein turnover occurs as the body adapts to a chronic reduction in protein intake by increasing the efficiency of nitrogen utilization to minimize loss (Golden *et al.*, 1977). Thus body tissues and organs, especially liver, degenerates and the activity levels of enzymes adjust to match their needs. Changes in plasma activity levels (usually increased) of lactate dehydrogenase (LD), alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) are commonly used in diagnosis (Smith *et al.*, 1998). Increased activity levels of serum alanine transaminase (ALT) and aspartate transaminase (AST) (indices of liver damage) have been observed in malnourished children (McLean, 1962).

The liver plays a major role in the digestion, metabolism and storage of nutrients. Carbon tetrachloride (CCl₄), a widely used hepatotoxin for evaluating hepatoprotective agents, induces liver damage by producing free radical intermediates (malondialdehyde and 4-hydroxy-2-nonenal) (Mitra *et al.*, 1998). CCl₄ can damage a number of tissues particularly the liver and kidney of many species (Drill, 1952). Administration of CCl₄ can cause cirrhosis (Cameron and Karunaratne, 1936) and ultimately lead to hepatic carcinoma (Reuber and Glover, 1970). CCl₄ by itself does not have toxic effects on the liver, but its metabolic products are responsible for the toxicity (Recknagel and Glende, 1973).

PEM induced liver damage is often alleviated by the feeding of high quality protein (animal protein) to the patient. Vegetable proteins are quickly replacing animal protein in combating PEM (Bianchi *et al.*, 1993).

There are a number of oil-seeds that are sources of vegetable proteins; soybeans are one of the lower cost sources and have the advantage of already developed technologies for processing (Schwarz, 1988). The soybean proteins have a good balance of essential amino acids except for methionine, which is the first limiting amino acid. Recent tests involving adult humans indicate that the quality of soy protein isolate is high and comparable with that of high quality animal proteins, such as milk and beef (Schwarz, 1988). Other seeds [e.g. sunflower seeds (Sodini and Canella, 1977); cotton seeds (Saeed and Cheryan, 1988) and other vegetable sources have high quality protein and can probably be considered as potential alternatives for meat proteins.

The cytological features of pumpkin seeds are to a large extent almost similar to those of typical oilseeds. The bulk of the cytoplasm consists of two organelles: spherosomes (the sites of lipid storage) and protein bodies (the sites of storage protein). Embedded within the protein bodies are globoids that contain metallic salts of phytic acid and crystalloids that contain storage globulin. Albumins and globulins constitute the protein portion. Like other oilseeds, pumpkin proteins are rich in arginine, aspartic acid and glutamic acid and are deficient in lysine and amino acids containing sulfur. The nutritional value of the protein is also very much similar in magnitude to other oilseeds (Bates *et al.*, 1983).

In view of the above-mentioned observations it was decided to ascertain the effects of pumpkin seed (*Cucurbita pepo*) protein isolate on the plasma activity levels of lactate dehydrogenase (LD), alanine transaminase (ALT), aspartate

transaminase (AST), and alkaline phosphatase (ALP) in CCl₄-induced liver injury in low-protein fed male Sprague-Dawley rats (*Rattus norvegicus*).

1.3 MATERIALS AND METHODS

1.3.1 Plant materials. Fresh flat white "Boer Ford" pumpkin seeds (*Cucurbita pepo*) were obtained from McDonalds Seeds, Pietermaritzburg, South Africa. The seeds were dehusked and dried at 50°C for 48 h in a thermostatically controlled oven. The dried dehusked seeds were then powdered (mesh size 2mm). The resulting powder was defatted with hexane, air dried and kept for further processing.

1.3.2 Preparation of protein isolate. The dried defatted powder was suspended in distilled water (adjusted to pH 10) and the slurry filtered to remove the leaf debris. The filtrate was centrifuged and the pH of the resulting supernatant adjusted to 5 (determined during preliminary studies). After centrifugation the residue was freeze-dried (Sodini and Canella, 1977).

1.3.3 Treatment of animals. Fifty-two male Sprague-Dawley rats *Rattus norvegicus* (3-4 weeks old; weighing between 80-90 g) were obtained from the animal facilities of the Department of Biochemistry and Microbiology, University of Zululand. All the animals (except four which were fed on a normal balanced

rat diet) were fed a low-protein diet (maize meal, 2mm mesh size and pelleted) for five days. After five days the four rats on a balanced diet (Normal group) and four of the rats on the low-protein diet (Malnutrition group) were sacrificed. The remaining 44 rats were divided into 3 groups. The rats in group 1 were intraperitoneally injected with 1.0ml/kg olive oil (Olive oil group) whereas groups 2 and 3 were subcutaneously injected with 1.0ml/kg body weight CCl_4 (dissolves in olive oil, 1:1) (Bruckner *et al.*, 1986) (group 2 designated as the CCl_4 Group) (Ohta *et al.*, 1998). Two hours after the CCl_4 intoxication the group 3 rats were administered 1ml/kg body weight of the pumpkin seed protein isolate in saline (20g/100ml) by stomach tube (Pumpkin seed protein isolate group) (Ohta *et al.*, 1998). At the same time the animals in groups 1 and 2 received the same volume of physiological saline. All the rats had access to water and food *ad libitum*. The rats in groups 1 and 2 were maintained on the low-protein diet had access to water and food (maize meal) *ad libitum* whereas the rats in group 3 were switched to a 1:5 pumpkin seed protein isolate:maize meal (w/w) diet.

Four rats each of the CCl_4 group and the Olive oil group were sacrificed two hours after CCl_4 intoxication and thereafter four rats of each of the three groups at 24, 48 and 72 h after the CCl_4 intoxication. The rats were anaesthetized with pentobarbital sodium (6mg/100g body mass) injected intraperitoneally and blood withdrawn from the abdominal aorta into K_2EDTA tubes. The blood was centrifuged (2000 rpm at $4\pm 1^\circ\text{C}$ for 10 min) to obtain plasma.

1.3.4 Analytical methods. Plasma samples were used to determine the activity levels of lactate dehydrogenase (LD) (Vassault, 1983), alanine transaminase (ALT) (Hørder and Rej, 1983), aspartate transaminase (AST) (Rej and Hørder, 1983) and alkaline phosphatase (ALP) was determined using commercial test kits comprising 1 mol/l diethanolamine pH 9.8, 0.5 mmol/l magnesiumchloride and 10 mmol/l of the substrate *p*- nitrophenylphosphate.

Enzyme assays were performed under conditions in which the reaction rate was proportional to the plasma concentration at 30°C in keeping with the recommendations of the Commission on Enzymes of the International Union of Biochemistry.

The enzyme activities are expressed as means \pm SD in U.ml⁻¹ at 30°C.

1.3.5 Statistical analysis. All data are expressed as the Mean \pm SD. The results were analyzed statistically using two-factor ANOVA with Bonferroni's post hoc tests, ($P < 0.05$ was regarded as significant).

1.4 RESULTS

In a preliminary study in our laboratories the pumpkin seed protein isolate was observed to effectively lower the increased levels of LD, ALT, AST and ALP in low-protein fed rats

1.4.1 Lactate dehydrogenase (LD). The activity levels of LD for the different treatments are presented in Fig. 1.

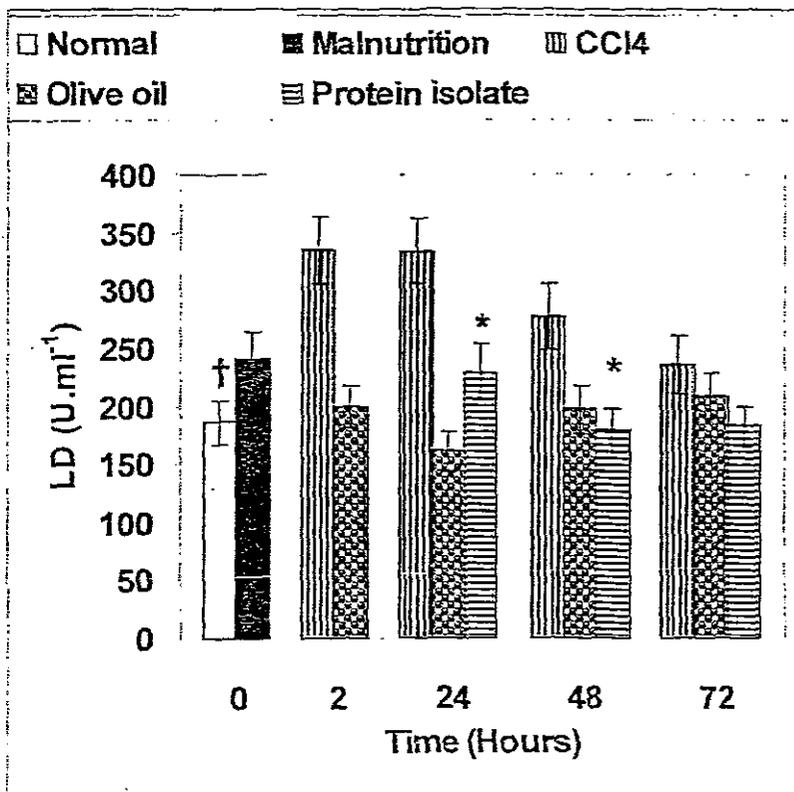


Figure 1. The effect of pumpkin seed protein isolate on plasma LD activity levels (U.ml⁻¹) of malnourished rats treated with olive oil followed after 2 hrs with CCl₄ and CCl₄+pumpkin seed protein isolate, after 24, 48 and 72 hrs. Values are means±SD for 4 rats. †Normal significantly lower than Malnutrition, P<0.05. *Pumpkin seed protein isolate significantly lower than CCl₄, P<0.05.

A low-protein diet as well as CCl_4 administration resulted in significantly elevated levels of plasma LD ($P < 0.05$). Pumpkin seed protein isolate administration resulted in significantly ($P < 0.05$) reduced levels of the enzyme at all intervals investigated. Pumpkin seed protein isolate treatment resulted in a complete neutralization of the detrimental effects of CCl_4 48 h after treatment.

1.4.2 Alanine transaminase (ALT). The activity levels of plasma ALT as affected by the different treatments are furnished in Fig. 2.

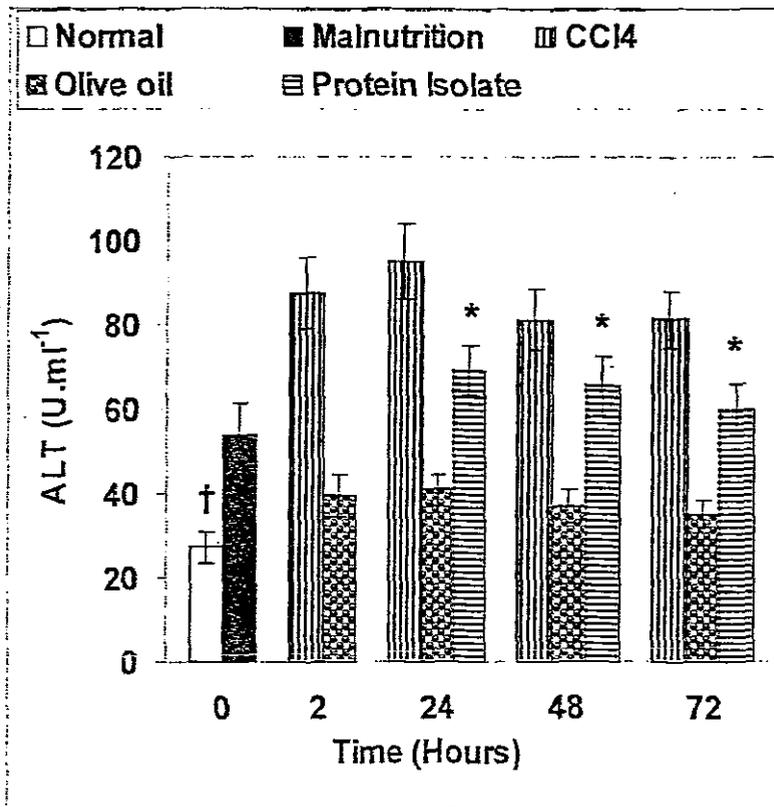


Figure 2. The effect of pumpkin seed protein isolate on plasma ALT activity levels (U.ml^{-1}) of malnourished rats treated with olive oil followed after 2 hrs with CCl_4 and CCl_4 +pumpkin seed protein isolate, after 24, 48 and 72 hrs. Values are means \pm SD for 4 rats. [†]Normal significantly lower than Malnutrition, $P < 0.05$. *Pumpkin seed protein isolate significantly lower than CCl_4 , $P < 0.05$.

The animals on the low-protein diet exhibited significantly higher ($P<0.05$) levels of ALT. Pumpkin seed protein isolate caused significantly decreased ($P<0.05$) levels of plasma ALT at all the intervals investigated in comparison with their CCl_4 treated counterparts. Even after 72 h the pumpkin seed protein isolate treated animals showed a progressive lowering of ALT activity levels in comparison with the animals on a balanced rat diet.

1.4.3 Aspartate transaminase (AST). The plasma AST activity levels are given in Fig. 3.

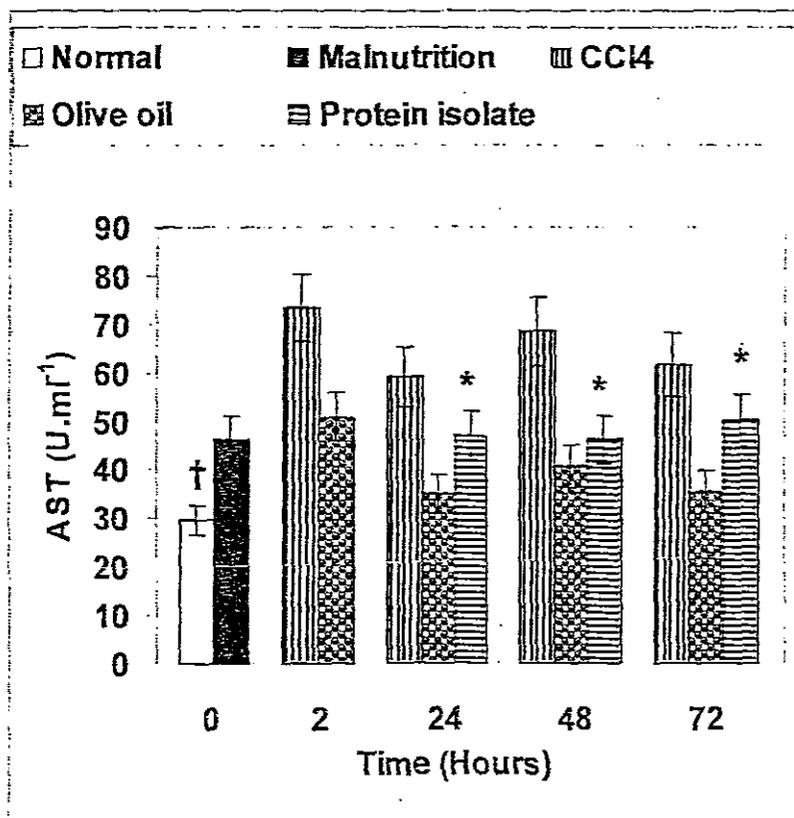


Figure 3. The effect of pumpkin seed protein isolate on plasma AST activity levels (U.ml^{-1}) of malnourished rats treated with olive oil followed after 2 hrs with CCl_4 and CCl_4 +pumpkin seed protein isolate, after 24, 48 and 72 hrs. Values are means \pm SD for 4 rats. †Normal significantly lower than Malnutrition, $P<0.05$. *Pumpkin seed protein isolate significantly lower than CCl_4 , $P<0.05$.

The results are to a large extent similar to those obtained for ALT (Fig. 2).

The plasma AST activity levels were significantly elevated ($P < 0.05$) as a result of the low-protein diet. Pumpkin seed protein isolate administration resulted in significantly reduced ($P < 0.05$) plasma AST activity levels even though these observed levels were almost still as high as the low-protein fed rats. The AST activity levels of the plasma of the olive oil treated animals were slightly elevated in comparison with the animals on a balanced diet.

1.4.4 Alkaline phosphatase (ALP). The plasma ALP activity levels of the different treatments are illustrated in Fig. 4.

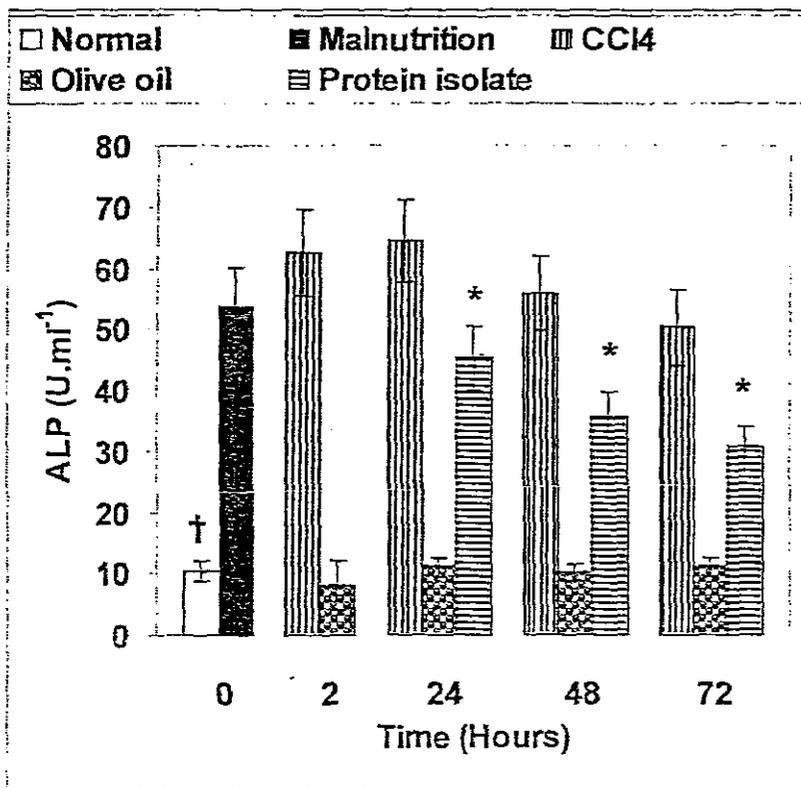


Figure 4. The effect of pumpkin seed protein isolate on plasma ALP activity levels (U.ml⁻¹) of malnourished rats treated with olive oil followed after 2 hrs with

CCl₄ and CCl₄+pumpkin seed protein isolate, after 24, 48 and 72 hrs. Values are means±SD for 4 rats. †Normal significantly lower than Malnutrition, $P<0.05$.

*Pumpkin seed protein isolate significantly lower than CCl₄, $P<0.05$.

The plasma activity levels of the enzyme ALP were dramatically affected by a low-protein diet as well as CCl₄ treatment. Both treatments resulted in significantly elevated ($P<0.05$) activity levels of the enzyme. Pumpkin seed protein isolate caused significant reductions ($P<0.05$) in the plasma ALP activity levels at all time intervals investigated.

1.5 DISCUSSION

In this study pumpkin seed protein isolate has been evaluated for its protective effect in CCl₄ induced hepatic damage. There were no observable changes in the physical appearance of the rats during the 4-day experiment. The CCl₄ injected rats were, however, inactive compared to the other treatment rats.

The enzymes LD, ALT, AST and ALP are known liver function indices in clinical diagnosis (McLean, 1962). The plasma activity levels of all four enzymes of the malnourished rats were significantly higher ($P<0.05$) than those of the rats on a balanced diet this could probably serve as an indication that the livers of the low-protein fed rats were already in a degenerative stage. This degeneration was accentuated by CCl₄ intoxication resulting in significantly elevated levels of LD, ALT, AST and ALP (Fig. 1.-Fig. 4.).

Olive oil treatment prevented the increase in the activity levels of all four enzymes as a result of the low-protein diet (malnutrition). UNICEF (1989)

suggests the fortification of PEM children's food with small amounts of oil (the energy value of oil is high). It is apparent from the results presented here that the administration of olive oil prevented the further degeneration of the liver as compared to the CCl₄ treatment. In fact, in most cases the olive oil improved the conditions of the low-protein fed rats.

The hepatic injury induced by CCl₄ resulted in significantly elevated ($P<0.05$) plasma LD activity levels. It is well known that LD activity levels increase in blood when heart muscle is damaged. Pumpkin seed protein isolate was able to ameliorate the activity levels of this enzyme as observed during the present study. Dinman *et al.* (1962) reported an elevation in the activity levels of serum LD in CCl₄ hepatotoxicity, however, the activity levels return to normal values 72 h after exposure. This observation is in agreement with the findings of the present study that the activity levels of the CCl₄ treated animals, although still slightly elevated, after 72 h it was not significantly different ($P<0.05$) from that of the animals on a balanced diet. However, the pumpkin seed protein isolate could lower the level of this enzyme to the normal value by 48h.

During the present study a low-protein diet also resulted in significantly elevated ($P<0.05$) activity levels of ALT and AST which were accentuated by CCl₄ intoxication (Fig. 2. and Fig. 3.). These results are in agreement with previous findings that the activity levels of serum ALT and AST were significantly elevated in rats as a result of CCl₄ administration (Lin *et al.*, 1998; Mitra *et al.*, 1998). In the present study treatment with pumpkin seed protein isolate significantly reduced the low-protein and CCl₄ elevated plasma activity levels of

all four enzymes ($P<0.05$). This is probably indicative of the hepato-protective effect of protein in the diet.

A low-protein diet resulted in significantly increased ($P<0.05$) activity levels of ALP. This is in agreement with a previous reported observation that the activity levels of ALP were significantly increased as a result of malnutrition (Gupta *et al.*, 1994). The increase in the activity levels of the enzyme became even more pronounced after the injection of CCl_4 (Fig. 4.). When pumpkin seed protein isolate was introduced the activity levels of ALP decreased significantly ($P<0.05$). The marked increased levels of plasma LD, ALT, AST and ALP observed in the present study as a result of CCl_4 intoxication probably reflects the degree of liver injury and leakage of cellular enzymes into the bloodstream as a result of the injury. Elevations of plasma transaminases (ALT and AST) as a result of CCl_4 -induced liver injury had been observed in several studies (Sodhi, 1997; Mitra *et al.*, 1998; Lin *et al.*, 1998).

LD and ALP are also two important enzymes for the diagnosis of liver diseases (Smith *et al.*, 1998). The elevated plasma activity levels of LD noticed during the present study probably confirms leakage from the damaged organ into the bloodstream (Van der Linde *et al.*, 1992; Terblanche *et al.*, 1998).

In the present study the activity levels of plasma ALT, AST and ALP, although significantly decreased ($P<0.05$) by the administration of pumpkin seed protein isolate in comparison with the CCl_4 treated animals were still significantly elevated ($P<0.05$) after 72 h in comparison with their counterparts fed a balanced diet.

From the results of the present study it could probably be concluded that pumpkin seed protein isolate administration was effective in alleviating the detrimental effects associated with protein malnutrition.

No matter the underlying factor, a child has to be malnourished for the known symptoms of PEM to manifest. An adequate protein diet still remains one of the best remedies for PEM because of their nutritive value as a source of essential amino acids and other nitrogenous compounds or possibly because of their anti-oxidative properties. The anti-oxidative properties of pumpkin seed protein isolate is currently under investigation.

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Section 2

Anti-oxidative Effects of Pumpkin Seed (*Cucurbita pepo*) Protein Isolate in CCl₄-Induced Liver Injury in Low-Protein Fed Rats

2.1 ABSTRACT

The effects of pumpkin seed (*Cucurbita pepo*) protein isolate on the plasma activity levels of catalase (CA), superoxide dismutase (SOD), glutathione peroxidase (GSHpx) and total antioxidant capacity (TAC) as well as Glucose-6-phosphatase (G6Pase) in liver homogenates and lipid peroxidation (LPO-malondialdehyde-MDA) levels in liver homogenates and liver microsomal fractions against carbon tetrachloride (CCl₄)-induced acute liver injury in low-protein fed Sprague-Dawley rats (*Rattus norvegicus*) were investigated. A group of male Sprague-Dawley rats maintained on a low-protein diet for five days were divided into three subgroups. Two subgroups were injected with carbon tetrachloride and the other group with an equivalent amount of olive oil. Two hours after CCl₄ intoxication one of the two subgroups was administered with pumpkin seed protein isolate and thereafter switched onto a 20% pumpkin seed protein isolate diet. The other two groups of rats were maintained on the low-protein diet for the duration of the investigation. Groups of rats from the different subgroups were sacrificed at 24, 48 and 72 hours after their respective treatments. After five days on the low-protein diet the activity levels of all the

enzymes as well as antioxidant levels were significantly lower than their counterparts on a normal balanced diet. However, a low-protein diet resulted in significantly increased levels of lipid peroxidation. The CCl₄ intoxicated rats responded in a similar way regarding all the variables investigated than their counterparts on a low-protein diet. The administration of pumpkin seed isolate after CCl₄ intoxication resulted in significantly increased levels of all the variables investigated, with the exception of the lipid peroxidation levels which were significantly decreased. From the results of the present study it is concluded that pumpkin seed protein isolate administration was effective in alleviating the detrimental effects associated with protein malnutrition and CCl₄ intoxication. It is therefore apparent that pumpkin seed protein isolate has components that have anti-peroxidative properties.

2.2 INTRODUCTION

In severely malnourished patients body tissues and organs, especially liver, degenerates and the activity levels of enzymes adjust to match their needs. In patients with end stage liver disease, the existence of protein energy malnutrition (PEM) is almost universal. In a detailed multivariate analysis by DiCecco *et al.* (1989) all of 74 patients evaluated for orthotopic liver transplantation were found to have evidence of malnutrition. According to Merli *et al.* (1996) it is conceivable that malnutrition may also condition the clinical outcome of cirrhotic patients.

The cell membrane contains a relatively high content of polyunsaturated fatty acid residues and often has heme and flavins as components of basic structure. The media on both sides of the membrane contain both oxygen and trace elements. Under these circumstances, the membrane is potentially more susceptible to free radical lipid peroxidation damage than cytoplasmic components (Chow, 1979). Extensive oxidation may lead to rupture of cellular membranes and release of destructive lysosomal enzymes and leading to death. It is thought that, if the *in vivo* activity of enzymes or scavengers is not enough to inhibit these radicals, various conditions such as arteriosclerosis, liver disease, diabetes, etc. may result (Lin *et al.*, 1998; Mitra *et al.*, 1998). A build up of free radicals damage tissues and explain many of the features of PEM e.g. bleached or reddish hair (Golden and Ramdath, 1987).

It is therefore not surprising that all oxygen-consuming organisms have developed complex antioxidant systems to counteract reactive oxygen species

and to reduce their damage (Cao *et al.*, 1995; Halliwell, 1996). The antioxidative enzymatic system consists largely of catalase (CA), superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and glutathione reductase (Schneider and Reed, 1985; Husain and Somani, 1997).

CCl₄ intoxication results in the peroxidation of lipids and lipid membranes of rats (Ohta *et al.* 1997). Ohta *et al.* (1997) observed an increase in lipid peroxidation (LPO) resulting from CCl₄ treatment. Lipid peroxidation products also contribute to the oxidative inactivation of glucose-6-phosphatase (G-6-Pase) (Ohyashiki *et al.* (1995).

PEM is often alleviated by the feeding of high quality protein (animal protein) to the patient. Cheaper and readily available vegetable proteins notably, oilseeds e.g. sunflower seeds (Sodini and Canella, 1977); cotton seeds (Saeed and Cheryan, 1988) and other vegetable sources that have high quality protein are quickly replacing animal protein in combating PEM (Bianchi *et al.*, 1993).

Based on the results of a previous study in which the effect of pumpkin seed protein isolate on the activity levels of certain plasma enzymes (lactate dehydrogenase, alanine transaminase, aspartate transaminase, alkaline phosphatase) in CCl₄-induced liver injury in low-protein fed rats were investigated (Nkosi *et al.*, in press), it was decided to also investigate the antioxidative properties of pumpkin seed protein isolate in CCl₄-induced liver injury in low-protein fed rats.

2.3 MATERIALS AND METHODS

2.3.1 Preparation of protein isolate and treatment of animals. Unless otherwise stated, the preparation of the protein isolates and the animal experiments were as previously described (Nkosi *et al.*, in press). Dehusked, defatted pumpkin seeds were extracted with water (pH 10). The proteins in the extract were obtained by adjusting the pH to 5, centrifuged, and freeze-dried (Sodini and Canella, 1977).

Fifty-two male Sprague-Dawley rats (except four which were fed on a normal balanced rat diet) were fed a low-protein diet (maize meal, 2mm mesh size and pelleted) for 5 days. After 5 days the four rats on a normal balanced diet (normal group) and four of the rats on the low-protein diet (malnutrition group) were sacrificed. The remaining rats were divided into 3 groups. The rats in group 1 were intraperitoneally injected with 1.0ml/kg olive oil (olive oil group) whereas groups 2 and 3 were subcutaneously injected with 1.0ml/kg body weight CCl₄ (dissolves in olive oil, 1:1) (Bruckner *et al.*, 1986) (group 2 designated as the CCl₄ Group) (Ohta *et al.*, 1998). Two hours after the CCl₄ intoxication the group 3 rats were administered 1ml/kg body weight of the pumpkin seed protein isolate in saline (20g/100ml) by stomach tube (pumpkin seed protein isolate group) (Ohta *et al.*, 1998). At the same time the animals in groups 1 and 2 received the same volume of physiological saline. All the rats had access to water and food *ad libitum*. The rats in groups 1 and 2 were maintained on the low-protein diet and

had access to water and food (maize meal) *ad libitum* whereas the rats in group 3 were switched to a 1:5 pumpkin seed protein isolate:maize meal (w/w) diet.

Four rats from each group were sacrificed 2, 24, 48 and 72 h after the CCl₄ intoxication. The rats were anaesthetized with pentobarbital sodium (6mg/100g body mass) injected intraperitoneally and blood withdrawn from the abdominal aorta into K₂EDTA tubes. The blood was centrifuged (2000 rpm at 4±1°C for 10 min) to obtain plasma.

The livers were immediately excised, washed in ice-cold 0.15M KCl and weighed after blotting out excess fluid. Liver homogenates were prepared using a glass Potter-Elvehjem homogenizer at 4±1°C in a medium containing 9 volumes 0.15M KCl-1.0mM EDTA. Portions of the liver homogenates were centrifuged at 4±1°C at 900xg for 15min. to remove the nuclei fraction; 1500xg for 15min. to remove the mitochondrial fraction; and then at 100 000g for 30min. to obtain the microsomal fraction). The microsomes were purified by 2 cycles of washing in buffer and centrifugation (Ohta *et al.*, 1998; Toda and Shirataki, 1998; Toda and Yase, 1998). The microsome pellets, the liver homogenate and the plasma were stored at -80°C.

2.3.2 Analytical methods. Plasma samples were used to determine the activity levels of catalase (CA), superoxide dismutase (SOD), glutathione peroxidase (GSHPx), as well as the total antioxidant capacity (TAC). G6Pase activity levels in liver homogenates and lipid peroxidation (malondialdehyde-MDA) levels were quantified in liver homogenates and liver microsomal fractions.

Catalase (CA) activity levels in plasma were determined according to the method described by Aebi (1983). The decomposition of hydrogen peroxide (H_2O_2) catalyzed by CA was recorded at 5-s intervals for 30 s in a Beckman DU 40 UV/VIS spectrophotometer at 240 nm.

Superoxide dismutase (SOD) activity levels in plasma were quantified according to the method described by Winterbourn *et al.* (1975).

Glutathione peroxidase (GSHPx) activity levels in plasma were determined according to the method described by Pütter and Becker (1983).

Total antioxidant capacity (TAC) in plasma were quantified with the aid of the kit provided by Randox, Crumlin, UK (Nemec *et al.*, 2000).

Glucose-6-phosphatase (G-6-Pase) activity of the microsomes was determined according to the method of Swanson (1955). The activity is the amount of enzyme required to release $1\mu\text{mol}$ of inorganic phosphate from glucose-6-phosphate (substrate) per min. Inorganic phosphate was determined by the method of Goldenberg and Fernandez (1966).

Lipid peroxide (LPO) content of the liver homogenate and the microsomal fractions was measured as malondialdehyde (MDA) level with the thiobarbituric acid (TBA) method (Aruoma *et al.*, 1993; Tripathi *et al.*, 1995).

Enzyme assays were performed under conditions in which the reaction rate was proportional to the plasma concentration at 30°C in keeping with the recommendations of the Commission on Enzymes of the International Union of Biochemistry.

2.3.3 Statistical analysis. All data are expressed as the mean \pm SD. The results were analyzed statistically using two-factor ANOVA with Bonferroni's *post hoc* tests, ($p < 0.05$ was regarded as significant).

2.4 RESULTS

A preliminary study in our laboratories on the nutritional quality of pumpkin seed protein isolate indicated a protein efficiency ratio of 2.17.

2.4.1 Catalase (CA). The plasma activity levels of CA ($K.I^{-1}$) for the different treatments are presented in Fig. 1.

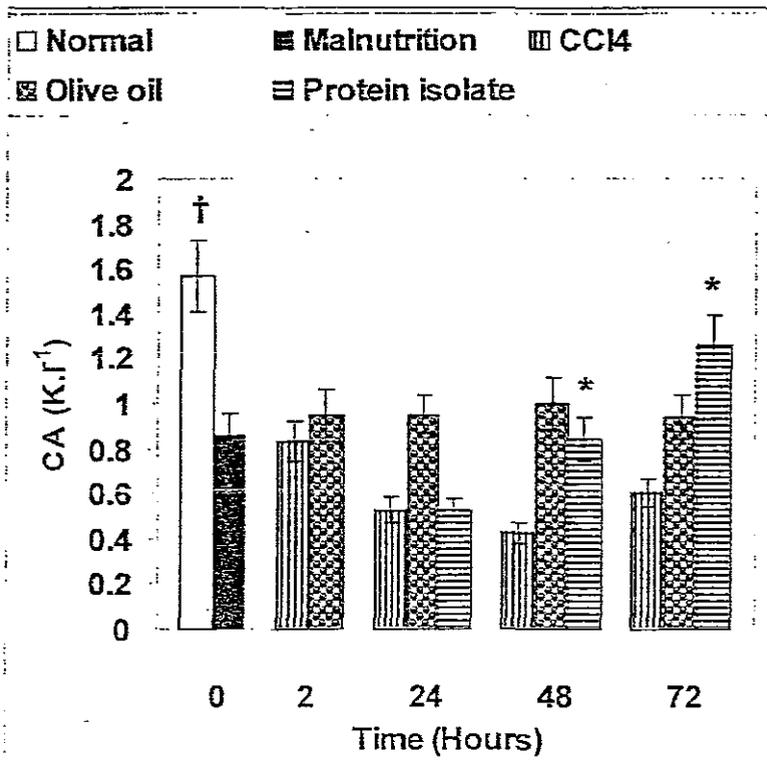


Figure 1. The effect of pumpkin seed protein isolate on plasma CA activity levels ($\text{K}\cdot\text{I}^{-1}$) of malnourished rats treated with CCl_4 and rehabilitated with pumpkin seed protein isolate over 72h. Values are mean \pm SD for 4 rats. † Normal significantly higher than Malnutrition, $p < 0.05$. * Pumpkin seed protein isolate significantly higher than CCl_4 , $p < 0.05$.

A low-protein diet as well as CCl_4 administration resulted in significantly reduced levels of plasma CA ($p < 0.05$). Pumpkin seed protein isolate administration resulted in significantly ($p < 0.05$) increased levels of the enzyme at 48 and 72 h after treatment.

2.4.2 Superoxide dismutase (SOD). The activity levels of plasma SOD ($\text{U}\cdot\text{ml}^{-1}$) as affected by the different treatments are furnished in Fig. 2.

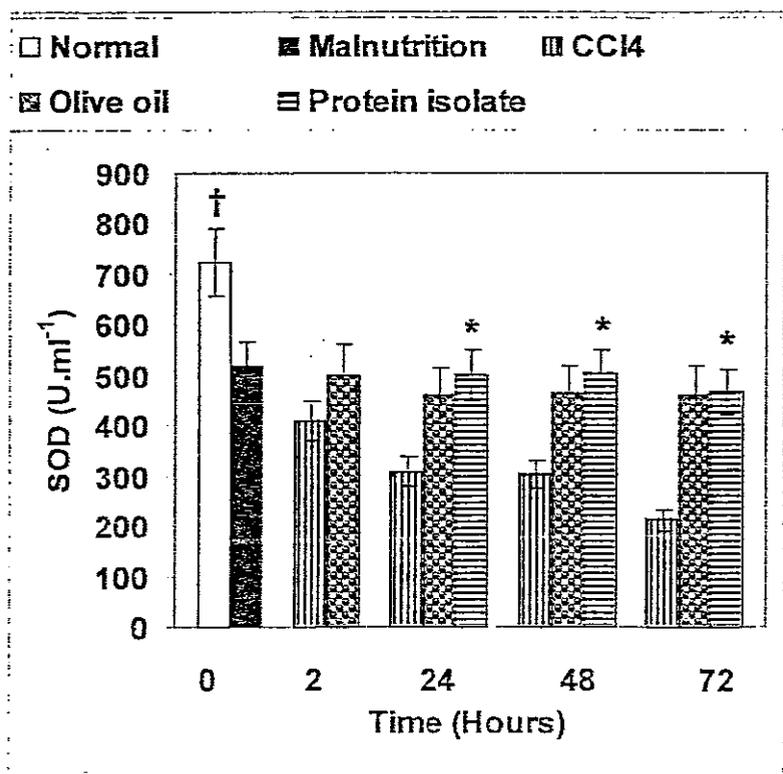


Figure 2. The effect of pumpkin seed protein isolate on plasma SOD activity levels (U.ml^{-1}) of malnourished rats treated with CCl_4 and rehabilitated with pumpkin seed protein isolate over 72h. Values are mean \pm SD for 4 rats.

† Normal significantly higher than Malnutrition, $p < 0.05$. * Pumpkin seed protein isolate significantly higher than CCl_4 , $p < 0.05$.

The animals on a normal diet exhibited significantly higher ($p < 0.05$) levels of SOD. Pumpkin seed protein isolate caused significantly increased ($p < 0.05$) levels of plasma SOD at all the treatment intervals investigated in comparison with their CCl_4 treated counterparts.

2.4.3 Glutathione peroxidase (GSHpx). The plasma GSHpx activity levels (U.l^{-1}) are given in Fig. 3.

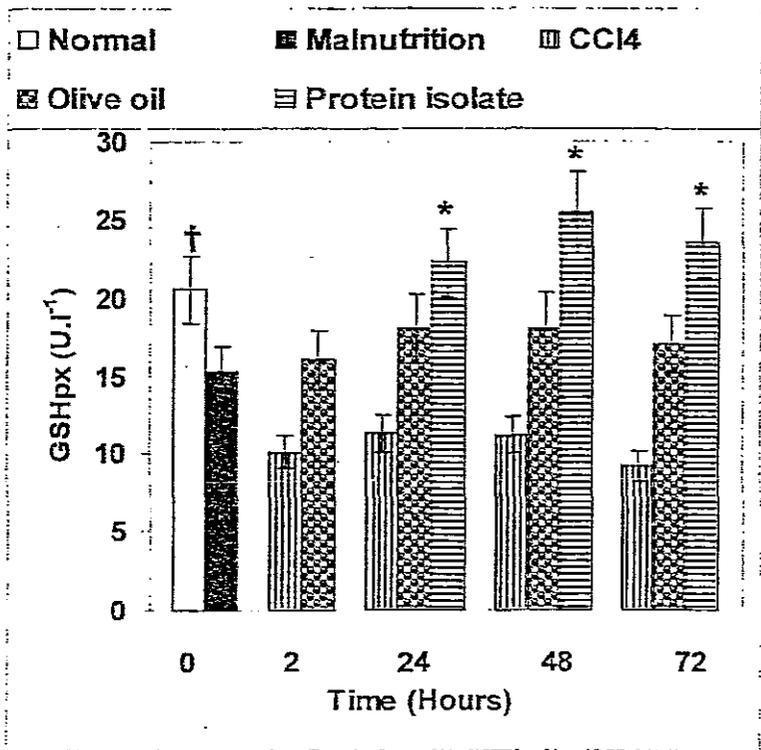


Figure 3. The effect of pumpkin seed protein isolate on plasma GSHpx activity levels (U.ml^{-1}) of malnourished rats treated with CCl_4 and rehabilitated with pumpkin seed protein isolate over 72 h. Values are mean \pm SD for 4 rats.

† Normal significantly higher than Malnutrition, $p < 0.05$. * Pumpkin seed protein isolate significantly higher than CCl_4 , $p < 0.05$.

The plasma GSHpx activity levels were significantly decreased ($p < 0.05$) as a result of the low-protein diet. Pumpkin seed protein isolate administration resulted in significantly increased ($p < 0.05$) plasma GSHpx activity levels. 24 h after treatment the GSHpx activity levels of the plasma of the pumpkin seed protein isolate treated animals were already higher than that of the animals on a balanced diet

2.4.4 Total antioxidant capacity (TAC). The plasma ALP activity levels (mmol.l^{-1}) of the different treatments are illustrated in Fig. 4.

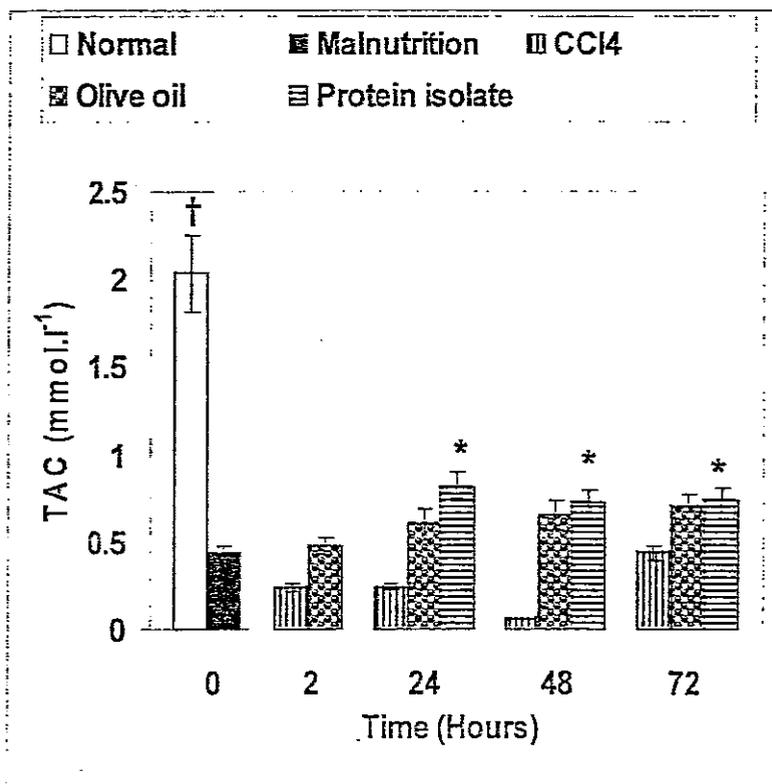


Figure 4. The effect of pumpkin seed protein isolate on plasma TAC (mmol.l^{-1}) of malnourished rats treated with CCl_4 and rehabilitated with pumpkin seed protein isolate over 72h. Values are mean \pm SD for 4 rats. † Normal significantly lower than Malnutrition, $p < 0.05$. *Pumpkin seed protein isolate significantly lower than CCl_4 , $p < 0.05$.

The plasma TAC was dramatically affected by a low-protein diet as well as CCl_4 treatment. Both treatments resulted in a significantly decreased ($p < 0.05$) TAC. Pumpkin seed protein isolate caused significant increases ($p < 0.05$) in the plasma TAC at all time intervals investigated, but even 72 h after treatment it was still significantly lower ($p < 0.05$) than that of the animals on a balanced diet.

2.4.5 Glucose-6-phosphatase (G-6-Pase). The G-6-Pase activity levels (U.g^{-1} tissue) in the liver homogenates are provided in Fig. 5.

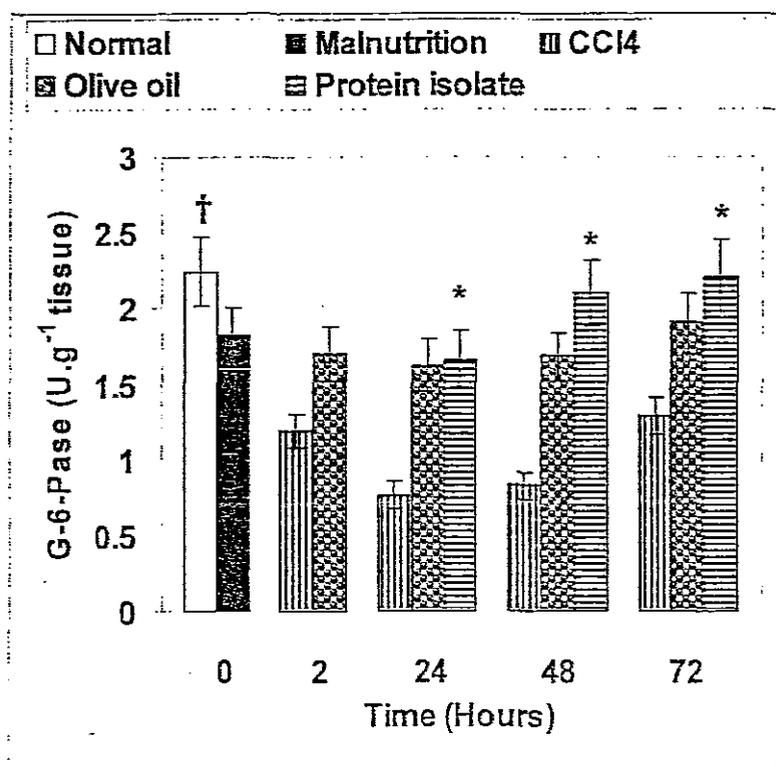


Figure 5. The effect of pumpkin seed protein isolate on G-6-Pase (U.g^{-1}) activity levels in liver homogenates of malnourished rats treated with CCl_4 and rehabilitated with pumpkin seed protein isolate over 72h. Values are mean \pm SD for 4 rats. \dagger Normal significantly higher than Malnutrition, $p < 0.05$. * Pumpkin seed protein isolate significantly higher than CCl_4 , $P < 0.05$.

Animals on a balanced diet exhibited significantly ($p < 0.05$) elevated G-6-Pase activity levels than their counterparts on a low-protein diet as well as those treated with CCl_4 . Pumpkin seed protein isolate treatment resulted in significantly elevated ($p < 0.05$) G-6-Pase activity levels in comparison with CCl_4 treatment. After 72 h the detrimental effects associated with a low-protein diet as well as CCl_4 treatment were completely neutralized.

2.4.6 Lipid peroxidation (LPO). The LPO levels (nmol MDA.g^{-1} tissue) of the liver homogenates are graphically illustrated in Fig. 6.

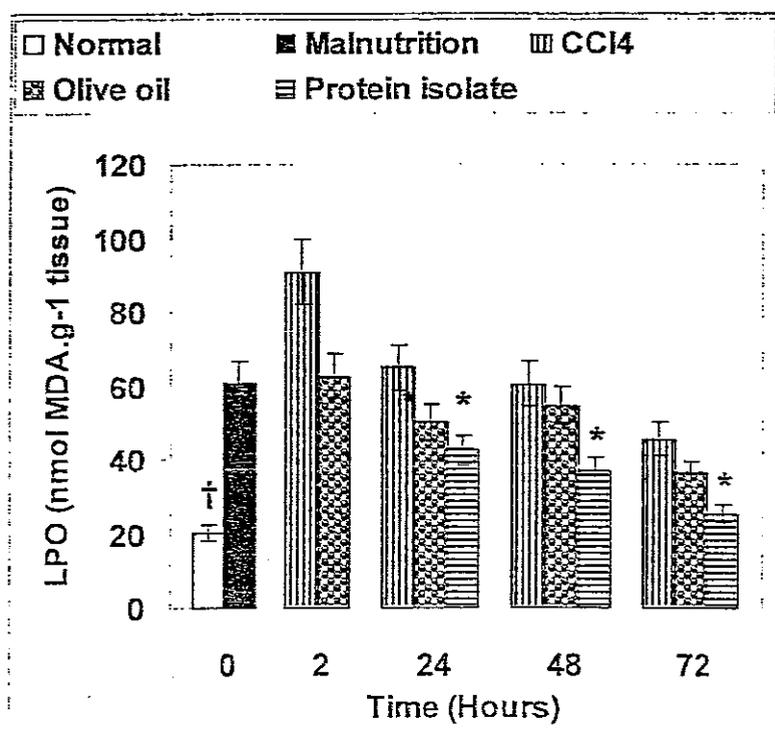


Figure 6. The effect of pumpkin seed protein isolate on liver homogenate LPO levels (nmol MDA.g⁻¹ tissue) of malnourished rats treated with CCl₄ and rehabilitated with pumpkin seed protein isolate over 72h. Values are mean ± SD for 4 rats. † Normal significantly lower than Malnutrition, $p < 0.05$. * Pumpkin seed protein isolate significantly lower than CCl₄, $p < 0.05$.

A low-protein diet as well as CCl₄ administration resulted in significantly increased levels of LPO in liver homogenates ($p < 0.05$). Administration of pumpkin seed protein isolate caused a significant ($p < 0.05$) progressive decrease in LPO levels over the duration of the investigation approaching normal values at 72h.

The LPO levels (nmol MDA.g⁻¹ tissue) of the liver microsomes are presented in Fig. 7.

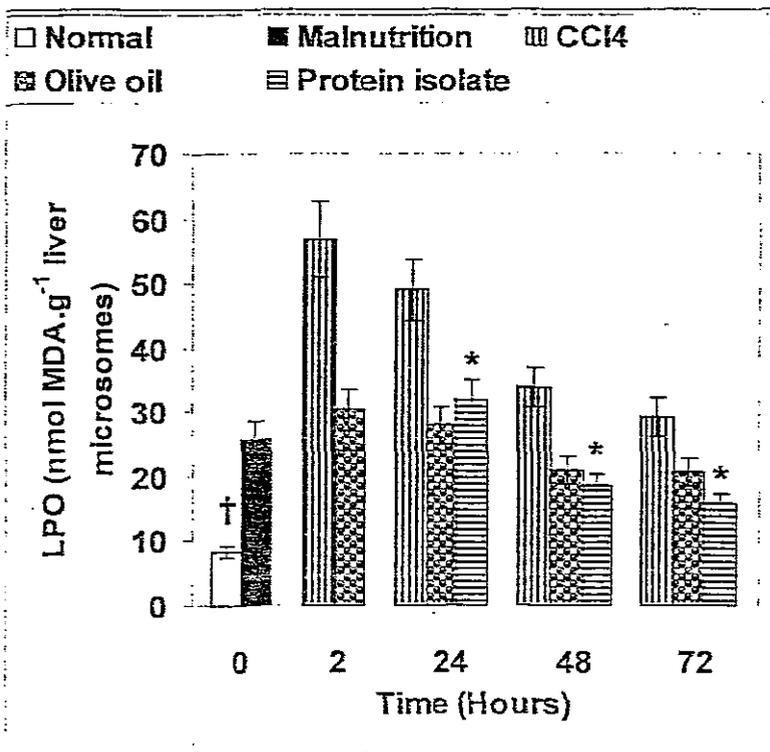


Figure 7. The effect of pumpkin seed protein isolate on liver microsomal LPO levels (nmol MDA.g⁻¹ liver) of malnourished rats treated with CCl₄ and rehabilitated with pumpkin seed protein isolate over 72h. Values are mean ± SD

for 4 rats. † Normal significantly lower than Malnutrition, $p < 0.05$. * Pumpkin seed protein isolate significantly lower than CCl_4 , $p < 0.05$.

The results are to a large extent similar to those obtained for the liver homogenates.

The liver microsomal LPO levels were significantly elevated ($p < 0.05$) as a result of the low-protein diet as well as CCl_4 treatment. Pumpkin seed protein isolate administration resulted in significantly reduced ($p < 0.05$) LPO levels.

2.5 DISCUSSION

Various substances, known as Reactive Oxygen Species (ROS), are constantly generated *in vivo* as an integral part of metabolism, as part of a controlled inflammatory reaction and by exposure to environmental factors (Nemec *et al.*, 2000). ROS circulate freely in the body with access to all organs and tissues. Free radicals within the membrane triggers the progressive destruction of polyunsaturated fatty acids (PUFA) ultimately leading to membrane destruction (Halliwell, 1991).

The enzymes CA, SOD, and GSHpx can be classified as enzyme antioxidants that function by catalysing the oxidation of other molecules (Chapple, 1997). The plasma activity levels of all three enzymes of the malnourished (low-protein diet) rats were significantly lower ($p < 0.05$) than those of the rats on a normal balanced diet.

This probably is an indication that the livers of the low-protein fed rats were already in a degenerative stage. This degeneration was accentuated by CCl₄ intoxication resulting in significantly decreased activity levels of CA, SOD and GSHpx (Figs. 1-3). It is understood that CCl₄ is enzymatically oxidized to a [•]CCl₃ radical.

The observed results are in agreement with the findings of Sodhi (1997) who reported a decrease in the activity levels of both CA and GSHpx in both liver and blood due to induced hepatic damage. Any decrease in activity of CA (breakdown of H₂O₂ into H₂O and O₂), SOD (conversion of superoxide to molecular oxygen and hydrogen peroxide) and GSHpx (reaction of hydroperoxides with reduced glutathione with the concomitant production of oxidized glutathione and the reduction product of the hydroperoxide), would probably result in a decreased anti-oxidative capacity. A low-protein diet and CCl₄ intoxication also resulted in a significant decrease in TAC ($p < 0.05$) (Fig. 4).

G-6-Pase activity levels were significantly ($p < 0.05$) lower in the rats fed a low-protein diet as well as in the CCl₄ administered rats than the rats on a normal balanced diet (Fig. 5). The results obtained during the present study regarding the G-6-Pase activity levels are in agreement with the findings of Ohyashiki *et al.* (1995) who reported that lipid peroxidation products are able to inactivate G-6-Pase.

The observed significantly ($p < 0.05$) increased LPO levels as reflected by the elevated levels of malondialdehyde in liver homogenates and liver

microsomes (Figs. 6 and 7) as a result of a low-protein diet and CCl₄ intoxication during the present study are in agreement with previous reported observations (Ohta *et al.*, 1997; Ohta *et al.*, 1998). It is apparent that the build up of free radicals contributed to the observed conditions of the rats.

Pumpkin seed protein isolate administration caused significant increases ($p < 0.05$) in the activity levels of all three enzymes especially GSHpx which could be indicative of an effective anti-oxidative capacity.

The administration of pumpkin seed protein isolate significantly ($p < 0.05$) improve the TAC of both the low-protein fed rats as well as those intoxicated with CCl₄ but the improvement was only approximately 50% when compared with the TAC of the rats fed a normal balanced diet.

Pumpkin seed protein isolate resulted in significantly ($p < 0.05$) increased activity levels of the enzyme G-6-Pase. The G-6-Pase activity levels of the pumpkin seed protein isolate treated rats were comparable with that of the rats on a normal balanced diet 72h after treatment.

Pumpkin seed protein isolate treatment resulted in significantly ($p < 0.05$) decreased LPO levels both in liver homogenates and liver microsomes. It is noted that when LPO levels were high, the G6Pase activities were low and that when the pumpkin seed proteins were administered, the order was reversed. This is in agreement with the observation by Ohyashiki *et al.* (1995) that lipid peroxidation products also contribute to the oxidative inactivation of G-6-Pase.

Golden and Ramdath (1987) reported a relationship between the build up of free radicals and the syndromes of PEM. Malnutrition has a negative balance on

the antioxidative profile of the rats. Damage by free radicals (CCl₄) in the rats was also confirmed by a decrease in the antioxidant enzymes and the elevation of LPO (MDA).

Hepatoprotective studies by Mitra *et al.* (1998) showed that plants have active ingredients that are capable of free radical scavenging in living systems.

From the results obtained it could be concluded that pumpkin seed protein isolate administration was effective in alleviating the detrimental effects associated with a low-protein diet and CCl₄ intoxication. It is therefore apparent that pumpkin seed protein isolate probably has components that have anti-peroxidative properties.

It is apparent that no matter the causative factors of PEM, a child has to be protein energy deficient before free radicals can damage his/her tissues.

2.6

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Section 3

***In Vitro* Anti-oxidative Activity of Pumpkin Seed (*Cucurbita pepo*) Protein Isolate and its *In Vivo* Effect on Alanine Transaminase and Aspartate Transaminase in Acetaminophen-Induced Liver Injury in Low Protein Fed Rats**

3.1 ABSTRACT

The antioxidative effects of pumpkin seed protein isolate (*Cucurbita pepo*) as reflected by free radical scavenging activity, Fe^{2+} chelating activity, and antixanthine oxidase activity were investigated *in vitro*. The pumpkin seed protein isolate exhibited about 80% radical scavenging activity, chelating activity of approximately 64% on Fe^{2+} ions and an inhibition of approximately 10% of xanthine oxidase. In a subsequent investigation the effects of pumpkin seed protein isolate on the plasma activity levels of alanine transaminase (ALT) and aspartate aminotransferase (AST) against acetaminophen (paracetamol) induced acute liver injury in low-protein fed male Sprague-Dawley rats (*Rattus norvegicus*) were ascertained. A group of male Sprague-Dawley rats maintained on a low-protein diet for five days were divided into three subgroups. Two subgroups were injected with acetaminophen and the other group with an equivalent amount of polyethylene glycol 400 (PEG 400). Two hours after acetaminophen intoxication one of the two subgroups was administered with

pumpkin seed protein isolate. All three subgroups of rats were maintained on the low-protein diet for the duration of the investigation. Groups of rats from the different subgroups were sacrificed at 24, 48 and 72 h after their respective treatments. After five days on the low-protein diet the activity levels of the two enzymes were significantly higher than their counterparts on a normal balanced diet. The enzymes levels were even higher in the acetaminophen intoxicated rats. The administration of pumpkin seed isolate after acetaminophen intoxication resulted in significantly reduced activity levels of the two enzymes. It is concluded that pumpkin seed protein isolate has promising antioxidative. Furthermore, the isolate administration was effective in alleviating the detrimental effects associated with protein malnutrition and acetaminophen intoxication.

3.2 INTRODUCTION

Various substances, known as Reactive Oxygen Species (ROS), are constantly generated *in vivo* as an integral part of metabolism, as part of a controlled inflammatory reaction and by exposure to environmental factors (Nemec *et al.*, 2000). ROS, and other free radicals, have long been recognized as causative agents of many human diseases. Golden and Ramdath (1987) reported a relationship between the build up of free radicals and the syndromes of protein energy malnutrition (PEM).

Prevalence of nutritional pathology is high in Africa, especially in the vulnerable groups of the population where PEM is high. Well nourished populations are better placed to resist tropical endemic diseases (e.g. malaria) and the associated side effects of the often abused chemotherapy. Vegetable proteins have been recognized as the cheapest and most abundant potential source of proteins for the vulnerable group.

In a previous investigation on the *in vivo* antioxidative effects of pumpkin seed protein isolate it was found that the administration of it was effective in alleviating the detrimental effects associated with protein malnutrition as well as that associated with carbon tetrachloride intoxication (Nkosi *et al.*, 2005). If pumpkin seed protein isolate is to be processed as food, then there is need to establish its antioxidative properties. Lipid peroxidation (as a result of ROS) contributes to the deteriorative changes in food during storage and processing.

Acetaminophen (paracetamol) is a commonly used antipyretic-analgesic drug, which is safe at therapeutic doses but has been reported to induce liver injury in man and experimental animals when used in large doses (Black, 1984). The toxin depletes antioxidant defenses. Lin *et al.* (1996) reported a significant elevation of plasma ALT and AST activity levels in paracetamol-induced hepatic injury.

Based on the above evidence it was decided to investigate some of the *in vitro* antioxidative effects of pumpkin seed protein isolate as reflected by free radical scavenging activity, chelating activity and antixanthine oxidase activity. Subsequently it was also decided to ascertain the effects of pumpkin seed protein isolate on the plasma activity levels of alanine transaminase (ALT) and aspartate aminotransferase (AST) against acetaminophen (paracetamol)-induced liver injury in low-protein fed male Sprague-Dawley rats (*Rattus norvegicus*).

3.3 MATERIALS AND METHODS

3.3.1 Preparation of protein isolate and treatment of animals. Unless otherwise stated, the preparation of the protein isolates and the animal experiments were as previously described (Nkosi *et al.*, 2005). Dehusked, defatted pumpkin seeds were extracted with water (pH 10). The proteins in the extract were obtained by adjusting the pH to 5, centrifuged, and freeze-dried (Sodini and Canella, 1977).

Twenty four male Sprague-Dawley rats *Rattus norvegicus* (3-4 weeks old; weighing between 80-90 g) (except four which were fed on a normal balanced rat diet) were fed a low-protein diet (maize meal, 2mm mesh size and pelleted) for 5 days. After 5 days the four rats on a normal balanced diet (normal group) and four of the rats on the low-protein diet (malnutrition group) were sacrificed. The remaining rats were divided into 3 groups. The rats in group 1 were intraperitoneally injected with 10ml/kg polyethylene glycol 400 (PEG 400) dissolved in physiological saline (PS) (PEG 400: PS) (1:1) (PEG group). Groups 2 and 3 were subcutaneously injected with 600mg acetaminophen/10ml PEG 400: PS (1:1)/ kg body weight (Black, 1984) (group 2 designated as the acetaminophen group). Two hours after the acetaminophen intoxication the group 3 rats were administered 1ml/kg body weight of the pumpkin seed protein isolate in saline (20g/100ml) by stomach tube (pumpkin seed protein isolate group). Simultaneously the animals in groups 1 and 2 received the same volume of physiological saline. All the rats had access to water and food *ad libitum*. The rats in groups 1 and 2 were maintained on the low-protein diet and had access to water and food (maize meal) *ad libitum* whereas the rats in group 3 were switched to a 1:5 pumpkin seed protein isolate:maize meal (w/w) diet.

Four rats from each group were sacrificed 2, 24, 48 and 72 h after the acetaminophen intoxication. The rats were anaesthetized with pentobarbital sodium (6mg/100g body mass) injected intraperitoneally and blood withdrawn from the abdominal aorta into K₂EDTA tubes. The blood was centrifuged (2000 rpm at 4±1°C for 10 min) to obtain plasma which was stored at -80°C.

3.3.2 Analytical methods. The total polyphenol content of the pumpkin seed protein isolate was determined (as gallic acid equivalent) according to the method of Swain and Hillis (1959).

Sulfhydryl content was measured fluorimetrically using O-phthalaldehyde condensation following the method of Cohen and Lyle, 1966.

Free radical scavenging activity of the pumpkin seed protein isolate was determined by the method of Navarro *et al.* (1992) as described by Opoku *et al.* (2002).

Chelating activity on Fe^{2+} of the pumpkin seed protein isolate was quantified by the method reported by Decker and Welch (1990) as outlined by Opoku *et al.* (2002).

The effect of the pumpkin seed protein isolate on anti-xanthine oxidase activity was ascertained by the method of Constantino *et al.* (1992) as reported by Opoku *et al.* (2002).

Plasma samples were used to determine the activity levels of alanine transaminase (ALT) (Hørder and Rej, 1983), aspartate transaminase (AST) (Rej and Hørder, 1983).

Enzyme assays were performed under conditions in which the reaction rate was proportional to the plasma concentration at 30°C in keeping with the recommendations of the Commission on Enzymes of the International Union of Biochemistry.

3.3.3 Statistical analysis. All data are expressed as the mean \pm SD. The results were analyzed statistically using two-factor ANOVA with Bonferroni's *post hoc* tests, ($p < 0.05$ was regarded as significant).

3.4 RESULTS

A preliminary study in our laboratories on the nutritional quality of pumpkin seed protein isolate indicated a protein efficiency ratio of 2.17.

3.4.1 Polyphenol and sulfhydryl content. The pumpkin seed protein isolate exhibited a polyphenol content of 2.3 mg.g⁻¹ (expressed as gallic acid equivalent). The sulfhydryl content was 1.39 $\mu\text{g mg}^{-1}$.

3.4.2 Free radical scavenging activity. The DPPH radical scavenging ability of the pumpkin seed protein isolate is illustrated in Fig.

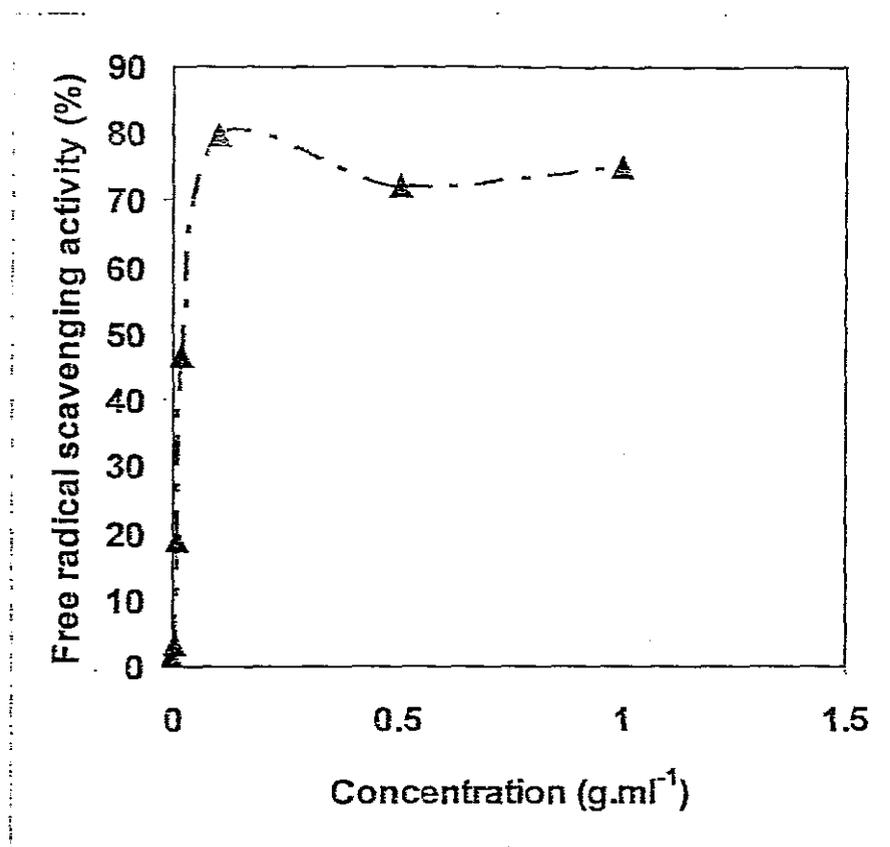


Figure 1. Free radical scavenging activity (%) of pumpkin seed protein isolate. Values are mean \pm SD for 4 rats.

At a concentration of 0.1g.ml⁻¹ the extracts exhibit about 80% radical scavenging activity.

3.4.2 Chelating activity on Fe²⁺. The chelating activity on Fe²⁺ is presented in

Fig.2.

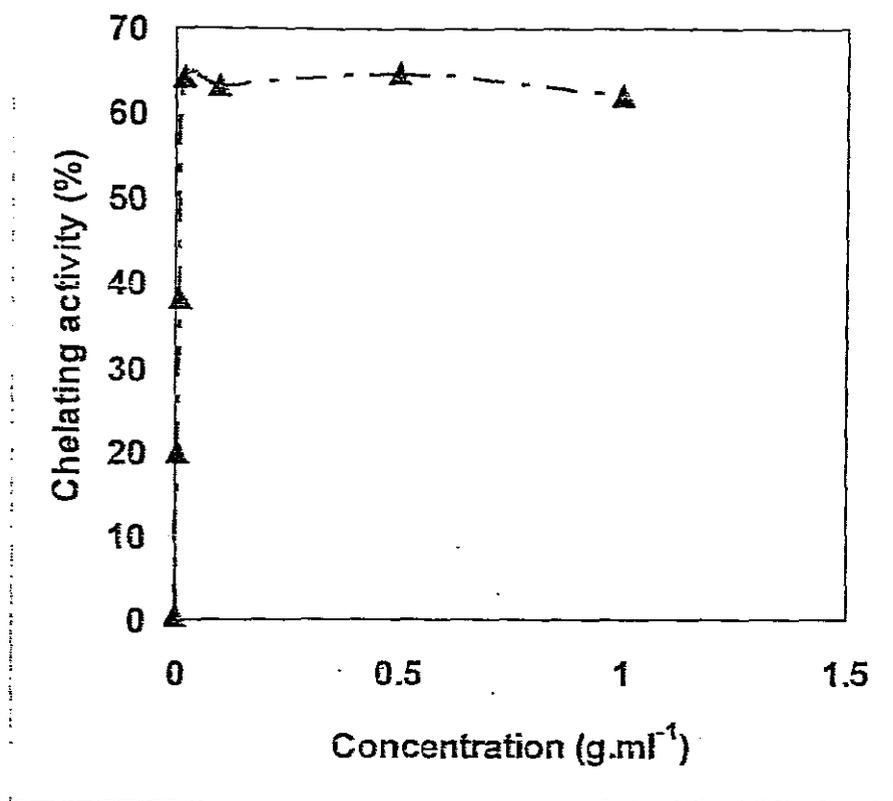


Figure 2. Chelating activity on Fe^{2+} (%) of pumpkin seed protein isolate. Values are mean \pm SD for 4 rats.

The pumpkin seed protein isolate showed a strong chelating activity of approximately 64% on Fe^{2+} ions at a concentration of 0.5g.ml⁻¹.

3.4.3 Anti-xanthine oxidase activity. Anti-xanthine oxidase activity (%) of the pumpkin seed protein isolate is given in Fig. 3.

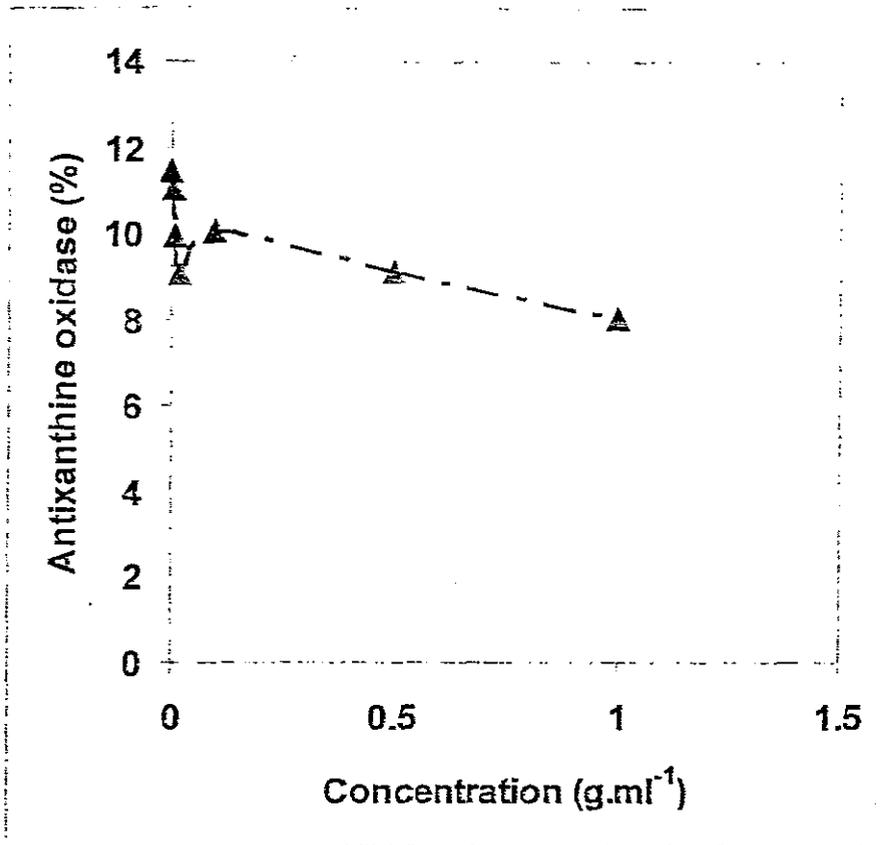


Figure 3. Antixanthine oxidase activity (%) of pumpkin seed protein isolate. Values are mean \pm SD for 4 rats.

The pumpkin seed protein isolate showed a maximum of approximately 10% inhibition at a concentration of 0.1g.ml⁻¹. At concentrations above 0.1g.ml⁻¹ the inhibitory effect of the pumpkin seed protein isolate was progressively reduced.

3.4.4 Alanine transaminase (ALT). The plasma activity levels of ALT (U.l⁻¹) for the different treatments are presented in Fig. 4.

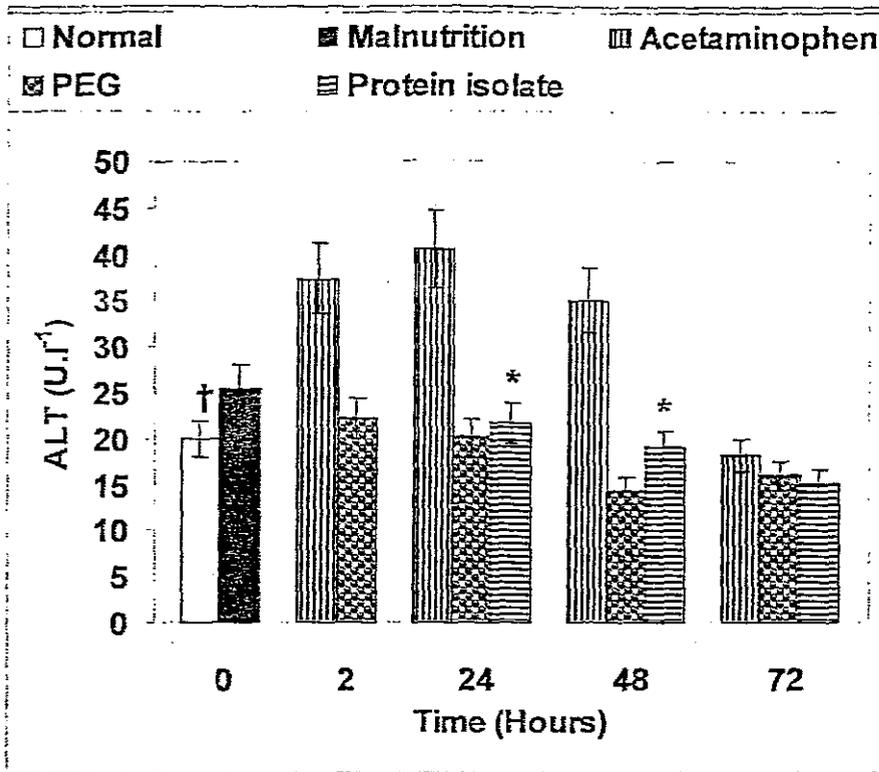


Figure 4. The effect of pumpkin seed protein isolate on plasma ALT activity levels (U.ml^{-1}) of malnourished rats treated with olive oil followed after 2 h with acetaminophen and acetaminophen+pumpkin seed protein isolate, after 24, 48 and 72 h. Values are means \pm SD for 4 rats. †Normal significantly lower than Malnutrition, $p < 0.05$. *Pumpkin seed protein isolate significantly lower than acetaminophen, $p < 0.05$.

The animals on the low-protein diet as well as those exposed to the acetaminophen treatment exhibited significantly higher ($p < 0.05$) levels of ALT. Pumpkin seed protein isolate caused significantly decreased ($p < 0.05$) levels of plasma ALT at 24 and 48h in comparison with their acetaminophen treated counterparts.

3.4.6 Aspartate transaminase (AST). The plasma AST activity levels are given in Fig. 5.

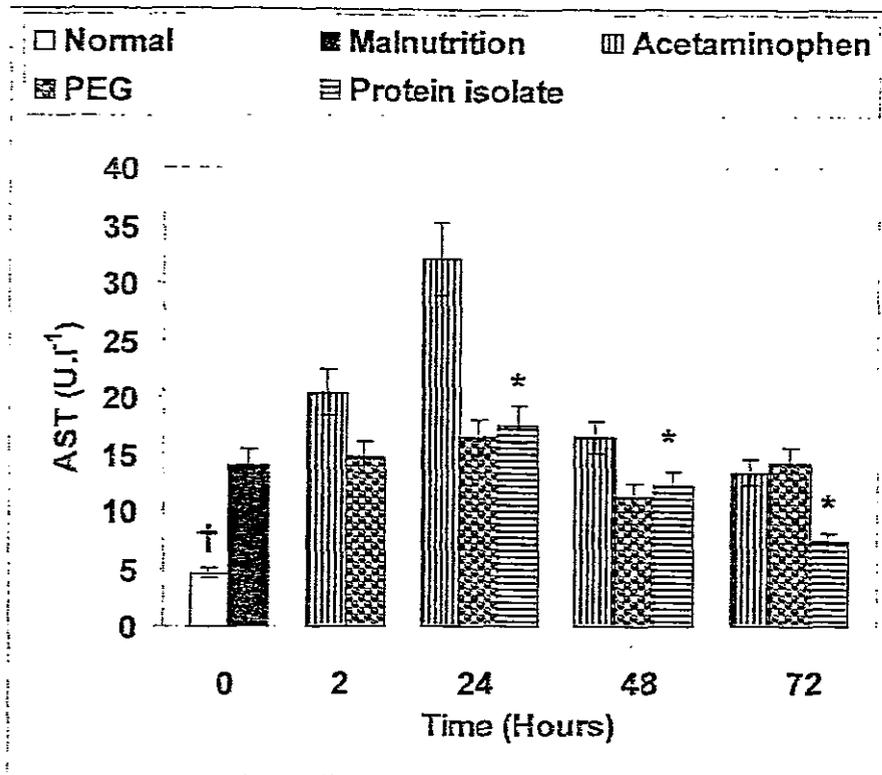


Figure 5. The effect of pumpkin seed protein isolate on plasma AST activity levels ($\text{U}\cdot\text{ml}^{-1}$) of malnourished rats treated with olive oil followed after 2 h with acetaminophen and acetaminophen+pumpkin seed protein isolate, after 24, 48 and 72 h. Values are means \pm SD for 4 rats. †Normal significantly lower than Malnutrition, $p<0.05$. *Pumpkin seed protein isolate significantly lower than acetaminophen, $p<0.05$.

The results are to a large extent similar to those obtained for ALT (Fig. 4).

The plasma AST activity levels were significantly elevated ($p<0.05$) as a result of the low-protein diet as well as acetaminophen treatment. Pumpkin seed protein isolate administration resulted in significantly reduced ($p<0.05$) plasma

AST activity levels at all the intervals investigated in comparison with their acetaminophen treated counterparts.

3.5 DISCUSSION

Hepatoprotective studies by Mitra *et al.* (1998) showed that plants have active ingredients that are capable of free radical scavenging in living systems. Most plants contain polyphenols as antioxidative compounds (Navarro *et al.*, 1992). The polyphenol content of $2.3\text{mg}\cdot\text{g}^{-1}$ observed for the pumpkin seed protein isolate is indicative of potential antioxidative properties. The observed 80% free radical scavenging activity, coupled with the 64% Fe^{2+} ion chelating ability indicates that although pumpkin seed protein isolate is poor in preventing the generation of free radical (10% inhibition of xanthine oxidase), yet it is good in the inhibition of the activity of free radicals when generated.

The enzymes, ALT and AST, are known liver function indices in clinical diagnosis (McLean, 1962). The plasma activity levels of both enzymes of the malnourished rats were significantly higher ($p < 0.05$) than those of the rats on a balanced diet this could probably serve as an indication that especially the livers of the low-protein fed rats were already in a degenerative stage. This degeneration was accentuated by acetaminophen intoxication resulting in significantly elevated levels of ALT and AST (Fig. 4 and Fig. 5). This is in agreement with the findings of Lin *et al.* (1996) who observed a significant

increase of plasma ALT and AST activity levels in paracetamol-induced hepatic injury. The marked increased levels of plasma ALT and AST observed in the present study as a result of acetaminophen intoxication probably reflects the degree of liver and kidney injury and leakage of cellular enzymes into the bloodstream as a result of the injury. However, the ALT and AST activity levels obtained during the present study were not elevated to the same extent as those observed in a previous study with carbon tetrachloride-induced hepatic injury (Nkosi *et al.*, 2005).

The treatment with pumpkin seed protein isolate significantly reduced the low-protein and acetaminophen elevated plasma activity levels of both enzymes ($p < 0.05$). It is also noted that in the previous study under similar conditions the activity levels of ALT and AST although significantly reduced ($P < 0.05$) were still elevated above that of rats fed a balanced diet 72h after carbon tetrachloride intoxication (Nkosi *et al.*, 2005). It is apparent that pumpkin seed protein isolate is more effective in the alleviation of cellular damage due to acetaminophen intoxication than in the case of carbon tetrachloride intoxication.

The metabolism of acetaminophen (paracetamol) by liver cytochrome P-450 generates a product (N-acetyl-p-benzoquinone imine) that reacts with and removes sulfhydryl substances such as glutathione or protein-thiols (Dahlin *et al.*, 1984; Potter and Hinson, 1986; Hoffmann *et al.*, 1985). Loss of glutathione causes secondary oxidative damage, which contributes to hepatic failure in acetaminophen overdose (Halliwell, 1994). The presence of sulfhydryl groups

(1.39 $\mu\text{g mg}^{-1}$) in the pumpkin seed protein isolate could have contributed to replenishing the depleted thiol groups.

It is apparent that pumpkin seed protein isolate has promising antioxidative properties as revealed by free radical scavenging activity, chelating activity and antioxidant activity. Furthermore it can be concluded that the isolate administration was effective in alleviating the detrimental effects associated with protein malnutrition and acetaminophen intoxication as revealed by its effects on ALT and AST. Pumpkin seed protein isolate has the potential to be used as a dietary antioxidant and protein supplier to the endemic protein under-nourished groups in most developing regions.

Dedicated to the memory of CZN.

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