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In Vivo Antioxidant Potential of Protein Hydrolysates of some Cucurbitaceae Seed

*** Dash Priyanka [1], Rath Goutam [2], Ghosh Goutam [2]**

[1] School of Pharmacy and Life Sciences, Centurion University of Technology & Management, Odisha, India

[2] Department of Pharmacognosy, School of Pharmaceutical Sciences, Siksha 'O' Anusandhan, (Deemed to be University), Khandagiri Square, Bhubaneswar, Odisha 751003, India

ABSTRACT

Nowadays, dietary proteins play a very crucial role against oxidation, which is a fundamental process in the occurrence of many diseases. The aim of this study was to hydrolyze globulin fractions from *C. moschata* (CMH), *C. lanatus* (CLH) and *L. siceraria* (LSH), and to evaluate their *in vivo* antioxidant potential. The *in vivo* antioxidant potential was conducted using *in vivo* catalase and lipid peroxidation (LPO) assay methods. The total protein content was also estimated using Bio-Rad protein assay and bovine serum albumin methods. For *in vivo* study, ethanol was induced into test animals for toxicity and oxidative damage for 15 days. The catalase and LOP of serum / liver homogenate were determined. The results showed that globulin hydrolysates decrease the MDA level and increase the catalase level in normal mice. Based on these findings, this study provides information about *in vivo* antioxidant activities of *C. moschata*, *C. lanatus* and *L. siceraria* hydrolysates. Globulin hydrolysates of *L. siceraria* showed effective antioxidant properties *in vivo* models, whereas globulin hydrolysates of *C. moschata* and *C. lanatus* exhibited remarkable antioxidant properties, as well. Thus, the results suggest that globulin hydrolysates might be used as a novel source in reducing the risk of oxidative stressed diseases and development of functional foods.

Keywords: cucurbitaceae, globulin, antioxidant, trypsin

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***Address for Correspondence:**

Priyanka Dash, School of Pharmacy and Life Sciences, Centurion University of Technology & Management, Odisha, India

INTRODUCTION

Worldwide, it is estimated that 1.7 million deaths occur due to insufficient consumption of green vegetables and fruits. Inadequate intake of vegetables and fruits causes deaths in gastrointestinal cancer (14%), heart diseases (11%) and stroke (9%) every year[1]. Today, the diet rich in vegetables and fruits are being very popular as it can reduce the risk of various complicated diseases like cardiovascular and cancer. Additionally, fruits and vegetables are rich sources of various types of bioactive compounds that can fight against life threatening diseases and maintain good health[2]. Indigenous healthcare practices are comparatively cheaper and safer than modern healthcare services, which are too expensive. In today's research field, traditional knowledge on medicinal plants is quite helpful for the development of many lead molecules[3].

A hypothesis, "Let food be thy medicine and medicine be thy food" was first grabbed by Hippocrates. In 19th century, the presumption "food as medicine" was doubtful with the advent of modern drug therapy. Hence, the researchers started identifying bio-active components from food sources, which may reduce the risk of various complicated chronic diseases. For betterment of physiological health benefits and to minimize the risk factor of chronic diseases, there is a need for extensive investigation of food[4].

Recently, food science has been highlighted on the sequences of active peptides present in food proteins. The peptides are found to be inactive within the parent protein and can be liberated by proteolytic reactions within the gastro intestinal system during digestion. Currently, it is found that food derived peptides are excellent source of antimicrobial, antiviral, anticarcinogenic, anti-inflammatory, antioxidant and antihypertensive activities. These peptides can also be used for the maintenance of human health[5]. Though, the seeds are rich in nutritional pack, they are commonly discarded as waste products and a very less attention has been paid to their dietary efficacy. Recently, it is found that food security has become a real challenge in the society. So, discard of any food nutrients seems to be criminal and a great attention is required for the assessment of various therapeutic values of food nutrients[6]. On the other

hand, proteins are thought to be utilized in the formulation of food products to overcome the shortage of animal proteins. Today, the preparations of soy-proteins[7] and oil seeds are being a good indicator for the use of plant sources, especially seeds[8]. Now a day, seed proteins like soya protein has been used for food formulations due to their good functional properties. The use of soya proteins by many people is going to be declined due to their disagreeable order, allergic reactions, and other dietary restrictions[9]. To overcome these complications, the new source of proteins from some low cost of seeds is coming in the limelight of research.

Generally, bioactivities of protein hydrolysates are enhanced during hydrolysis of their peptide bond[10]. Protein hydrolysates are the mixture of amino acids and oligopeptides[11]. In comparison to native proteins, hydrolytic bioactive peptides are more effective in terms of digestibility, solubility, and absorption affinities due to their low molecular weight and less complex structure. The solubility of these bioactive peptides increases gradually during removal of peptide bonds from the polypeptides under enzymatic hydrolysis[12]. Low-molecular weight peptides are absorbed as a whole than hydrolysable proteins, but oligopeptides and polypeptides are commonly hydrolyzed before absorption in the intestinal mucosa. In comparison to the other naturally bioactive peptides, bioactive peptides obtained from food sources showed more multiplicity in their mode of action.

In India, the seeds of *Cucurbit moschata*, *Citrullus lanatus* and *Lagenaria siceraria* are edible, and available plentily. Nowadays, food safety confers real challenge in current and coming days. So, wasting of any food products may be treated as most illegal. That is why; special attention is needed to these seeds as a dietary component. In our previous research work, globulin was found most potent than other protein fractions, such as albumin, prolamin and glutelin. Therefore, the further investigation was performed using enzymatic hydrolysis of globulin fractions by trypsin, and their antioxidant properties were evaluated. Therefore, the objective of this study was to investigate *in vivo* antioxidant potential of protein fractions of globulin hydrolysate obtained from the seeds of three species of Cucurbitaceae (*C. moschata*, *C. lanatus* and *L. siceraria*).

MATERIALS AND METHODS

Extraction and purification of globulin fractions from *C. moschata*, *C. lanatus* and *L. siceraria* seeds

The fresh seeds were dried at 40°C and ground into a fine powder. The powdered sample (500 mg) was macerated with n-hexane (50 ml) for 48 h. The product was filtered using whatman filter paper (0.45µm) to get residue. The residue (200mg) obtained was homogenized in magnetic stirrer for 1 h using Tris HCl (50ml) at pH 8. It was then centrifuged at 10,000g for 20 min. The globulin fraction was separated as supernatant. All the globulin fractions were prepared from the rest seed powder following the same procedure and purified using acetone. All the fractions were lyophilized and stored at −20 °C for future use[13].

Preparation of hydrolysates of globulin fraction

The globulin hydrolysates were prepared as per the method described earlier[13]. The 5 mg/ml of globulin fraction from each *C. moschata*, *C. lanatus* and *L. siceraria* was treated with Tris-HCl buffer (50 mM, pH 7.5) and trypsin (0.08 mg/ml) at the ratio of 50:1. The solution was kept in incubator for 4 h, and then heated in water bath for 15 to 20 min. for inhibition of enzymatic hydrolysis. The supernatant was then collected after centrifugation at 10,000 g for 10 min., and stored at -20 ºC for future use.

Estimation of protein contents

The total protein content was determined as per the method previously described by Bradford (1976)[14]. In this method, bovine serum albumin (BSA) was used as standard. All the assay reagents along with test proteins were allowed to stand for 5 min at room temperature. The absorbance of this mixture was then measured at 595 nm for estimation of total protein content.

Experimental design

Wistar albino rats of either sex weighing 170-220 gm were obtained from the Department of Pharmacology, Siksha 'O' Anusandhan (Deemed to be University), Bhubaneswar. All animal procedures and protocols were approved by the institutional animal ethical committee (IAEC/SPS/SOA/09/2018). All the animals were kept in polypropylene cages and acclimatized at 25 ± 2 °C at 50–60% relative humidity to the laboratory environment for 1 week. The rats were maintained on normal laboratory food and water *ad libitum*, under controlled room temperature (25°C ± 2°C, 60-70% humidity) and 12-12 h light-dark cycle. The animals were feed normal laboratory food and water *ad libitum*. The rats were randomly divided into 6 groups (n=6 in each group). After administration of ethanol (50%), globulin hydrolysates (1 gm/kg BW) were administered orally to rats once in a day for a period of 15 days as presented below:

Group I: normal control,

Group II: negative control (50% ethanol)

Group III: ethanol + globulin hydrolysates of *C. moschata* (CMGH)

Group IV: ethanol + globulin hydrolysates of *C. lanatus* (CLGH)

Group V: ethanol + globulin hydrolysates of *L. siceraria* (LSGH)

Group VI: ethanol + ascorbic acid (0.2 gm/kg BW**)**

In this study, ethanol was induced into test animals for toxicity and oxidative damage for 15 days. After 15 days of treatment, all the rats were fasted for 12 h and then sacrificed by cervical dislocation. The blood samples were collected through direct heart puncture and centrifuged at 4000g at 4 °C for 15 min to separate serums. The liver of the anesthetized rats were dissected and liver was mixed with Tris–HCl buffer (50 mmol/l, pH 7.4) and homogenized. It was then centrifuged at 5000 g at 4 °C for 15 min. The supernatant was collected immediately and kept at −20 °C for further study[15].

In vivo catalase assay

The catalase activity of serum obtain from blood sample and liver homogenate was evaluated according to the previously reported method[16]. The reaction mixture was prepared by adding serum or liver homogenate (50 µL) to phosphate buffer (2µl) and hydrogen peroxide (1 ml). The catalase activity was measured at 240 nm for 1 min. One unit of activity is equal to 1 mmol of H_2O_2 degraded per minute and is expressed as units per milligram of protein.

In vivo lipid peroxidation (LPO) assay

Thiobarbituric acid (TBA), when reacts with malondialdehyde (MDA), it forms a pink colored diadduct, thiobarbituric acid reactive species (TBARS) which can be

measured at 532 nm. The LOP of serum / liver homogenate was determined according to the method described by Onoja *et al*., 2014[16]. The reaction mixture was prepared by adding 100 µL of plasma or liver homogenate to trichloro acetic acid (15 %), thiobarbituic acid (0.37 %) and HCl (0.25 N). The reaction mixture was heated at 80 ºC for 1h, and then it was cooled for 10 min. The cooled reaction mixture was centrifuged at 3000 g, and the absorbance was measured at 532 nm against blank. The MDA content was determined from the standard curve and expressed as nmol/L.

Biochemical assessment

At the end of 15 days of experimentation, the blood samples were collected by heart puncture method. Total lipids, Triglycerides, Total cholesterol, HDL-Cholesterol and LDL-cholesterol were estimated.

Statistical analysis

All the results were expressed as mean values ± standard deviation of three independent determinations. Statistical analysis was done using SPSS 11.0 (SPSS Inc., Chicago, IL, USA). The data were analyzed using ANOVA, followed by Duncan's test.

RESULTS AND DISCUSSION

Estimation of globulin contents

In *C. moschata, C. lanatus* and *L. siceraria,* globulin fraction was found as the major protein fraction and it was found to be 46 ± 0.3, 39 ± 0.1, 49 ± 0.3% respectively.

***In vivo* antioxidant activity of potent globulin hydrolysates**

Catalase enzyme checks the formation of hydroxyl radical and catalyzes the liberation of water and oxygen from hydrogen peroxide.

$$2H_2O_2 = 2H_2O + O_2$$

In the present study, changes in the activities of antioxidant enzymes in rat were evaluated. The level of catalase was significantly ($P < 0.05$) decreased in the negative control group (serum 0.50 ± 0.02 mM/lt; liver 0.41 ± 0.05 mM/gm), than that of normal group rats (serum 1.33 ± 0.01 mM/lt; liver 0.71 ± 0.06 mM/gm). These data clearly showed that the ethanol induced oxidative stress at *in vivo* level, which correspond to the alteration in antioxidant enzymes activity due to the presence of excess ROS. The level of catalase in serum and liver homogenate of rats, treated with CMGH was found to be 1.14 ± 0.02 mM/lt and 0.74 ± 0.02 mM/gm, respectively. Similarly, catalase level in serum and liver homogenate of rats treated with CLGH was registered 0.91 ± 0.02 mM/lt and 0.66 ± 0.04 mM/gm, respectively. The rats treated with LSGH exhibited a significant ($P < 0.05$) increase in the activities of these enzymes in the serum (1.31 ± 0.32 mM/lt) and liver (0.78 ± 0.01 mM/gm) when compared with the negative control rats. The results depicted in Table 6.5 revealed that ethanol induced oxidative stress reduced the enzymatic activity of antioxidant enzymes. So, the reduced enzymatic activity showed an oxidative stress in defense system of rats. These data are also consistent with the findings of Farbiszewski *et al.*, 1991[17] and Schisler *et al*., 1989[18]. Moreover, catalase can also oxidize trace amount of ethanol in the presence of a hydrogen peroxide to produce acetaldehyde resulting protection of vital parts of the body from oxidative stress[19]. Thus, globulin hydrolysates prevent oxidative stress by restoring antioxidant potential of catalase and maintain the normal cellular function.

***In vivo* lipid peroxidation (LPO) assay**

Reactive oxygen species are susceptible to react with polyunsaturated fatty acids which lead to lipid peroxidation (LPO). Malondialdehyde (MDA) is an endogenous genotoxic product and also the end products of LPO[20]. The amount of malondialdehyde (MDA) in serum and other vital parts of the body indicates the extent of lipid peroxidation. After acute alcohol administration, MDA levels was found to be increased in liver and blood contributing enhanced peroxidation and interruption of the antioxidant defense mechanisms. In the present study, LPO is evaluated by measuring the amount of MDA present in serum and liver homogenate. A significant increase in MDA levels in the rats of negative control group (serum 16.36 ± 1.44 nM/lt; liver 19.69 ± 0.15 nM/gm) than that of normal group (serum 6.80 ± 1.02 nM/lt; liver 10.21 ± 0.69 nM/gm). In CMGH treated rats the MDA level in serum and liver homogenate was found to be 9.84 ± 0.97 nM/lt and 14.14 ± 0.98 nM/gm, respectively. In case of LSGH the amount of MDA in serum was registered 6.59 ± 1.69 nM/lt and in liver it is found 10.68 ± 1.07 nM/gm. Similarly, in CLGH treated rats, the MDA level in serum and liver homogenate was found 13.05 ± 0.60 nM/lt and 17.32 ± 0.99 nM/gm, respectively. In the present study, Table 6.5 showed that MDA level was increased in control groups, whereas in all globulin hydrolysates treated groups MDA level was found to be decreased.

The results of this study are also in agreement with the findings of other investigators[17]. The muscle protein hydrolysate (*Otolithes ruber*) and soya protein are reported to enhance the *in vivo* antioxidant capacity [15,21], which is in consistent with our results. Ethanol is commonly known as the abused substance by the society. It induces a significance increase in levels of ROS in plasma and tissues. In the present investigation, it was found that ethanol significantly increases the MDA levels in serum and liver and impaired the antioxidant mechanism. Globulin hydrolysates significantly reversed these changes causing a significant reduction of MDA levels both in serum and liver and showing its defensive effects against ROS. The findings of this are also similar to the report published earlier by Oyenihi *et al.*, 2016[22] showing a significant decrease in MDA levels in organs after administration of watermelon juice against ethanolic induced oxidative damage.

Table 1 Effect of CMGH, CLGH and LSGH on antioxidant enzyme activities in rats exposed to ethanol

Group	Catalase activity		Lipid peroxidation	
	Serum (mM/lt)	Liver (mM/gm)	Plasma (nm/lt)	Liver (nM/gm)
Normal	1.33±0.01[a]	0.71±0.06[a]	6.80±1.44[d]	10.21±0.69[d]
Toxic control	0.50±0.03[c]	0.41±0.05[c]	16.36±1.44[a]	19.69±0.87[a]
CLGH	0.91±0.02[b]	0.66±0.04[b]	13.05±0.6[b]	17.32±0.99[b]
CMGH	1.14±0.02[a]	0.74±0.02[a]	9.84±0.97[c]	14.14±0.98[c]
LSGH	1.31±0.32[a]	0.78±0.01[a]	6.59±1.69[d]	10.68±1.07[d]
Ascorbic acid	1.32±0.03[a]	0.77±0.06[a]	6.78± 1.07[d]	10.28±0.78

Values are expressed as mean ± SD for 6 animals. Values within column with different superscript letters are significantly different ($P < 0.05$)

The results showed that the globulin hydrolysates are antioxidant in action that react with free radicals, and may reduce the occurrence of free radical damage living organisms. Li et al, 2003[23] reported that phenylalanine is responsible to enhance the antioxidant potential of peptides as phenylalanine basically reacts with OH free radical to form a stable compound[23]. This finding is found to be correlated to the previous study described by Fan *et al.* (2012)[24]. The presence of sulfhydryl group of cysteine in globulin hydrolysates may be responsible for reduction of free radicals.

Hydrophobic amino acids such as glycine, alanine, valine and leucine exhibit significant antioxidant activity as they easily pass through the cell membrane[25]. It is previously reported that LSH is rich in hydrophobic amino acids with compared to CMH and CLH[13]. Alkaline amino acids including histidine, arginine and lysine reported to have a strong antioxidant activity[26].

Biochemical assessment

Fig. 1 showed that administration of alcohol caused a significant increase in total lipids, as compared to that of control group. Total lipid concentration of toxic control was found to be 510.54±1.6 (mg/dL). This increase in total lipid content may be due to the administration of alcohol. The effect of CLGH, CMGH, and LSGH on serum lipid concentration was significantly decreased in treated rats. These results suggest that LSGH might preserve the liver against lipid accumulation and peroxidation.

In this study, serum triglycerides of rats treated with alcohol showed a significant increase as compared to the control group. The increase level in triglycerides may be due to the degradation of phospholipids which results increase in lipid peroxidation[27, 28].

On the other hand, after alcohol intoxication, the rats treated with the CLGH, CMGH, LSGH showed a significant decrease in serum triglycerides concentration as compared to the control group. This finding suggested that the protein hydrolysates may preserve the liver against lipid accumulation.

In the present work, it was found that serum total cholesterol concentration of rats treated with alcohol increases significantly. This finding suggested that alcohol exerts significant toxic effect on serum total cholesterol. Regarding the effect of the protein hydrolysates on serum total cholesterol concentration of rats, the present study revealed a significant decrease in serum total cholesterol of rats treated with CLGH, CMGH and LSGH. Hypercholesterolemia is known to be a risk factor for coronary artery disease (CAD), and LDL-cholesterol also plays a crucial role in the pathogenesis of CAD[28].

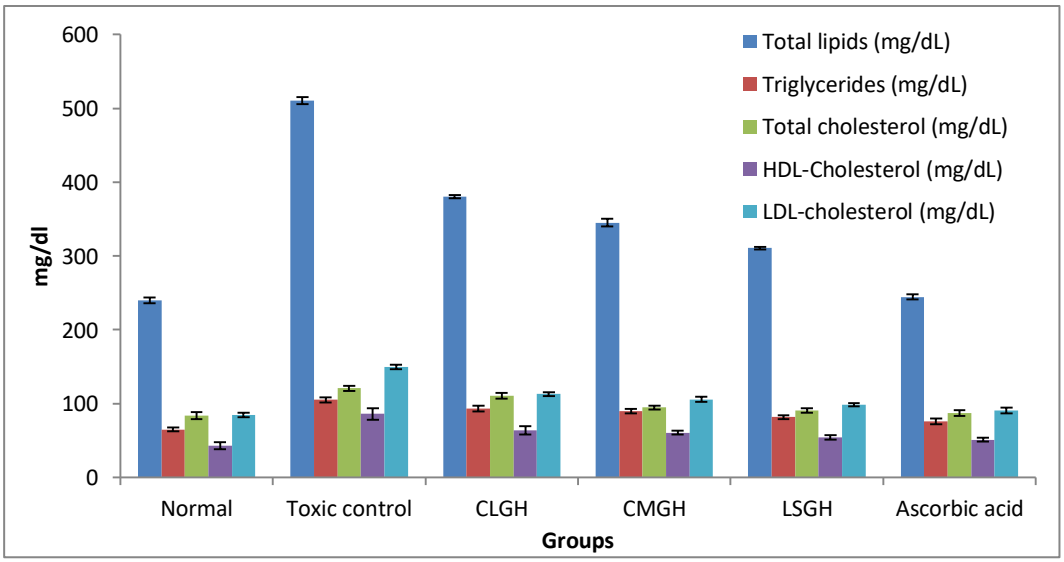

Fig. 1 Effect of the CMGH, CLGH and LSGH on post-treatment on lipids profile parameters of rats

It the present study, rats treated with alcohol showed a significant increase in serum HDL and LDL-cholesterol as compared to the control groups, whereas the rats treated with the CLGH, CMGH and LSGH showed a significant decrease in serum HDL and LDL-cholesterol levels.

CONCLUSION

The study indicated that, hydrolysates of *C. moschata*, *C. lanatus* and *L. siceraria* seed were prepared, and their *in-vivo* antioxidant effects were evaluated. It was showed that administrations of hydrolysates reduce the MDA level and increases the catalase level in normal mice. Therefore, the present study provides important information about the *in vivo* antioxidant activity of *C. moschata*, *C. lanatus* and *L. siceraria* hydrolysate. So, it can be suggested that the hydrolysate prepared from *C. moschata*, *C. lanatus* and *L. siceraria* has potential for applications in pharmaceutical and food industries.

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