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Toxicity Assessment of Fungal Chitin obtained from *Aspergillus niger* and Evaluation of its Antioxidant Potential in BALB/c Mice

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ABSTRACT: N-acetyl glucosamine, a component of the fungal cell wall and a biopolymer with several industrial uses, including the food industry, makes up chitin. Therefore, in the present study, the fungal chitin obtained from *Aspergillus niger* intended to be used in the food industry was assessed for its toxicity and evaluated for antioxidant potential in a BALB/c mouse model. For this, the test chitin was administered orally to six groups of mice, with five mice in each group. Animals were divided into several groups and given variable amounts of chitin, with one group being designated as the "control". The study was conducted for 14 days, and various affecting parameters, including clinical, behavioral, and physical patterns, mood, sleep, eating pattern, weight, fur, and skin, were observed along with histopathology studies. The results obtained revealed no adverse effect of chitin on mice. Alanine aminotransferase (ALT) and aspartate transaminase (AST) examinations also revealed that chitin is within safe limits to be used in various food applications. The maximum dose of chitin administered was 1000mg/kg body weight of mice, while the minimum dose was 100mg /kg body weight of mice. According to histopathology findings, even the maximum dose of chitin (1000 mg/kg body weight) was safe and had no adverse effects on the gut, liver, or kidney. An *in vivo* study of the blood sample revealed that the test chitin had good antioxidant properties concerning standard ascorbic acid. No challenges were faced while conducting the study. Chitin serves as an good antioxidant agent. This can be used to replace the synthetic antioxidants as it shows also similar effect.

Keywords: *Aspergillus niger*, chitin, antioxidant, *in vivo*, toxicity.

Abbreviations: IAEC: Institutional animal ethical committee, CPCSEA: Committee for the purpose of control and supervision of animals, PU: Panjab University, PBS: Phosphate buffered saline, ALT: Alanine aminotransferase, AST: Aspartate transaminase, IFCC: International Federation of Clinical Chemistry SOD: Superoxide dismutase, CAT: Catalase, GSH: Glutathione reductase.

INTRODUCTION

Chitin is the second-most abundant polysaccharide after cellulose. It is present in the cell wall of fungi and in the exoskeleton of insects, molluscs, cnidaria, brachiopods, and crustaceans. Chitin is unique, as it is biodegradable and biocompatible. Because of these properties, chitin is widely used in the pharmaceutical, medicine, food, agriculture, textile, and paper industries. The traditional source of chitin is the exoskeleton of crustaceans and insects. However, due to issues with seasonal constraints, environmental pollution, and restricted supply in some areas, the industrial isolation of this polysaccharide is diminished (White *et al.*, 1979).

The intestine, liver, and kidney are the most vital organs that play a pivotal role in physiological functions. The various metabolic processes performed by these organs are secretion, storage, retention, filtration, excretion, and absorption. The liver also has the ability to detoxify poisonous chemicals (Sahani, 1999). The main causes of liver disease, which affects people all over the world, are toxic chemicals (such as certain antibiotics, chemotherapeutics, peroxidized oil, chlorinated hydrocarbons, etc.), excessive alcohol use, infections, and autoimmune diseases (Muriel and Rivera 2008). The majority of hepatotoxic substances harm liver cells by causing lipid peroxidation and other oxidative liver damage. In the absence of reliable liver protective drugs in modern medicine, there exists a challenge for pharmaceutical scientists to explore the potential of hepatoprotective activity using natural sources (Raghuveer and Tandon 2009).

According to various studies, the natural, non-toxic bioactive compound chitin is essential in preventing oxidative damage brought on by an excess of free radicals. Oxidative stress (OS) is essentially an imbalance between the body's capacity to neutralize free radicals such as superoxide (O-2), hydroxyl (OH), and peroxyl (ROO) to counteract or detoxify their negative effects. Cancer, atherosclerosis, Parkinson's, Alzheimer's, diabetes, neurological illnesses, and aging are just a few of the chronic and degenerative diseases (Yu, 1994) that cause oxidative stresses due to the overproduction of these radicals. Various synthetic antioxidant substances, including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), and propyl

gallate (PG), have demonstrated effectiveness in preventing oxidative damage brought on by free radicals in human tissue (Issa *et al.*, 2006; Kaliora *et al.*, 2006). However, the use of synthetic antioxidants is now limited because of their toxicity and undesirable effects on human health (Bajpai *et al*., 2014; Jonker *et al.*, 2010). It is a challenge for pharmaceutical scientists to investigate various natural sources of drugs to protect the intestine, liver, and kidney because there are no effective protective drugs for them. These vital organs play a crucial role in various physiological functions such as secretion, storage, retention, filtration, excretion, detoxification, and absorption. Therefore, in the present study, an attempt has been made to isolate natural chitin from a fungal source using *Aspergillus niger*. Additionally, its non-toxic and antioxidant effects on various important organs in animal models (BALB/c mice) have been studied as per standard CPCSEA and IAEC guidelines, following good laboratory practices.

MATERIALS AND METHODS

Experimental Animals. Female albino BALB/c mice approximately 6–8 weeks weighing 20-25 gm were obtained from Central Animal House, Panjab University, Chandigarh, India. Animals were housed in standard cages with paddy husk as bedding and fed a solid diet and water *ad libitum.*

Mice were exposed to a 12-h light/dark cycle at room temperature. They were handled by established protocols for the care and use of laboratory animals (CPCSEA guidelines) and were identifiable by color marks on their bodies. Mice were acclimatized to the above- mentioned conditions for one week before toxicity studies. Ethical approval and necessary permissions for handling mice were obtained from Institutional Animal Ethical Committee (IAEC), Panjab University, Chandigarh, India (Approval No. PU/45/99/CPCSEA/IAEC/2022/667).

Chitin Production. Chitin was produced under submerged fermentation using *Aspergillus niger* isolated from the soil sample and identified using 18srRNA analysis. The extraction of chitin was done as per the modified method (Johney *et al.*, 2016) in potato dextrose broth. The medium was autoclaved for 15 minutes at 15 psi pressure, and after cooling it was inoculated with pure cultures of *Aspergillus niger* and incubated at 28±0.5ºC for 10 days. After incubation, the fermentation broth was centrifuged at 5000 rpm for 10 min to separate the mycelial biomass, which was then washed and dried. The extraction of chitin was done using 1gm of the dried mycelial biomass in a clean conical flask and treated with 10 ml of 60% aqueous NaOH solution and incubated at 130º C for 2-3 hours to remove proteins, lipids and alkali soluble polysaccharide present in the mycelium. After being treated with NaOH, the remaining insoluble material (chitin) was cleaned with ethanol, dried, and weighed before being mixed with PBS (phosphate-buffered saline) in varying amounts and fed to mice.

Acute Toxicity Study. A total of 30 mice obtained from Central Animal House, Panjab University Chandigarh, India were used in the experiment. These were divided into 6 groups with 5 mice in each group. Mice in Group I (weighing average) 20.1 gm were kept as the control group which was fed with 100 µl PBS. Mice in Group II weighing 20.8 gm (average weight) were fed 100 mg /kg body weight of chitin soluble in PBS (100µl). Mice in Group III weighing 22.44 gm (average weight) were fed 500 mg /kg body weight of chitin soluble in PBS. Mice in Group IV weighing 20.58 gm (average weight) were fed 1000 mg /kg body weight of chitin soluble in PBS. Mice in Group V (weighing on average) 22.7 gm were fed 100 mg /kg body weight of ascorbic acid to be used as standard in the antioxidant study. Mice in Group VI (weighing on average) 21.56 gm were fed 75 mg /kg body weight of paracetamol to give them oxidative stress. The administration was done orally. All groups were kept in different cages with full air ventilation, feed and water *ad libitum*. Dosing was done daily and weight was taken every day for 14 days. After the experiment, the animals were observed daily for clinical and behavioral changes, physical patterns, mood, sleep, eating pattern, weight, fur, and skin.

Collection of liver, kidney, small intestine and serum. At the end of the experiment, all feeds were removed 14 h before anaesthesia. All animals were anaesthetised using ketamine 50 mg /kg of body weight, sacrificed and the liver, kidney and small intestine of each animal were dissected out, washed in ice-cold saline, patted dry and weighed. All organs were preserved in 10% formalin for further histopathological examinations. The blood samples were obtained using retroorbital puncture, collected in tubes and allowed to clot by leaving them undisturbed for 10-15 min at room temperature. The tubes were centrifuged at 3000g for 10 min to obtain serum as supernatant, which was stored at -20◦C for further use in *in vivo* antioxidant studies.

Assessment of liver functioning. The estimation of alanine aminotransferase (ALT) and aspartate transaminase (AST) was performed as per methods recommended by the International Federation of Clinical Chemistry (IFCC) using ERBA test kits (ERBA Diagnostics, Manheim, Germany). A tissue homogenate of the liver was prepared for assessing liver functioning (Kumari *et al.*, 2016).

Determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity. 0.1 ml of liver tissue homogenate was added to 1.0 ml of ALT/AST reagent, mixed well, and a change in absorbance per min (ΔA/min) was read at 340 nm. The enzyme activity was expressed in international units/ liter (IU/L) (Gudipati *et al.*, 2015).

Calculations. The change in absorbance was converted into an international unit (IU) of activity using the formula (1):

$$\text{Activity of ALT/AST} = \frac{(\Delta A/\text{min}) \times \text{T.V.} \times 10^3}{\text{S.V.} \times \text{Absorptivity} \times \text{P}} \quad (1)$$

Where:
T.V. = Total reaction volume
S.V. =Sample volume

Absorptivity = Millimolar absorptivity of NADH at 340 nm = 6.22
P = Cuvette path length (cm) = 1 cm
The activity of AST/ALT (IU/L) = (ΔA/min) × Factor (1768)

Histopathological examination of the liver, kidney, and small intestine. The organs separated from mice (liver, kidney and small intestine) were then processed for histopathological examination (Garg *et al.*, 1987; Liang *et al.*, 2011). Tissues were dehydrated in different grades of alcohol, i.e., 70%, 80%, 90%, and absolute alcohol twice for 30 min, 40 min, 45 min, and 1 h at room temperature. Finally, tissues were dipped in molten paraffin wax and were quickly cooled to prevent crystallization. Thin sections (5.0 µm) of the tissues were cut with a fine razor attached to a Spencer microtome (Sipcon, SP 1120) and embedded tissue sections were kept in a water bath at 50◦C to remove the wax. Sections were placed on a separate slide coated with Mayer's adhesive fixative and were dried by keeping them in a hot air oven at 60◦C for 20 min. Slides were treated with xylene to remove wax and alcohol, rinsed in water and stained in hematoxylin (15-30 min) followed by rinsing in water again. Thereafter, slides were differentiated in acid alcohol till only nuclei were stained and rinsed in water. Then, they were kept under running water for 20 to 30 min, stained in eosin till sections were bright red, and washed in running water till eosin was differentiated and blot dried. The sections stained with hematoxylin and eosin stains (H & E) were mounted in DPX. After mounting in DPX, slides were examined under a microscope for histopathological changes. The results of treated animals were compared with those of control animals.

***In vivo* antioxidant assay.** Three assays of biomarkers of oxidative stress were conducted. The superoxide dismutase (SOD) assay was conducted as per Kakkar *et al.* (1984). SOD catalyzes superoxide radicals that result in oxygen and hydrogen peroxide. In this 20 µl serum sample, sodium pyrophosphate buffer (0.1µmol/l, pH 8.4), phenazine methosulphate (186 µmol/l), nitrosonium tetrazolium (100 µl/l) were added in the test tube. Reduced nicotinamide adenine dinucleotide (500 µmol/l), of which 200 µl was added to the mixture to initiate the reaction. The mixture was then incubated at 30◦C for 90 seconds. Glacial acetic acid (1 ml), was added to stop the reaction. The amount of chromogen formed was measured at 560 nm against the blank. Results were expressed in U/ml.

Catalase (CAT) assay was conducted as per Claiborne, (1985). The assay is based on the degradation of hydrogen peroxide (H_2O_2) using catalase enzyme. 50 mM of potassium phosphate buffer (pH 7.4), 20 mM of H_2O_2 and 20 µl of serum sample were mixed and the reaction proceeded. The consumption of H_2O_2 was monitored spectrophotometrically at 570 nm for 1 min and the enzymatic activity was calculated according to the formula (2):

$$K = 2.303/T \times \log (A1/A2) \quad (2)$$

where K is: Rate of reaction, T is: Time interval, A1: Absorbance at time zero and A2: Absorbance at 60-second interval. Enzyme activity was expressed in U/min.

Glutathione reductase (GSH) was performed as per Ellman, (1959). The assay was based on the oxidation of GSH using 5,5'-disthiobis (2-nitrobenzoic acid) (DNTB). The two compounds reacted and generated 2-nitro-5-thiobenzoic acid (NTB), which is yellow. The GSH concentration was measured at 412 nm. For the assay, 50 µl of serum was diluted in 10 ml of a phosphate buffer (0.1M, pH 8). In this, 20 µl of DNTB (0.01M) was added. Absorbance was taken at 412 nm and expressed as U/ml.

Statistical analysis. The values were expressed as the mean ± standard deviation of two observations. A one-way ANOVA was used for the statistical analysis. $P < 0.05$ was considered significant.

RESULTS

Clinical and Behavioral Observations. Adult mice used in an acute toxicity investigation of chitin showed no differences in their physical patterns, sleep, eating habits, fur, or skin. From the result of the acute toxicity study of chitin in adult mice, no difference in physical patterns, mood, sleep, eating pattern, fur, and skin was observed. No change was observed in their morphology and all mice appeared to be normal with no hair loss and swelling on their bodies. However, the weight of mice slightly increased with an increase in the dose of chitin. All the mice were active till the last day and none of the mice died untimely (Fig. 1).

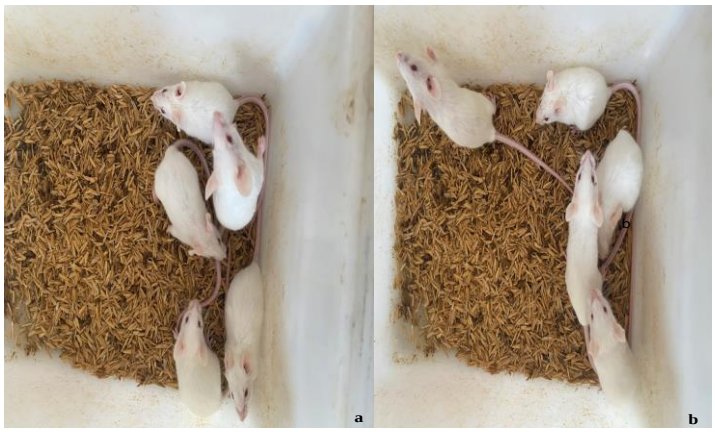

Fig. 1. The mice on day 1 (a) and day 14 (b) were equally active. No signs of swelling, abrasions, or hair loss were observed.

Histopathology study of the mice. Histopathology results showed that none of the vital organs (kidney, liver and small intestine) showed any toxic effect of chitin. The test samples showed the results of histopathology with the highest dose of chitin, i.e., 1000 mg/kg body weight of mice. There was no sign of slight degeneration or necrosis in the liver sample. No signs of inflammation were observed in the hepatic parenchyma. In the kidney, no signs of damage or renal tubulointerstitial fibrosis were observed. Similarly, no sign of lesions was observed in the small intestine. The results showed no change in the sections of these organs (Fig. 2).

Assessment of liver functioning. The effect of chitin toxicity on liver function was also assessed after continuous oral administration of chitin for 14 days. Two well-known liver-functioning markers were assessed, i.e., ALT and AST. Because both ALT and AST were unaffected by chitin in the treatment group, the findings of the present investigation indicated that liver physiology was normal (Table 1).

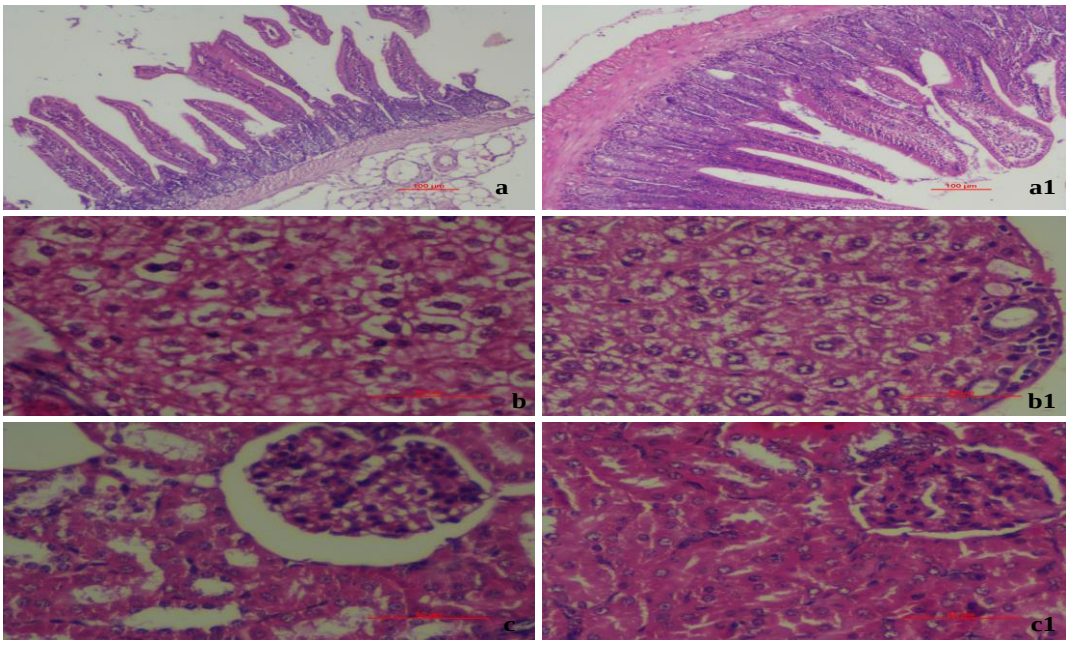

Fig. 2: Histopathology findings for the following animal organs: a and a1 represent the small intestine control and test samples; b and b1 represent the liver control and test samples; and c and c1 represent the kidney control and test samples.

Table 1: The AST and ALT results of control (Group I) and test (Group IV) sample of mice liver. Each value is a mean average of 5 mice. No significant difference between control and test sample were observed. P-value < 0.05 is significant.

	OD at 340 nm (0min)	OD at 340nm (1min)	ΔT×1768 (IU/l)	Normal Range (IU/l)
AST				
Control group I	1.229 ± 0.00068	1.202 ± 0.001	47.736 ± 0.25	7-56
Test group IV	1.224 ± 0.001	1.200 ± 0.24	42.432 ± 0.001	7-56
ALT				
Control group I	1.719 ± 0.25	1.708 ± 0.14	19.448 ± 0.028	5- 40
Test group IV	1.704 ± 0.27	1.695 ± 0.001	15.912 ± 0.001	5- 40

AST – Aspartate aminotransferase ALT- Alanine aminotransferase

***In vivo* antioxidant effect of chitin in mice's serum.** Oxidative stress markers such as SOD, CAT, and GSH were evaluated in mouse serum. Chitin at 1000 mg and 500 mg showed a significant increase in the biomarkers for all three enzymes (Fig. 3). The chitin at 100 mg showed increased oxidative marker activity but was less than the standard ascorbic acid. Group VI mice that were given a dose of paracetamol only showed the least number of markers compared to the control. The results showed that there was an increase in the oxidative stress markers with an increase in the dose of chitin. Hence, chitin showed a good antioxidant effect. The markers increased in response to the reactive oxygen species attempts to destroy them and inhibit the production of free radicals.

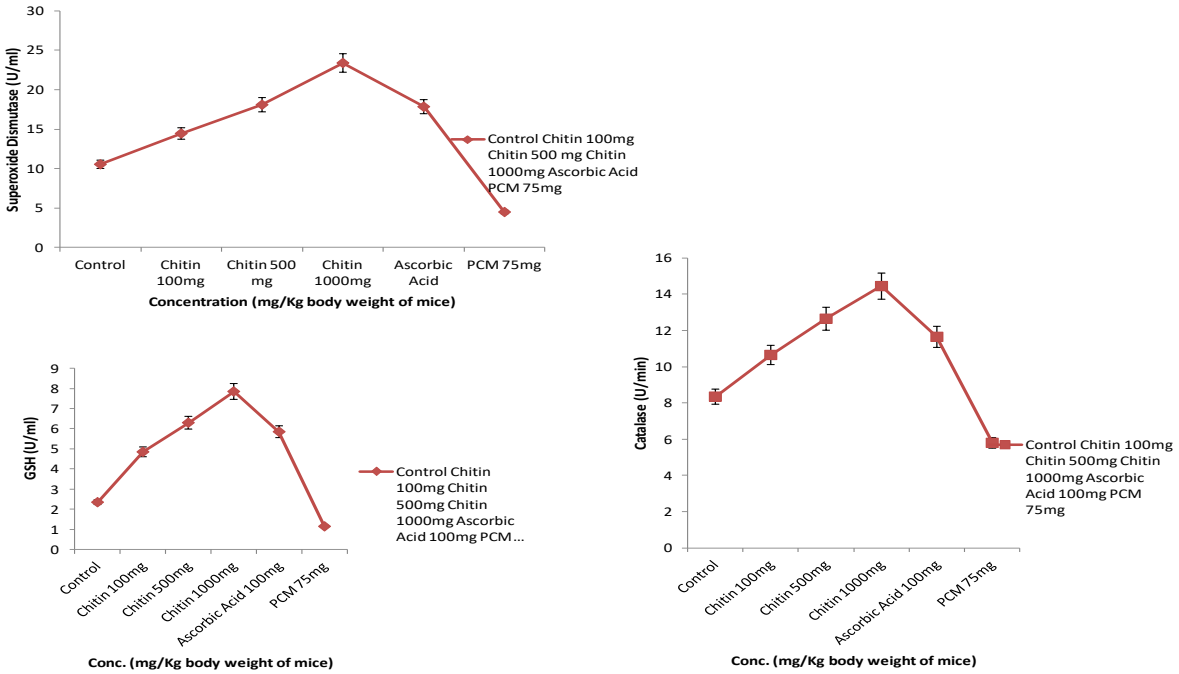

Fig. 3. The impact of various chitin dosages on SOD, CAT, and GSH. The mean minus the standard deviation of two observations was used to express all values. A P-value of 0.05 is significant.

DISCUSSION

The chitin used in the present study was extracted from *Aspergillus niger*, which has numerous advantages over crustaceans' chitin. Chitin synthesis from fungi has several advantages over sea waste. The availability of fungal mycelium can be obtained through a convenient fermentation process that is not geographically or seasonally constrained. While the supply of crustacean wastes is constrained by the seasons and locations of the fishing business, fungus mycelia contain a lower amount of inorganic elements than crustacean wastes, so demineralization treatment is not required during processing. Fungal chitin has very stable qualities due to regulated fermentation conditions and can find usage in numerous industries, including the food industry, unlike crustacean chitin, which might fluctuate in physicochemical properties. Therefore, in this study, the toxicity of the test chitin was evaluated. The result obtained revealed no toxicity, and therefore, it can be used in various food applications. The results also showed that the oral administration of chitin has no toxic effect on the behavior or physiology of mice. The results also indicated that the chitin given to the study's animals decreased oxidative stress and markedly improved the antioxidant activity of glutathione reductase (GSH), catalase, and superoxide dismutase (SOD). Additionally, it also stimulated the defense systems of the organisms. Therefore, the rise in these enzyme activities suggested that chitin, which has strong antioxidant activity, can counteract the reactive oxygen species and free radicals created inside of our bodies.

The results of the acute toxicity study are in correspondence with another study of the oral toxicity of the chitin glucan complex in rats (Jonker *et al.*, 2010). Amelia *et al.* (2021) studied *in vivo* chitin toxicity for 14 days and results revealed that no deaths and abnormalities in behaviour, bodyweight or organ weight were observed. However, another study conducted by Tanaka *et al.* (1997) said that mice in the chitin-administered group had poor appetites for several days at the start and then normalized. In one study, twenty overweight and hyperglycemic men were given chitin capsules containing 4.5 g of chitin per day before meals for one month. On days 0, 14, and 28, blood samples were analyzed for cholesterol, triglycerides, glucose, and antioxidant parameters such as glutathione and oxidized LDL. Compared with the placebo group (ten subjects consuming 4.5 g kaolin /day), oxidized LDL and oxidized glutathione decreased at the end of the treatment period. Importantly, the treatment was well tolerated, and no adverse events were reported (Deschamps *et al.*, 2009).

Many studies have been conducted on the antioxidant activity of chitin and chitosan. The antioxidant activity of chitosan was studied in vitro and *in vivo* (Liu, 2008). In that study, chitosan was added to crude rapeseed and lard oil, and it was observed that chitosan significantly increased the activities of SOD, CAT, and GSH enzymes, indicating that chitosan regulated the antioxidant enzymes activities and decreased lipid peroxidation. Animal studies have shown that Chitooligosaccharides (COS) can significantly increase rats' overall antioxidant capacity and superoxide dismutase (SOD) activity as well as significantly decrease serum levels of malondialdehyde (MDA) (Yuan *et al.*, 2009). In another study, one more group was added to chitin, i.e., the propyl group, and its antioxidant activity was evaluated in vitro (Zhang *et al.*, 2012). -carotene-linoleic acid values of propyl chitin and propyl chitosan at 0.8 mg/mL were up to 91% and 96%, respectively, while that of chitosan was 40%.

CONCLUSIONS

In conclusion, this study was conducted to investigate the toxicity effect of chitin in BALB/c mice and its antioxidant activity *in vivo*. The results showed that oral administration of chitin has no toxic effect on the behavior or physiology of mice. In addition to it, chitin has good antioxidant activity as it increases the levels of superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) significantly. Histopathology examination revealed that chitin has no signs of necropsy, abrasions, or any toxic effects. The AST and ALT levels revealed good liver functioning with chitin.

FUTURE SCOPE

There is an increased demand of natural antioxidants because of their non toxic nature. Also limited natural antioxidants are present in the market. Hence fungal chitin appears to be a good alternative for synthetic antioxidants.

Ethical Approval. Ethical approval and necessary permissions for handling mice were obtained from Institutional Animal Ethical Committee (IAEC), Panjab University, Chandigarh, India (Approval No. PU/45/99/CPCSEA/IAEC/2022/667).

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Conflict of Interest. None.

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