

Encapsulation of Nano Beta-Glucan for Preservation of Functionality and Targeted Delivery of Bioactive Food Component

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Abstract: *Pleurotes ostreatus*, provides bioactive compounds in which several polysaccharides are claim to posses cancerous and immune modulatory properties. Encapsulation enhanced bioavailability, stability and shelf-life of these sensitive ingredients. The purpose of this study is to analyse the physiochemical properties of its nano β -glucan encapsulated in nano form that possesses bioactivity as lowering cholesterol levels. Biological evaluation showed that oral administration of the nano beta-glucan capsules at levels 100 & 200 mg for 4 days consecutive days indicating its hepatoprotective agent, and have a great potential for use in industrial scale as emulsifier and flocculating agent as well. So, nanotechnology could also help the consumers to modify the food depending on their own nutritional needs and tastes, and in the creation of functional foods. However, from health viewpoint, the potential risks of nanoscale materials and the possibility of the accumulation and translocation of nanoparticles in the body should be avoided and needs more studies to be addressed.

Key Words: *Pleurotus ostreatus* beta-glucan, nanotechnology, capsulation, thermal properties, hepatoprotective agent.

Introduction:

In the last years, it has been suggested that the daily use of products with anti -mutagenic and anti-carcinogenic activities may be an efficient way for preventing cancer. This approach is known as chemoprevention^(1,2).

In the diet, several products or compounds of different origins (e.g. cereals and fungi) contain chemopreventive properties. There are different medical indications:

It is used against stress, to stimulate the immune system, for improving the life of diabetics, to lower cholesterol, and against osteoporosis, among several other bene-fits.

Mushroom, possessing spores without chlorophyll, grows between living and dead creatures. This plant has been recognized to exhibit some living characteristics, such as heterotrophy, saprophyte, mutualistic and parasite⁽³⁾.

Some harmless mushrooms, called edible mushrooms, have been widely cultivated as foodstuff with high economical value. Many edible mushrooms, such as, *Auricularia* sp., *Flammulina velutipes* *Lentinula*

edodes, *Pleurotus* sp., *Tremella fuciformis* dan *Volvariella* sp., are extensively developed. Some of which has been utilised for health improvement, since they exhibit many pharmacologically active compounds⁽⁴⁾.

One of the mushroom's special qualities is its capability to convert cellulose and lignin to polysaccharide and cholesterol free-proteins. With this interesting capability, the phyto-protein contents in mushroom are almost equal to or greater than of chlorophyll plants and its fat and calorie content are less than that of meats, making it very suitable as foodstuffs for diet purposes. Moreover, consuming certain mushrooms regularly is able to prevent hypercholesterolemia, thus, reducing the risk of hypertension. Considering those benefits, therefore, most people are accustomed to consuming various edible mushrooms as daily food⁽⁵⁾.

Polysaccharides of the β -glucan type are attracting attention due to their antitumor⁽⁶⁾, antiproliferative⁽⁷⁾, antigenotoxic and antioxidant^(8, 9), and anti-mutagenic^(8, 10, 11) activities and their capacity to increase the expression of the transcription factors c-Jun/AP1 in breast cancer cells⁽¹²⁾. Glucans are reported to elicit immune response through activation of macrophages by a specific interaction of β -glucan with an immune cell-specific (1,3)- β -glucan receptor or Dectin-1 receptor⁽¹³⁾. Beta-glucans are a heterogeneous group of natural polysaccharides mostly investigated for their immunological effects. Due to the low systemic availability of oral preparations, it has been thought that only parenterally applied beta-glucans can modulate the immune system⁽¹⁴⁾.

Most mushroom polysaccharides are polymer from glucose with glycosidic bond such as glycosidic β -(1-3), (1-6) or β -(1-4) with branch at β -(1-6). This polymer form is distinctive for interconnection mechanism with either amino acids in proteins or nucleotides in nucleic acids which can only interconnect in one way, whereas, polysaccharide in some positions, making it is able to bring different biological information^(15, 4).

The mushroom's cell-wall is composed of carbohydrates with β -glucan bond, proteins and lipids, however, its structure is different than that of in higher species, including in mammals^(16, 17).

The aim of this research was to extract β -glucan in nano particles from oyster mushroom by using supercritical fluid CO₂, studying its physical properties as well as identified its functional groups and testing its hepatoprotective agents.

Materials & Methods:

Sample preparation:

Oyster mushroom (Mycelia & Whole) was obtained from the special unit of "Production, Cultivation & Utilization of Mushroom", NRC, Egypt.

Oyster mushroom (Mycelia & Whole) was treated with 1 % sodium metabisulphite for 3 min and homogenized using a Sonicator (Ultrasonic processor) XL No. 2015-010 in dark place, then placed in petri dish and stored in a refrigerator at -50 °C for 1 day until freeze. Then, mushroom was freeze-dried (LABCONCO, Kansas City, USA) at (-50 °C & 0.014 mbar) for 2 days to reach moisture content 4 %. Mushroom powder was ground and stored in a refrigerator at 80 °C until use.

Supercritical extraction of Nano β -Glucan

Extraction of β -Glucan by Supercritical CO₂ from mushroom was carried out by using supercritical fluid at 60°C temperature & 300 bar pressure⁽¹⁸⁾.

Physical & chemical Properties

1-Transmission Electron Microscopy (TEM)

The morphology of extracted Nano β -Glucan was measured using the TEM (JED 1230, JEOL Ltd., and Tokyo, Japan) using freeze-fraction replica method⁽¹⁹⁾.

2-Fourier Transform Infrared Spectroscopy

It is used to study the materials that are uniformly transparent or opaque in the visible spectrum, but have significant absorption transmission bands in the 700 nanometre plus wave length region. It is used to identify the functional groups and the extracted nano β – Glucan was subjected to FT / IR - 600 Fourier Transform Infrared Spectroscopy (Jasco, Japan) and the structure of polysaccharides were prepared by using potassium bromide disks ⁽²⁰⁾.

3-Flocculating test

Flocculating test was carried out in a test tube containing a mixture of 10 ml of 0.5 % activated carbon (SD fine- Chem. Limited, MUMBAI, India) and 100 μ l of 1 % CaCl_2 by the method of Nakamura *et al.* ⁽²¹⁾. To the test tube, 100 μ l of 0.02 % nano poly saccharine solution β –Glucan was added and mixed well to make a suspension. The resulting suspension was observed during incubation at room temperature. A similar experiment was carried out using water, xanthan gum, guar gum, Arabic gum and locust gum as a control.

4- Lipid Emulsifying Test:

Emulsifying effect of Nano polysaccharide (β –Glucan) was tested by the method of Yasumatsu ⁽²²⁾ with some modifications.

Equal volumes of various oil and polysaccharide solution in distilled water were shaken for 10 min at 150 rev min^{-1} on a rotary shaker to make a lipid emulsion. After the emulsion was centrifuged at 2000g for 5 min using a swing-out rotor, the height of the emulsified layer was measured. The lipid emulsifying activity was expressed as percentage of the height of emulsified layer per the height of whole layer.

5-Functional Properties (Thermal Properties) of Nano β – Glucan

The thermal properties of extracted Nano β –Glucan were analyzed using Diferential Scanning Colorimeter (DSC) 821 (Mettler Toledo, Switzerland) equipped with a thermal analysis data station ⁽²³⁾. Sample dispersions containing about 2.4-3.6 mg dry matter of nano β –Glucan (4% w/w) were sealed hermetically into high volume stainless steel DSC pans and stored at 25°C for 72h. The samples were then heated at heating rate of 5 °C /min from 25 to 100 °C.

6- Nano β -Glucan Microencapsulation

Microencapsulation of extracted Nano β -glucan was done with sodium alginate (6 % (w/v), $dp = 3$ mm) by using standard ionotropic gelation through a syringe as described by Kubic *et al.* ⁽²⁴⁾.

7- Transmission Electron Microscope

The morphology of microencapsulated β -glucan nanoparticle was measured as described previously.

8-Thermal stability of Nano Encapsulated β -glucan

The thermal stability of microencapsulated β -glucan nanoparticle was determined according to the methods described by Pérez-Alonso *et al.* ⁽²⁵⁾. β -Glucan extracted samples between 4 and 5 mg were placed in the furnace of the TA Instruments DSC model 2010 (New Castle, DE.USA), and were subjected to heating rates (β) of 4,6,8 and 10 °C min^{-1} from 30 to 230 °C or 400 °C, when required, using an oxygen flow rate of 25 $\text{cm}^3 \text{min}^{-1}$. A blank was run using N_2 in order to determine if the exothermic peaks of the samples were due to oxidation. Measurements were done in duplicate.

9- Biological experiments:

Male albino rats (150-200 g) produced from the Central Animal House, National Research Centre, Giza, Egypt were used. The animals were acclimated for 5 days prior to dosing, during which time; they had free access to food and water at libitum.

Thirty six of acclimated rates were randomly divided into six groups. 1st group (normal) received only vehicle. Second and third groups received suspension of nano β -glucan capsules equivalent to 100 and 200 mg Arabic gum respectively for 7 days. The fourth group (Toxin control) received vehicle and 50% tetra chlorocarbon CCL₄ in paraffin oil (v/v). 5th and 6th groups received oral suspension equivalent to 100 and 200 mg. Nano β -glucan capsules respectively and addition of CCL₄ 50% (2,5 ml in paraffin oil for 48 hrs). Rats were scarified and blood samples were collected from the retro-orbital venous plexus and livers were removed. The following assay was carried out:

1-Determination of enzyme levels in serum:

Aspartase amino transferase (AST) and alanine aminotransferase (ALT) activities were measured using standard assay kits biomerieux diagnostics; France.

2-Preparation of liver supernatant:

Liver supernatant was prepared according to the procedure described by Jayakumar *et al.*,⁽²⁶⁾. In the liver supernate; lipid peroxidation was determined as malonaldehyde (MAD) content (mmole/g.liver.tissue) in the form of thiobarbituric acid-reactive substance (TABRS) by the method of Okahawa *et al.*⁽²⁷⁾. The mean reduced glutathione concentration (μ moles glutathione.mg protein-1) in the liver homogenate was determined by the method of Moron *et al.*⁽²⁸⁾.

3-Histopathological examination

Rat livers were fixed in 10% neutral formalin routinely processed for the light microscopic examination. Slices were stained with H&E⁽²⁹⁾.

Results & Discussion:

Supercritical extraction of Nano β - Glucan:

The yield of extracted Nano β -Glucan by supercritical after modifications was 1310 μ g/gm. The obtained results indicated that the yield of Nano β -Glucan extracted by supercritical CO₂ was increased due to the modification of the used method. The increase was reached to 63.3 times that extracted by conventional method.

Transmission Electron Microscope

The morphology of extracted Nano β -Glucan by supercritical CO₂ after freeze-drying was obtained by transmission electron using Transmission electron microscopy.

Figures (1) show the TEM of Nano β -Glucan nanoparticles with diameter range from 11.5- 24 nm.

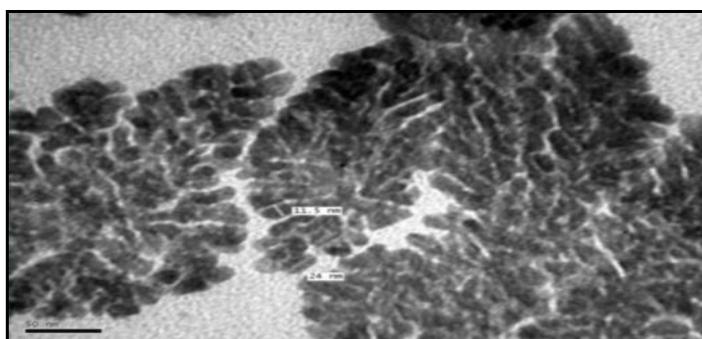


Figure (1): TEM of extracted Nano β -Glucan nanoparticles

FT-IR analysis:

Infrared microscopy of Nano β -Glucan nanoparticles extracted by Supercritical was presented in (Fig.40).The FTIR spectrum showed a strong band at 3425 cm^{-1} (O-H stretching), referring to a polysaccharide, with bands at around 2928 cm^{-1} (C-H stretching) due to lipid hydrocarbon chains, bands at 2861 cm^{-1} (C-H

stretching sym/asym) and at 1736 and 1638 cm^{-1} corresponding to amide and amide carbonyl groups. Thus, FTIR spectroscopy offers the possibility to probe both structure and content modifications of nanomolecules like β (1 \rightarrow 6) and β (1 \rightarrow 3) glucans.

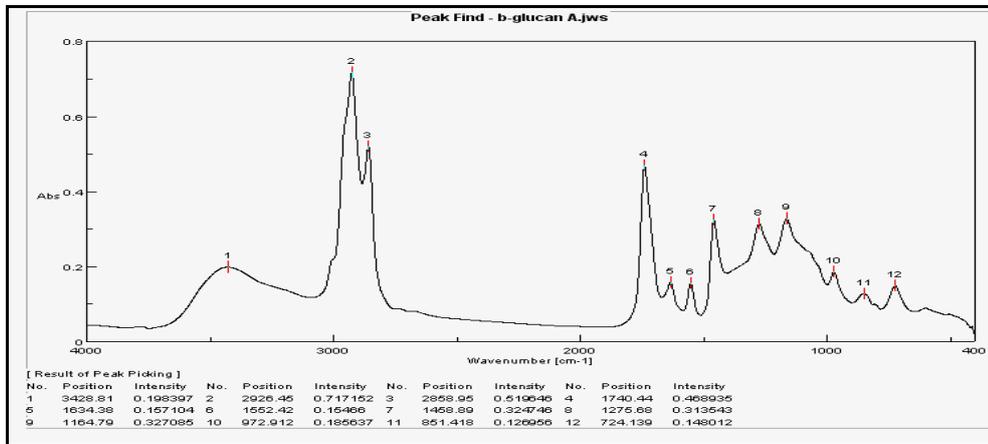


Figure (2): FT-IR of extracted Nano β -glucan by supercritical

Lipid Emulsifying test:

Lipid emulsifying test Nano β -Glucan of was measured as was seen in (Fig.3)

SunFlour oil:

$$\text{Lipid emulsifying activity} = \frac{\text{Height of emulsified layer}}{\text{Height of whole layer}} \times 100$$

$$\text{Lipid emulsifying activity} = \frac{1.9}{6.2} \times 100 = 30.65 \%$$

Corn oil:

$$\text{Lipid emulsifying activity} = \frac{2.1}{6.2} \times 100 = 33.87 \%$$

Olive oil:

$$\text{Lipid emulsifying activity} = \frac{1.8}{6.2} \times 100 = 29.03 \%$$



Figure(3): Lipid Emulsifying Test of Nano β -Glucan

Microbial and plant gums as well as some plant and animal proteins have been known to posses lipid emulsifying effects. Therefore , the emulsifying effect of the extracted polysaccharides on olive,corn and

sunflower oils- water was evaluated (Fig. 3). Addition of mushroom extract polysaccharides resulted in the stability of the oil-water emulsion for 11 days . The emulsifying activity of the extracted polysaccharides was tested using various vegetable oils and compared their activities. In the case of all the vegetable oils , including olive, corn and sunflower oils, the polysaccharides showed higher emulsifying activity. The most effective concentration was about 0.06 % (w/v) except for the sunflower oil . In sunflower oil-water emulsion , the most effective concentration was 0.1%. When high concentrations of polysaccharide were used 0.7% , the emulsifying activity decreased in case of sunflower and corn oils. However , the olive oil emulsion was very stable at cocentration up to 0.2 % . From these results, the nano polysaccharides extracted from Oyster mushroom by Supercritical was expected to have a great potential for use in industrial scale as an emulsifier.

Flocculating test:

Recently, many studies have been reported on the flocculating effect of microbial polysaccharides to replace synthetic flocculants, which are industrially used. The flocculating effect of the purified nano polysaccharides against a suspension of activated carbon water was determined (Fig. 4). Other polysaccharides including Arabic and guar gums as a control to compare its flocculating activity were used. The nano oyster mushroom polysaccharide showed a much higher flocculating activity than Arabic and guar gums. Flocculating occurred after 3 min incubation (Fig. 4). The flocculating activity of the studied nano polysaccharides of oyster mushroom was higher than other polysaccharides suggesting that the polysaccharide has a great potential as a flocculating agent as well.



Figure (4): Flocculating test of Nano β -Glucan Where: A: sample, B: water, C: guar gum, D: Acacia (Arabic) gum

Thermal Stability (Differential Scanning Coliremetry) DSC:

The apparent melting enthalpy values calculated from the DSC curves is a measure of energy required for disruption of the H-bonding within the junction zones. The onset melting temperature and the broadness of the endothermic peaks also provide information on gel properties. Extracted nano β -Glucan exhibited no thermal transition, but gel network structure developed progressively upon aging⁽³⁰⁾. Two peaks were observed in Figure (5) corresponding to melting points for Nano β -Glucan compounds being 172.84 and 226.45°C.

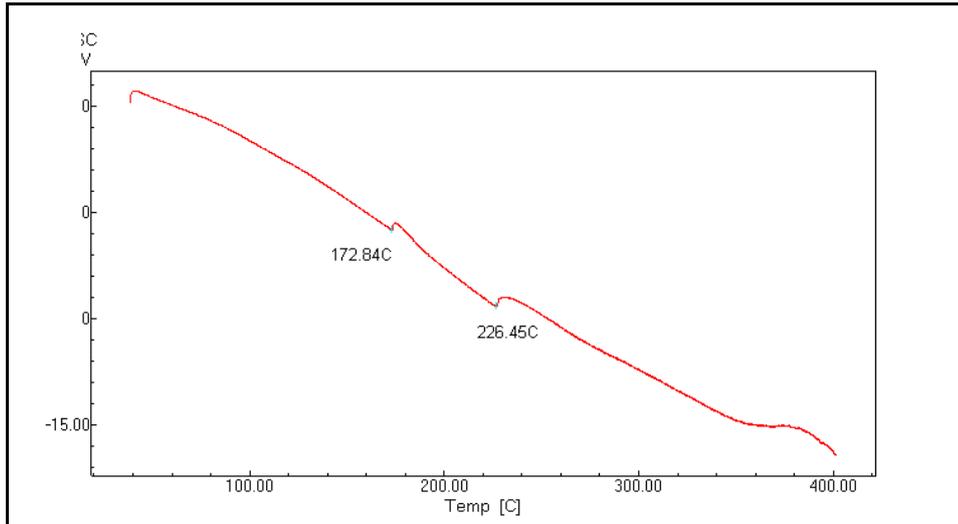


Figure (5): DSC of nano β -Glucan

Glucan Microencapsulation

Microencapsulation of Nano β -glucan by using sodium alginate beads provide protection against degradative reaction, oxidation and enhance the stability

Transmission Electron Microscope:

The morphology of the encapsulated nano β -glucan was obtained by using Transmission electron microscopy.

Figure (6) show the TEM of the encapsulated β -glucan nanoparticles (core) and the shell with diameter range from 15 to 459 nm.

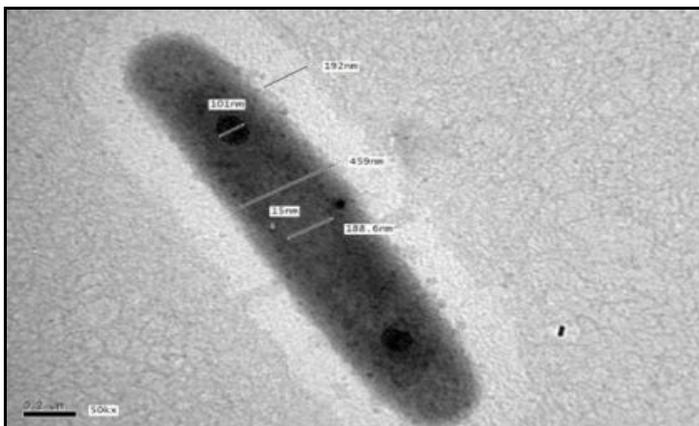


Figure (6): TEM of the nanoencapsulated β -glucan

Thermal Stability of the encapsulated Nano β -glucan(Differential Scanning Coliremetry):

DSC of encapsulated Nano β -glucan Fig. (7) Showed the melting point of Nano β -glucan compounds being 297.02 and 383.87 °C which are higher than the extracted ones indicating the protection of microencapsulation to the reduction in thermal stability.

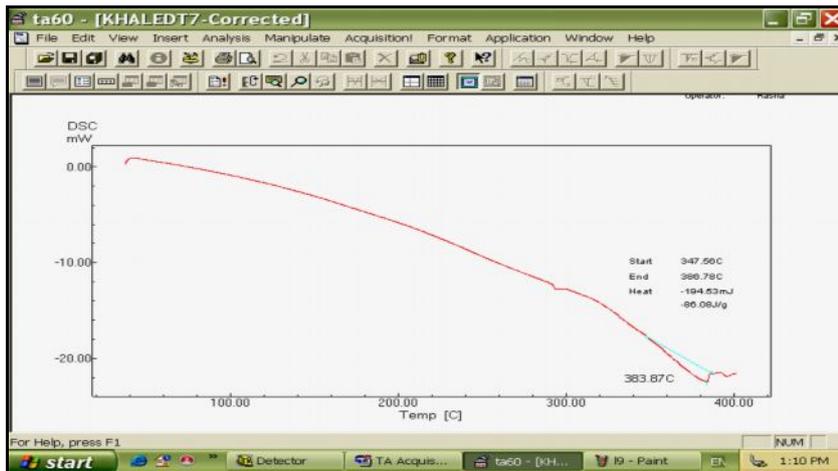


Figure (7): DSC of encapsulated Nano β -glucan

Results of biological evaluation in Table (1) showed that oral administration of the nano β -glucan capsules (polysaccharides) at levels of 100 mg and 200 mg for 4 consecutive days caused a significant reduction in the previously mentioned parameters AST, ALP and LP, while the level of reduced glutathione GSH did not change significantly.

Regarding to the other groups; CCL₄ mediated hepatotoxicity which was chosen as an experimental model, the ability of β -Glucan to reduce the injurious effect or to preserve the normal physiological mechanisms have been taken as an index of the protective effect Yadav and Dixit⁽³¹⁾. In group 4, where CCL₄ was used to induce hepatotoxicity, significant changes in the previous markers were noticed i.e the levels of AST, ALT and LP are getting higher indicating hepatotoxicity. The hepatotoxicity induced by CCL₄ is due to its metabolite CCL₃, a free radical that alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids in the presence of oxygen to produce lipid peroxides leading to liver damage Bishayee *et al.*,⁽³²⁾. Hepatocellular necrosis leads to elevation of the serum marker enzymes, which are released from the liver into blood. The increased levels in AST, ALT and LP are the conventional indicators of the liver injury Achliya *et al.*⁽³³⁾.

Oral administration of the Nano β -glucan (polysaccharides) at levels of 100 mg and 200 mg for 4 consecutive days caused a significant reduction in the previously mentioned parameters AST, ALT and LP were lowered from (153.54 to 92.54); (89.29 to 52.34) and (755.68 to 394.24), respectively; indicating hepatoprotective effect. It appears that the major nano beta-glucan capsules (NBC) were potent hepatoprotective agents in rats treated toxic chemical such as CCL₄. The hepatoprotective effects were perhaps related to the ability to promote the activity of scavenging enzymes for hepatic free radicals in mice and thus to raise the ability of autooxidation in mice Wang *et al.*⁽³⁴⁾. Our results could support this hypothesis; since the level of reduced glutathione are gradually enhanced when doses of 100 mg and 200 mg of NBC were used during treating animals with CCL₄.

Histopathological studies

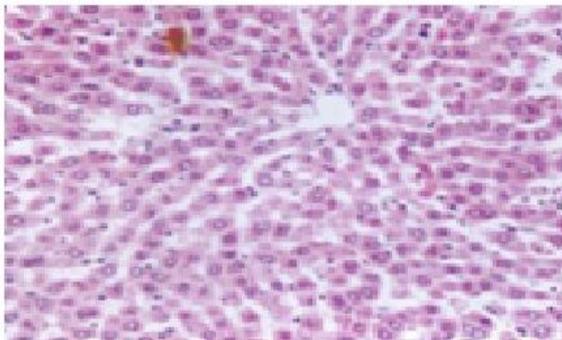
were also performed to provide; direct evidence of hepatotoxicity of CCL₄ and the hepatoprotective effect of NBC compared to normal liver (Fig. 8a).

Histopathological observation

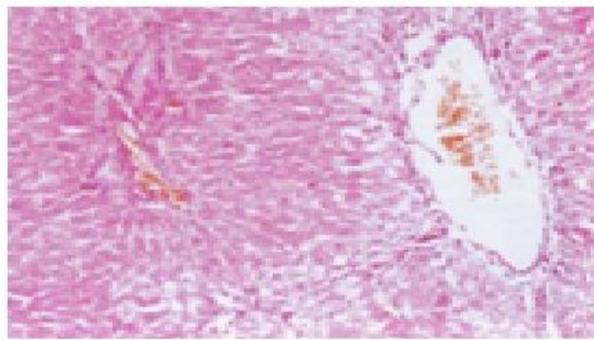
Induced by CCL₄ showed an amount of intense centrilobular necrosis, remarkable microvascular and macrovascular fatty changes as well as moderate inflammatory infiltration (Figs. 8_b and _c). The sections of liver taken from the animals treated with CCL₄ and NIC showed a near normal hepatic architecture. These sections showed a significant liver protection against the toxicant as evident by the presence of normal hepatic cord, absence of necrosis and laser fatty infiltration. (Figs. 8_d and _e) showed that effect; meanwhile accumulation of fatty changes was noticed in the section of animals treated with 100 mg NBC.

Table (1): The effect of oral administration of NBC (100 mg and 200 mg /kg b.wt.) in normal or CCL₄ – hepatic damaged rats on serum AST, ALT, GSH and LP of liver tissue (mean± SE of means , n= 6 rats / group), - one way (the different capital letters are significantly different at p> 0.05)

	IU/m AST	IU/ml serum ALT	Mmol/g liver tissue GSH	Mmol/g liver tissue LP
Normal	43±0.02	29±0.04	13±0.06	322
Normal + 100 mg NBC	24±0.01	26±0.05	14±0.05	302
Normal + 200 mg NBC	23±0.04	19±0.05	15 ±0.02	282
CCL ₄ -Hepatic damage NBC	151±0.02	90±0.02	5±0.01	742
CCL ₄ -Hepatic damage + 100 mg NBC	151±0.02	61±0.04	5.8±0.02	432
CCL ₄ - Hepatic damage + 200 mg NBC	98±0.04	51±0.03	10.2±0.04	402



Figure(8a) :photomicrograph of a section of the liver of a rat showing the architecture of the hepatic lobule. The central vein lies at the center of the lobules surrounded by cords of hepatocytes(H&X150)



Figure(8c): photomicrograph of a section of the liver of a rat treated with CCL₄ at dose (50%) showing centrilobular necrosis and cellular infiltration (H&Ex150)

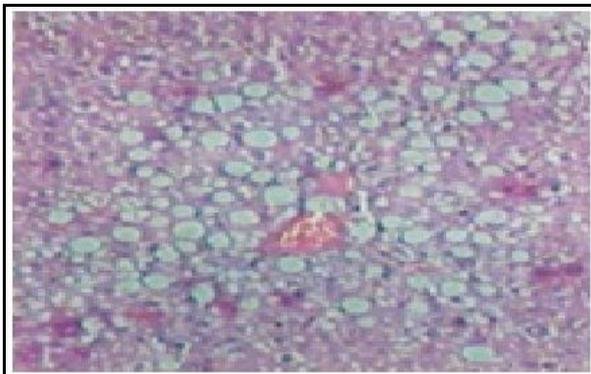


Figure (8b): photomicrograph of a section of the liver of a rat treated with CCL₄ AT Dose (50%) showing necrosis and fatty change (H&E x 150)



Figure (8d): photomicrograph of a section of the liver of a rat treated with CCL₄ at dose (50%) and NBC (100mg) showing dilated and congested of central vein (H&Ex150)

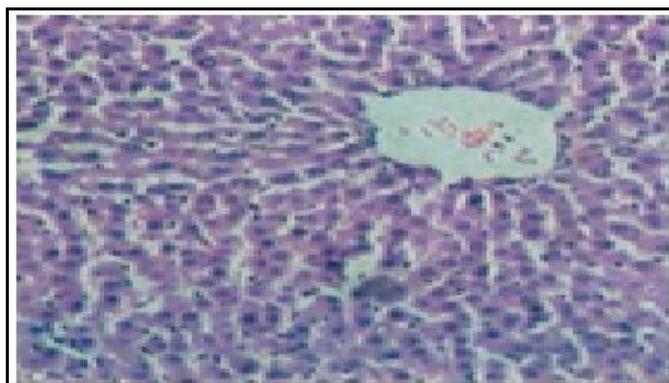


Figure (8e): photomicrograph of a section of the liver of a rat treated with CCL₄ at dose (50%) and NBC (200mg) showing nearly the same control (H7Ex150)

Figure (8): photomicrograph of a section of the liver of a rat

Conclusion:

The present study shows that β -glucan could be effectively extracted from oyster mushroom (*Pleurotus aurrateus*) by using supercritical fluid CO₂ extraction and transferred to nano-particles after ultrasonication treatment, then capsulated. Its purity and safety aside with microencapsulation and stability exerting additionally a substantial protective effect that could be present in human diets.

Nano Polysaccharides (β -glucan) extracted from Oyster mushroom was expected to have a great potential for use in industrial scale as an emulsifier and was higher than other polysaccharides suggesting that the polysaccharide has a great potential as a flocculating agent as well.

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