



ISSN: 0067-2904

GIF: 0.851

Evaluation of Antioxidant Activity of Acoholic Extracts from *Pleurotus ostreatus* (P2) In Vivo

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Abstract

In this study found treatment mice exposed to CCl₄ with the alcoholic extract of *Pleurotus ostreatus* (P2) at (25) mg/ml appeared to exert a beneficial effect since the activities of catalase, was significantly (p<0.05) higher in liver treatment with alcoholic extract of *P. ostreatus* (P2) (37.290±2.638) μmol/mg than in aqueous extract for the same fungus (36.557±2.231) μmol/mg. Super oxide dismutase (SOD) was significantly (p<0.05) higher in liver treatment with alcoholic extract of *P. ostreatus* (P2) at (25) mg/ml (17.863±0.454)U/mg than in aqueous extract (17.863±0.454) U/mg for the same fungus and same concentration. Glutathione peroxidase (Gpx) was significantly (p<0.05) higher in liver treatment with alcoholic extract of *P. ostreatus*(P2) at (25) mg/ml (29.260±1.666)μmol/mg than in aqueous extract (24.660±1.130)μmol/mg for the same fungus and same concentration. Alcoholic extract of *P. ostreatus* (P2) at (25) mg/ml induce a non-apoptotic, apoptosis was (0.0657±0.0047%) ,in aqueous extract was (0.0770±0.0060%). Histopathological study appeared liver cells exposed to CCl₄, CCl₄ made severe degeneration and necrotic change in liver parenchyma by evidence of apoptosis in some hepatocytes , in sinusoids showed slight congestion with blood vessel and thrombus formation. Liver cells of mouse exposed to CCl₄ and treated with alcoholic mushroom extract and appeared the liver cells returned like control. the tissue treatment by aqueous extract of *P.ostreatus* (P2) at (25) mg/ml appeared mononuclear cells aggregation.

Keywords: *Pleurotus ostreatus* (P2)

تقييم الفعاليه المضادة للاكسدة للمستخلص الكحولي لفطر *Pleurotus ostreatus* (P2) في

جسم الكائن الحي

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الخلاصة

في هذه الدراسة وجد علاج الفئران التي عرضت الى رابع كلوريد الكاربون بالمستخلص الكحولي للفطر *Pleurotus ostreatus* (P2) اعطت نتائج افضل من المستخلص المائي فقد كانت فعالية انزيم الكاتلاز (37.290±2.638) مايكرومول لكل ملغرام للمستخلص الكحولي *Pleurotus ostreatus* (P2) بتركيز (25) ملغم لكل مليلتر اما فعالية الانزيم للمستخلص المائي لنفس الفطر وبنفس التركيز كانت (36.557±2.231) مايكرومول لكل ملغرام اما فعالية انزيم سوبر اوكسايدي ديسميبيز للمستخلص الكحولي *Pleurotus ostreatus*

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(P2) بتركيز (25) ملغم لكل مليلتر (17.863 ± 0.454) وحدة بروتين لكل ملغرام من الانزيم اما في المستخلص المائي كانت الفعالية لنفس الفطر وبنفس التركيز (17.863 ± 0.454) وحدة بروتين لكل ملغرام انزيم اما فعالية الانزيم كلونثاايون بيروكسيد في المسخلص الكحولي (P2) *Pleurotus ostreatus* بتركيز (25) ملغرام لكل مليلتر (29.260 ± 1.666) مايكرومول لكل ملغرام في المستخلص المائي كانت فعالية الانزيم لنفس الفطر وبنفس التركيز (24.660 ± 1.130) مايكرومول لكل ملغرام اما الايبيتوساس للمستخلص الكحولي ($0.0657 \pm 0.0047\%$) اما بالمسخلص المائي ($0.0770 \pm 0.0060\%$).
 اما الدراسة النسيجية اوضحت ان خلايا الكبد التي تعرضت لرابع كلوريد الكاريون اصابها تنخر وتخر وتغيرت في بركيما الكبد وظهر بعض خلايا الكبد اصببت بالموت المبرمج .عند المعالجة بالمستخلص الكحولي تظهر خلايا الكبد وكأنها رجعت لحالتها الطبيعية اما العلاج بالمستخلص المائي نلاحظ تجمع للخلايا وحيدة النواة .

Introduction

The liver is the most complex organ in the body. It plays a vital role in regulating metabolism processes, performing many essential functions in order to maintain life, such as glycogen storage, production of necessary biochemicals for digestion, plasma protein synthesis and detoxification. These functions are carried out generally by hepatocytes especially for the process of blood filtration, for chemical digestion of medications, but also against environmental pollution toxins, and alcohol intoxication which can have largely damaging effects over long periods of exposure or abuse [1].

Recently, many natural agents possessing antioxidative properties have been reported to prevent and treat liver damages caused by free radicals induced by CCl_4 in experimental animal's model, Catalase is one of the important antioxidative enzymes with a heme structure that can catalyze the decomposition of $2 H_2O_2$ to $2 H_2O$ and O_2 , SOD are enzymes that catalyze the dismutation of superoxide (O_2^-) into oxygen and hydrogen peroxide. Thus, they are an important antioxidant defense in nearly all cells exposed to oxygen [2]. Gpx provides a mechanism for detoxification of peroxides in living cells and plays a crucial role in protecting cells from damage by free radicals, which are formed by peroxide decomposition, lipid components of the cell are especially susceptible to reactions with free radicals, resulting in lipid peroxidation [3].

Materials and methods

Experimental animals

Forty mice aged (8) weeks and weight (20) gm males and females were provided by the national center of drug control. The animals used to determine the antioxidant activity for aqueous and ethanolic extract of *Pleurotus ostreatus* (P2) at (25) mg/ml, mice reared at an optimal room temperature ranged between $(22-25)^\circ C$. Animals were fed on standard diet provided from the national center of drug control.

Animals were divided into five groups, each group contains (8) mice. The groups were as follows:

Group 1: Animals were received (2 ml / kg body wt) of D.W daily for 16 days by oral as aqueous control.

Group 2: Animals without any treatment for 16 days, only on the 14th day were given CCl_4 (2 ml/kg body wt) in olive oil (2% v/v) by subcutaneous injection as negative control.

Group 3: Animals were received (2ml / kg body wt) of aqueous extract (25mg/ml) of *P. ostreatus* (P2) daily for 16 days by oral, on the 14th day they were given CCl_4 (2 ml/kg b.wt) in olive oil (2% v/v) by subcutaneous injection.

Group 4: Animals were received (2 ml / kg body wt) of ethanol daily for 16 days by oral as alcoholic control.

Group 5: Animals were received (2 ml / kg body wt) of ethanolic extract (25mg/ml) of *P. ostreatus* (P2) daily for 16 days by oral, on the 14th day they were given CCl_4 (2 ml/kg b.wt) in olive oil (2% v/v) by subcutaneous injection.

Detection of enzymes activity in mice liver

Tissue Homogenate: Prior to dissection, perfuse tissue or rinse with a phosphate buffered saline (PBS) solution. This is to remove any red blood cells and clots. Weigh and homogenize the tissue on ice in (5-10) ml cold (PBS) with (1) mM EDTA per gram of tissue. Centrifuge at $(10,000 \times g)$ for (15)

min at (4)°C. Remove the supernatant and store on ice. Store any unused supernatant at (-70) °C for up to one month.

ELISA Procedure

ELISA technique was performed using ELISA kit

- 1- The test kit was brought to the room temperature (20-25°C) for at least 30 minutes.
- 2- The required micro-well strips were putted into plate frames.
- 3- Washing buffer was returned to room temperature before use.
- 4- The micro-wells were numbered according to samples and standard solution (each sample and standard solution was performed in duplicate and their position was recorded).
- 5- Fifty microliter of the sample or the standard solution was added into separate duplicate wells , (50µl/ well) of enzyme conjugate was added, then (50 µl / well) of antibody working solution was added , The mixture was shook manually and the micro-plate was sealed with cover membrane and incubated at room temperature for (30 min) in the dark .
- 6- The micro-plate was washed and the cover membrane was opened carefully , the liquid was poured out of micro-well , then (300 µl) of washing buffer which prepared was added , the micro-well was washed fully for 5 times (30 s each time), then the washing buffer was taken out and flapped to dry with absorbent paper.

Histopathological Study.

All mice were sacrificed after (16) days of the treatment. Liver were dissected out. Organ was fixed in plastic containers containing (100) ml of formalin (10%).After that organ sample was dehydrated in progressively more concentrated alcohols , then embedded in paraffin and cut into section of(4-5) µm thickness and stained with haematoxylin and eosin (H & E) [4] as follows:

- 1- The sections were deparaffinized by hot xylene for (5-10) min, this step was repeated twice.
- 2- Graded alcohol (100 %, 90 % and 70 %) was used for dehydration, (5)min in each alcohol grade.
- 3- The section was stained with haematoxylin for (2-3) min.
- 4- Washed with tap water for (5-10) min.
- 5- Differentiated a few second in (1%) acidic alcohol (1% HCl in 70% alcohol) until the section looks red, usually (5-15) seconds .
- 6- Washed well in running tap water for (3-5) min to remove the acid.
- 7- Stained in (1%) eosin for (10) min.
- 8- Graded alcohol (70 %,90 %,100 %, and100%) was used for dehydration, (5) min in each alcohol grade.
- 9- The section was cleared by xylene through three changes (15, 15 and 30) min.
- 10- Mounting by using Disterne-Plastcizer Xylene (DPX) and cover slide.
- 11- For microscopical examination, the slides were examined at (200 X) magnifications using an optical microscope.

Apoptosis

Apoptosis was determined according to[5] induce apoptosis in cells using the desired method.

Include negative control experiment.

- 1- Harvest cells by centerfugation, discard supernatant. Resuspended pellet in cold PBS and wash cells by gentle shaking or by up and down mixing in pipette tip. Recenterifuge washed cells again and discard supernatant.
- 2- Resuspend pellet in 1X Binding Buffer and adjust cell density to (2-5x10⁵) cells/ml, preparing a sufficient volume of cell suspension (100) µl per assay.
- 3- Add (5) µl of Annexin V-FITC and (5) µl of PI to each (100) µl of cell sustention, mix gently.
- 4- Incubate stained cells for (15) minute in dark at room temperature.
- 5- After the incubation period, centrifuge cells and resuspend pellet in (100)µl of binding buffer (or in an appropriate volume according to a method of sample acquisition).
- 6- Analyze the stained cells by flow-cytometry as soon as possible.

Statistical analysis

All analyses were performed in triplicate. Analysis of variance (ANOVA) was performed using Duncan's multiple range test to compare treatment means at (P < 0.05) using SPSS software version 16 (SPSS Inc., USA)[6].

Results and discussion

Catalase

In table -1 showed treatment with the alcoholic extract of *P. ostreatus* (P2) at (25) mg/ml appeared to exert a beneficial effect since the activities of catalase, superoxide dismutase and glutathione peroxidase were significantly ($p < 0.05$) higher in liver treatment with alcoholic extract of *P. ostreatus*(P2) than in aqueous extract for the same fungus. The liver is a highly sensitive organ which plays a major role in maintenance and performance of homeostasis in the body. It is the chief organ where important processes life metabolism and detoxification take place [7]. Living tissues are endowed with innate antioxidant defense mechanisms, such as the presence of the enzymes catalase, superoxide dismutase and glutathione peroxidase. A reduction in the activities of these enzymes is associated with the accumulation of highly reactive free radicals, leading to deleterious effects such as loss of integrity and function of cell membranes ,the extract possibly confers this protective effect by dampening the generation of free radicals that is induced by CCl_4 , the utilization of multiple isoforms of enzymes is believed to be one of the primary control mechanisms that regulates cellular metabolism [8].

Superoxide dismutase (SOD)

In table -1 showed significant decrease in SOD activity was observed in the liver of CCl_4 compared to normal,the activities of SOD was significantly ($p < 0.05$) higher in liver treatment with alcoholic extract of *P. ostreatus* (P2) at (25) mg/ml than in aqueous extract for the same fungus and same concentration. SOD catalyzes the dismutation of superoxide radicals in to oxygen and hydrogen peroxide plus, thus participating with other antioxidant enzymes in the enzymatic defense against oxidative injury [9].This result agree with [10] finding that SOD is the most sensitive enzymatic index in liver injury caused by ROS and oxidative stress, SOD is one of the most abundant intracellular antioxidant enzymes present in all aerobic cells and it has an antitoxic effect against ROS.

Glutathione peroxidase (GPX)

In table -1 showed significant decrease in GPX activity was observed in the liver of CCl_4 compared with normal, the activities of GPX was significantly ($p < 0.05$) higher in liver treatment with alcoholic extract of *P. ostreatus* (P2) at (25) mg/ml than in aqueous extract for the same fungus and same concentration. GPX acts synergistically with other endogenous antioxidants and acts as a co-factor with the enzyme GPX to scavenge free radicals antioxidants and detoxify xenobiotics [11] .This result agree with [12] that reported the increase in the serum level of this enzyme GPX might be due to the presence of various phenolic and flavonoid compounds in the mushroom extract that enhanced the liver's regeneration ability, equilibrium between ROS and enzymatic antioxidant like GPX is crucial and liver could play a central role in the maintenance of systemic lipid homeostasis and is especially susceptible to ROS damage, this mechanism has been suggested to play role in preventing toxicity [13].

Table 1- Catalase , SOD,GPX activity for alcoholic mushroom extract of *P. ostreatus* (P2)at (25) mg/ml

Groups	Catalase $\mu\text{mol/mg}$	SOD U/mg	GPX $\mu\text{mol/mg}$
Control water	A 40.207 \pm 2.792	A 16.810 \pm 1.717	A 32.877 \pm 2.584
CCl_4	B 25.060 \pm 1.449	B 11.880 \pm 0.363	B 15.923 \pm 1.631
CCl_4 +aqueous extract	A 37.557 \pm 2.231	AB 13.823 \pm 0.866	C 24.660 \pm 1.130
Control coholic	A 39.820 \pm 1.503	A 17.963 \pm 2.241	A 31.960 \pm 1.749
CCl_4 +Alcoholic extract	A 37.290 \pm 2.638	A 17.863 \pm 0.454	A 29.260 \pm 1.666

Apoptosis

In table (2) showed that both extracts did not induce apoptosis however; alcoholic extract of *P. ostreatus* (P2) at (25) mg/ml induce a non-apoptotic, is revealed in the significant increase in the

number of cells and a decrease when treated with aqueous extract for the same fungus and concentration. Taken together these results indicate that both extracts cause non-apoptotic. Programmed cell death plays critical roles in a wide variety of physiological processes during fetal development and in adult tissues. In most cases, physiological cell death occurs by apoptosis as opposed to necrosis, defects in apoptotic cell death regulation contribute for many diseases [14].

Table (2): Apoptosis for alcoholic and aqueous mushroom extract of *P. ostreatus* (P2) at (25) mg/ml

Groups	Apoptosis %
Control water	A 0.0567±0.0379
CCl ₄	B 2.3333±0.5774
CCl ₄ ±aqueous extract	A 0.0670±0.0060
Control coholic	A 0.0690±0.0079
CCl ₄ ±Alcoholic extract	A 0.0757±0.0047

Histopathological study

Liver from control animal, showed normal structure as central vein, hepatocytes and sinusoids as figure-1, when liver cells exposed to CCl₄, CCl₄ is a well-known hepato-toxic agent and made severe degeneration and necrotic change in liver parenchyma by evidence of apoptosis in some hepatocytes, in sinusoids showed slight congestion with blood vessel and thrombus formation as figure2, liver cells of mouse exposed to CCl₄ and treated with alcoholic mushroom extract and appeared the liver cells returned like control (3).. The results of the present study suggest that an extract of the oyster mushroom *P. ostreatus* (P2) at (25) mg/ml is able to confer protection against acute hepatotoxicity induced by CCl₄. Since free radicals play such an important role in CCl₄ induced hepatotoxicity, it seems logical that compounds in mice receiving the mushroom extract and CCl₄ near normal levels of hepatic constituents and showed mononuclear aggregation as figure-4. The use of some halogenated alkanes such as carbon tetrachloride (CCl₄) classified as a potential human carcinogen, increases the frequency of liver tumors in experimental animals. It's widely used as a model for the study of agents that cause liver damage by formation of trichloromethyl and trichloromethylperoxy radicals by a free radical mechanism. Recently, many natural agents possessing antioxidative properties have been reported to prevent and treat liver damages caused by free radicals induced by CCl₄ in experimental animal's model, since free radicals play such an important role in CCl₄ induced hepatotoxicity, it seems logical that compounds that neutralize such radicals may have an hepatoprotective effect. Indeed, various natural products have been reported to protect against CCl₄-induced hepatotoxicity [15].

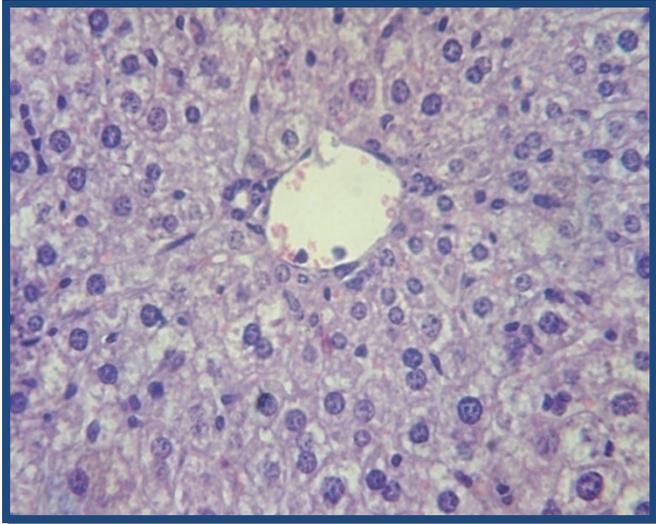


Figure 1-: Mouse liver from control animal central vein (V), hepatocytes (H) and sinusoid (s)(400x)

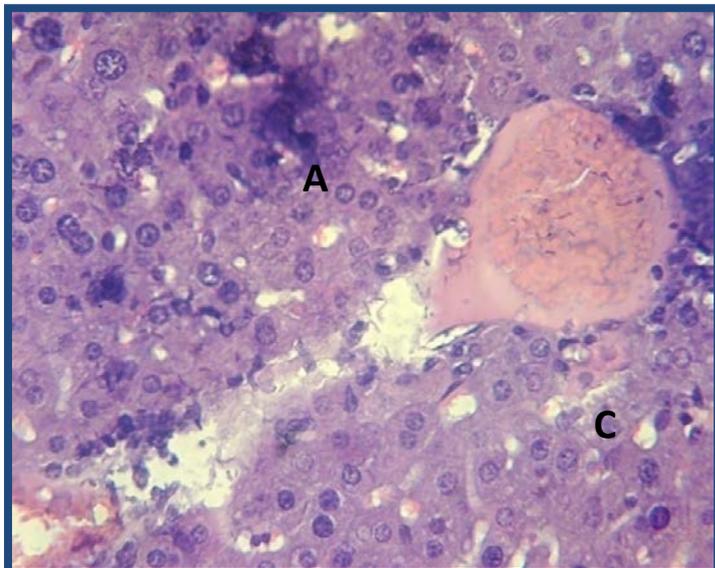


Figure 2- Liver cells of mice exposed to CCl₄, congestion and thrombus in blood vessel (C) , apoptosis (A) (400x)

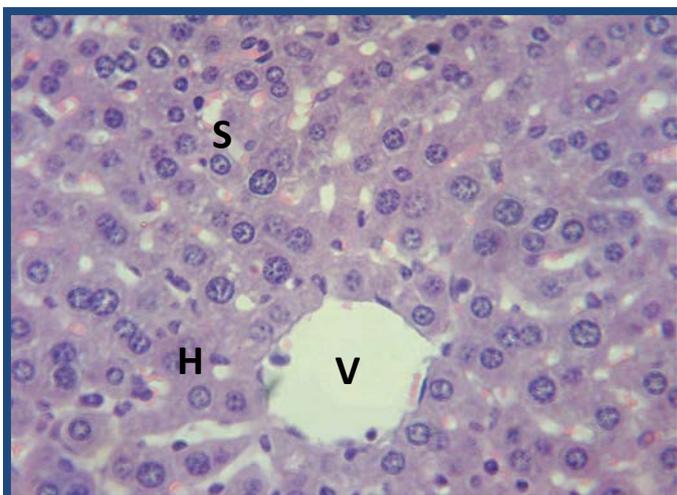


Figure 3- Liver cells of mice exposed to CCl₄ and treated with alcoholic mushroom extract (40x) central vein (V), hepatocytes (H) and sinusoid (s)

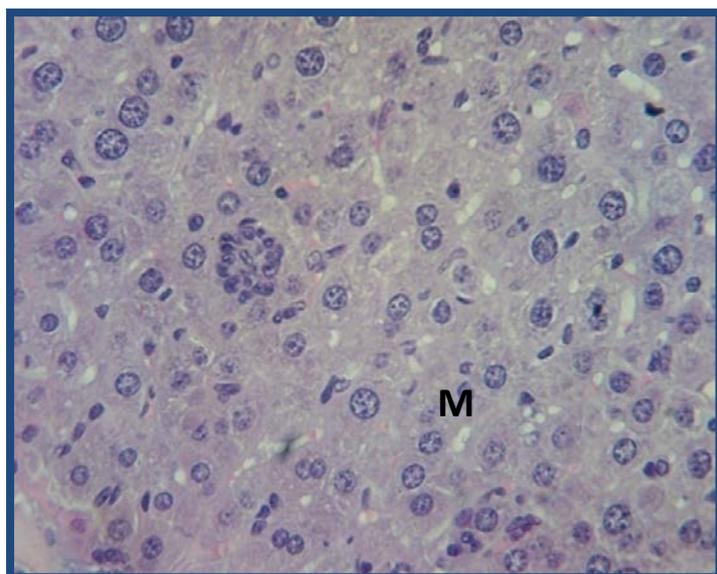


Figure 4- Liver cells of mouse exposed to CCl₄ and treated with aqueous mushroom extract, mononuclear aggregation (M) (400x)

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