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Research Article

HEPATOPROTECTIVE EFFECT OF A WILD EDIBLE MUSHROOM ON CARBON TETRACHLORIDE-INDUCED HEPATOTOXICITY IN MICE

KRISHNENDU ACHARYA[1*], SOUMYA CHATTERJEE[1], GUNJAN BISWAS[1], ANIRUDDHA CHATTERJEE[2], GOUTAM KUMAR SAHA[2]

[1]Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany, [2]Department of Zoology, University of Calcutta, 35, Ballygunge Circular Road, Kolkata- 700019, India. Email: krish_paper@yahoo.com

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ABSTRACT

This study assessed hepatoprotection by ethanolic extract of a wild edible mushroom (*Macrocybe gigantea*) towards carbon tetrachloride (CCl_4) intoxicated hepatic damage in mice. The extract was orally administered to the animals with hepatotoxicity induced by CCl_4 at a dose of 150 mg/kg once daily. Serum glutamate pyruvate tranaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP) and bilirubin content which was elevated due to CCl_4 intoxication was significantly reduced by the extract. Standard drug Silymarin was used as reference. In CCl_4 alone treated animals, lipid peroxidation was increased with decrease in superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) levels which represents the hepatic antioxidant status. The hepatic antioxidant status was restored with the extract treatment. Administration of the extract of *Macrocybe gigantea* indicated significant suppression of CCl_4 induced hepatotoxicity as confirmed by histopathological studies. Free radical scavenging and antioxidant activities may be the possible mechanism of hepatoprotection and may be conferred by the presence of high amount of phenolics compounds and flavonoids.

Keywords: *Macrocybe gigantea*, Carbon tetrachloride, Hepatoprotective activity, Antioxidant activity, Lipid peroxidation.

INTRODUCTION

The liver is an amazingly complex organ which virtually affects every physiological process of the body. Our body is protected from various injurious substances and toxic metabolic byproducts by the liver, which has been absorbed from intestinal tract[1]. Xenobiotics are often reported to cause potential hepatic damage. Carbon tetrachloride (CCl_4) is a xenobiotic introduced into the water mainly as industrial wastes from its primary use in manufacture of chlorofluorocarbons[2], dry cleaning fluids, fire extinguishing agents, etc, producing hepatotoxicity in human beings and animals[3]. Centrilobular necrosis followed by hepatic fibrosis is the common characteristic of CCl_4 mediated hepatocyte injury[4]. The metabolism of CCl_4 in the liver leads to hepatic damage. CCl_4 binds to cytochrome P_{450} reductase. The enzyme substrate complex then loses a chloride ion and a free radical (CCl_3·) intermediate is generated which reacts with oxygen or takes a hydrogen from a donor to yield a secondary radical or reacts with lipids or proteins[5]. The lipid radicals thus formed add on molecular oxygen to generate lipid peroxyl radicals, which steals the hydrogen atoms from other lipid molecules and the process of lipid peroxidation propagates[6]. Trichloromethyl (CCl_3·) radical even reacts with reduced glutathione (GSH) and causes various pathological and toxicological manifestations[7]. CCl_4 increases intercellular Ca^{2+} concentration and activates Kupffer cells, thereby releasing harmful cytokines that leads to the death of the hepatic tissue and oxidative stress[8]. Although modern medicine has made tremendous advancements, effective drugs that offer protection from liver damage, stimulate liver function or help to regenerate liver cells are still not available[9]. As an alternative approach, a number of medicinal preparations are recommended for the treatment of liver disease and offer significant relief[10].

Asian countries have a long tradition of using mushrooms as medicine whereas in western hemisphere their use has increased since last decade[11]. Besides being a healthy food, mushrooms could be used as medicine for treatment of cancer, inflammation, heart ailments, diabetes, high blood pressure, hepatic damage, constipation, renal failure etc[12-17]. Antioxidants play a crucial role in hepatoprotective ability and hence the search for crude drugs of natural origin with this property has become a central focus of study of hepatoprotection today[18]. Mushrooms have been known to be potential source of antioxidants and capable of strong inhibition of lipid peroxidation[12, 19-21].

Macrocybe gigantea of the Family Tricholomataceae, a wild edible mushroom is most conspicuous in the tropical region during rainy season. They are robust in size and popular among the people of these areas because they are a gastronomic and nutritional delicacy. Our earlier investigation showed that ethanolic extract of *M. gigantea* possessed significant *in vitro* antioxidant activities[22]. Different antioxidants such as vitamin E, vitamin E- like compounds, 5-methylthioadenosine, colchicines, desferrioxamine was found to improve hepatic conditions significantly when treated in animals with CCl_4 induced damage[23]. Here, an attempt has been made to investigate hepaprotective activity of ethanolic extract of *Macrocybe gigantea* basidiocarp (MGEE) against CCl_4 induced liver damage in mice.

MATERIALS AND METHODS

Sample collection and preparation

Basidiocarps of *M. gigantea* were collected from the forest and local market of Kolkata and adjoining area. Fresh mushrooms were randomly divided into three portions of 150 g each was dried at 40°C for 48 h in hot air oven. Dried mushroom powder was the extracted with 200 ml of ethanol at 30°C for 24 h at 150 rpm and filtered through Whatman No. 4 filter paper. The residue was then again extracted with another 200 ml of ethanol as described earlier. The total filtrate was then rotary evaporated to dryness at 40°C and redissolved in ethanol at a concentration of 10 mg/ml and stored at -20°C for further use[13].

Phytochemical analysis

Total phenolic content in the MGEE were measured according to the method of Slinkard[24] using Folin-Ciocalteu reagent and pyrocatechol was kept as the standard. The total phenolic concentration was expressed as mg pyrocatechol equivalents (PE)/100 g dry weight. Flavonoid concentration of MGEE was also determined using quercetin as standard[25]. Total flavonoid concentration was expressed as mg quercetin equivalents (QE)/100 g dry weight.

Animals

Healthy Swiss albino mice (male) of approximately same age weighing about 20 g were used for the study. They were sheltered in polypropylene cages maintaining standard condition (12 h light/dark cycle; 25 ± 3°C, RH 35-60%) and were fed with standard diet and water *ad libitum*. The animals were maintained according to the guidelines recommended by Animal Welfare Board and approved by our institutional ethics committee. All procedures complied with the Declaration of Helsinki, as revised in 1996.

Acute toxicity studies

Swiss albino mice were used for acute toxicity study for MGEE. The standard conditions were maintained during the experiment with animals fasted overnight prior to the experiment. MGEE was fed orally with increasing dose upto a dose of 3000 mg/kg body wt.

CCl_4 induced hepatotoxicity and assessment of liver damage

The animals were divided into 4 groups of 6 animals each. Group I was given saline (5 ml/kg body wt/day, p.o.) serving as the normal control set. The positive control set designated as Group II received CCl_4 in paraffin oil (1:1, 2.5 ml/kg body wt/day, p.o.) once daily for seven consecutive days. Standard drug Silymarin (100 mg/kg body wt, p.o.) was given to animals of Group III once daily for 7 days and simultaneously administered with CCl_4 with equal volume of paraffin oil. Group IV received MGEE (150 mg/kg body wt, p.o) once daily for seven consecutive days, simultaneously with equal mixture of CCl_4 and paraffin oil. After 24 h of last treatment of CCl_4, animals were sacrificed. Blood was collected and serum was separated from clotted blood by centrifugation at 2500 rpm for 15 min and biochemical investigations were performed. The liver of the animals of all sets were excised and their antioxidant status were determined. For histopathological assessment of liver damage, liver tissue was cut and fixed in 10% buffered formalin.

Biochemical determinations

Serum hepatic marker enzymes namely, serum glutamate pyruvate transaminase (SGPT)[26], serum glutamate oxaloacetate transaminase (SGOT)[26], total and direct bilirubin[27] and alkaline phosphatase (ALP)[28] were measured using assay kits (Span Diagnostic, Surat). The extent of hepatocyte necrosis was determined with these activities as markers.

Evaluation of antioxidant status

Dissected out liver samples were washed immediately with ice-cold saline to remove excess blood on it. Liver tissue was homogenized in cold PBS (50 mM, pH 7) at a concentration of 10% (w/v). The homogenate was then centrifuged at 5000 × g for 10 minutes at 4°C to obtain the supernatant, which was used for the assay of superoxide dismutase (SOD)[29], catalase (CAT)[30], malondialdehyde (MDA)[31] and estimation of GSH[32]. Determination of protein was done by the method of Lowry et al.[33].

Histopathological examination

Fresh Liver tissue that was fixed in 10% formalin, was dehydrated in ethanol gradient (30-100%), cleared in xylene and infiltrated with wax at 57°C. The tissues thus cleared were embedded in paraffin. 4µm thick were prepared from each liver and stained by hematoxylin-eosin (H&E). Assessment of necrosis, fatty infiltration, fibrosis, lymphocyte infiltration and so forth were done by examining the sections under bright field microscope.

Statistical analysis

All data are represented as mean ± SD. One-way analysis of variance (ANOVA) followed by Duncan multiple range test was done to determine significant differences in all parameters. Values were considered statistically significant at P values <0.05 compared to the CCl_4 group.

RESULTS

Phytochemical analysis

MGEE was found to have high concentration of phenolic compounds and flavonoids as 400 mg PE/100 g dry weight and 158 mg QE/100 g dry weight respectively.

Acute toxicity studies

MGEE when administered upto 3000 mg/ kg body wt dose does not exhibit signs and symptoms of toxicity and mortality.

Effects of extract on serum enzyme parameters

The hepatoprotective effects of MGEE on CCl_4 induced hepatic injury in mice are shown on Table 1. The CCl_4 receiving group (II) as expected, revealed significantly higher increase in liver function indices such as SGPT, SGOT and ALP ($P<0.05$) compared to the normal group. The increased activity of serum enzymes may explain cell membrane break down and death[34]. CCl_4 intoxication even produced a significant ($P<0.05$) rise in serum bilirubin thereby indicating hepatic damage[35]. Treatment with ethanolic extract in group (IV) significantly ($P<0.05$) lowered the activities of serum marker enzymes, bilirubin, comparable to standard drug silymarin and towards normalization. Serum transaminases (SGPT and SGOT) were inhibited by 55.02% and 62.59% respectively compared with the control group animals whereas the extract showed inhibition of 39.26% in ALP level with respect to the control set.

Effects of extract on MDA, GSH, SOD, CAT levels

A marked increase in the levels of MDA was found in the livers of animals in CCl_4 intoxicated group (II) when compared to the normal set (Table II). Treatment with the extract in Group IV mice resulted in significant ($P<0.05$) lowering of MDA when compared with positive control set. A significant decrease in SOD, CAT, GSH status were observed on CCl_4 intoxication, whereas treatment with the extract appeared to exert a beneficial effect since the hepatic antioxidant level is restored to a extent comparable to normal and drug control groups. In the CCl_4 intoxicated group (II), GSH, SOD and CAT were depleted to 0.247 µg/mg, 4.69 U/mg and 56.7 U/mg respectively. The reduced levels were ameliorated to values as 0.324 µg/mg ($P<0.05$), 6.41 U/mg ($P<0.05$) and 59.9 U/mg ($P>0.05$) in the extract treated groups.

Table 1: Effect of MGEE on biochemical parameters of serum in mice

Group	Treatment	SGPT (IU/ l)	SGOT (IU/ l)	ALP (KA)	TB (mg/dl)	DB (mg/dl)
Group I	Normal	68.5 ± 15^a	98 ± 26^a	7.82 ± 0.28^a	1.219 ± 0.43^a	0.131 ± 0.031^a
Group II	CCl_4	235.67 ± 35^b	270 ± 40^b	13.13 ± 0.94^b	2.114 ± 0.38^b	0.635 ± 0.088^b
Group III	Silymarin + CCl_4	78 ± 12^a	96.4 ± 14^a	7.695 ± 0.65^a	1.296 ± 0.26^a	0.139 ± 0.045^a
Group IV	Ethanolic extract + CCl_4	106 ± 24^a	101 ± 26^a	7.975 ± 0.48^a	1.43 ± 0.22^a	0.219 ± 0.034^a

Values carrying different superscript for each parameter are significantly different ($P<0.05$). SGPT, serum glutamate pyruvate tranaminase; SGOT, serum glutamate oxaloacetate transaminase; ALP, alkaline phosphatase; TB, total bilirubin; DB, direct bilirubin; KA, King-Armstrong Unit.

Table 2: Effect of MGEE on liver MDA, SOD, CAT and GSH

Group	Treatment	MDA (nmol/mg)	GSH (µg/mg)	SOD (U/mg)	CAT (U/mg)
Group I	Normal	185.4 ± 1.1^a	0.495 ± 0.051^a	7.42 ± 0.42^a	80.7 ± 6.4^a
Group II	CCl_4	477.3 ± 5.6^b	0.247 ± 0.044^b	4.69 ± 0.19^b	56.7 ± 4.5^b
Group III	Silymarin + CCl_4	253.4 ± 3.6^c	0.399 ± 0.048^c	6.98 ± 0.42^c	69.4 ± 2.3^c
Group IV	Ethanolic extract + CCl_4	289.5 ± 5.4^d	0.324 ± 0.032^c	6.41 ± 0.43^c	59.9 ± 1.4^b

Values carrying different superscript for each parameter are significantly different ($P<0.05$). MDA, malondialdehyde; GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase

Histopathological examinations

Histopathological observations showed extensive damage characterized by severe necrosis, damaged cell membrane, degenerated nuclei, fibrosis, broad infiltration of lymphocytes in the hepatocyte of CCl_4 exposed animals (Figure 1). In group IV mice (exposed to CCl_4 and ethanolic extract), the histoarchitecture of liver sections showed minimal disruption. More or less normalized lobular pattern was observed with prominent cytoplasm and visible central veins.

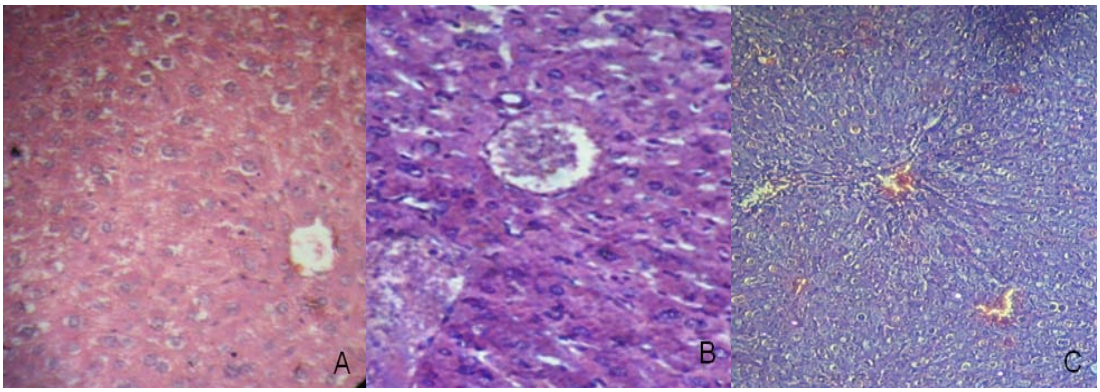

Fig. 1: (A) Liver section of normal control mice: normal hepatic cells with well preserved cytoplasm, well brought out central vein and prominent nucleus. (B) Liver section of CCl_4 treated mice showing necrotic lesions, fatty infiltration, fibrosis, infiltration of lymphocytes and loss of cellular boundaries. (C) Liver section of mice treated with CCl_4 and extract showing well brought out central vein, hepatic cell with well preserved cytoplasm and prominent nucleus.

DISCUSSION

Hepatic damage by CCl_4 exposure is the most common model for hepatoprotective drug development[36]. CCl_4 induces hepatotoxicity by metabolic activation; therefore, it selectively causes toxicity in liver cells maintaining semi-normal metabolic function[37]. In the present study, MGEE when administered orally exhibited hepatoprotective actions. The evidence of hepatic damage was noted by the level of increased serum enzymes (SGPT, SGOT and ALP), serum bilirubin content[38] and by histopathological studies. The experimental damage produced by CCl_4 intoxication resembles viral hepatitis histologically[39]. Treatment with the extract lowers serum transaminases and alkaline phosphatase indicating stabilization of plasma membrane as well as repair of hepatic injury. Reduction of serum transaminases near normal levels suggested regeneration of hepatocytes with healing of hepatic parenchyma[40]. Even pathological alteration in biliary flow is reflected by the enzyme ALP[41]. Marked increase in serum bilirubin content is also in relation with CCl_4 induced elevation of serum enzymatic activity. Depletion of raised bilirubin with concurrent suppression of increased ALP as may be induced by the ethanolic extract suggests the ability of the extract to stabilize biliary dysfunction during CCl_4 induced liver injury in mice. The histopathological studies was performed to provide direct evidence of the possibility of the extract being able to minimize disruption of structure of hepatocytes and accelerates hepatic regeneration thus decreasing the leakage of SGPT, SGOT and ALP into the circulation.

Our study also coincides with earlier works that demonstrated hepatoprotective potential of various extracts and purified compounds of several mushrooms. CCl_4 induced chronic hepatotoxicity in rats is protected by ethyl acetate extract of *Phellinus rimosus*[1]. *In vitro* and *in vivo* protective effects of *Ganoderma lucidum* proteoglycan were exhibited on CCl_4 induced hepatic tissue damage[23]. *Lentinus edodes, Grifola frondosa*, *Tricholoma lobayense*, *Ramaria botrytis*, *Calocybe indica* and *Astraeus hygrometricus* are also reported to have significant hepaprotective activites[15, 17, 42, 43].

The antioxidant and hepatoprotective activities of the extract may be due to the presence of flavonoid and phenolic compounds, which has been shown by the preliminary phytochemical analysis of the extract[44]. Our earlier works show strong *in vitro* free radical scavenging activity and inhibition of lipid peroxidation by MGEE[22]. Here, an investigation has been made to find out the correlation between antioxidant status of the liver and hepatoprotective activity. In the CCl_4 alone treated animals, alterations were evident in the antioxidant status of the hepatocytes. The treatment of MGEE effectively protected the decline of antioxidant activity. Estimation of hepatic constituents, MDA, GSH, SOD and CAT levels were done to assess the *in vivo* antioxidant status. SOD and catalase are important enzymes, which project against the free radical injury mediated. Low levels of the antioxidant enzymes SOD and CAT in CCl_4 treated animals might be due to the overwhelming effects of free radicals, as evidenced by the elevated levels of lipid peroxidation[45]. One of the major mechanisms to inhibit the process of lipid peroxidation is scavenging of free radicals derivatives. Significant decrease in MDA level in MGEE treated group reveals diminished lipid peroxidation. Simultaneously, significant increase in GSH, SOD and CAT content of liver suggested antioxidant activity of MGEE. Thus, it can be concluded that MGEE is able to confer protection against hepatotoxicity induced by CCl_4 in mice model. The possible mechanism may be due to its antioxidant property and ability to scavenge free radicals, which can be correlated to the presence of flavonoid and phenolic compounds in MGEE.

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