FREE RADICAL SCAVENGING AND HEPATOPROTECTIVE ACTIVITIES OF STANDARDIZED METHANOLIC EXTRACT OF *MAYDIS STIGMA*

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Summary

The herbal drug *Maydis stigma*, style of female flower of *Zea mays* L. (Family-*Gramina*e), is used for the treatment of variety of diseases such as in urinary tract diseases, gonorrhea, benign prostatic hyperplasia, hypertension, hepatobiliary diseases etc. The present study was carried out to evaluate the free radical scavenging and hepatoprotective activities of standardized methanolic extract of *Maydis stigma* against CCl_4-induced decreased level of antioxidant enzymes, hepatotoxicity respectively and also to correlate the role of free radicals in hepatic injury. Methanolic extract of the *Maydis stigma* was standardized for total polyphenol, tannin, flavonoid, and flavonols contents and evaluated for the free radical scavenging activity against CCl_4 induced lipid peroxidation and reduction in the levels of SOD, GSH, peroxidase and catalase. Hepatoprotective activity was evaluated against CCl_4-induced hepatotoxicity by estimation of the level of hepatic enzymes ALT, AST, ALP and total bilirubin in the serum. Methanolic extract of *Maydis stigma* showed significant free radical scavenging and hepatoprotective activity against CCl_4-induced toxicity and was found to be comparable with the standard drugs. Histopathological observations of the H-E stained liver sections of different animal groups supported the hepatoprotective activity of the plant.

Keywords: Free radical scavenging activity; Hepatoprotective activity; *Maydis stigma*.

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Introduction

The herbal drug *Maydis stigma*, style of female flower of *Zea mays* L. (Family- *Graminae*), which is commonly known as "Corn Silk" grows in the tropical and subtropical Asian countries, America and is cultivated in subtropical countries of the world. It is used as a diuretic in acute and chronic cystitis in the bladder irritation of uric acid and phosphatic gravel, gonorrhea and hepatobiliary diseases [1]. The style of *Zea mays* has been used in the folk medicine as a decoction for diuretic treatment in Japan and China [2-3]. Anti-hyperglycemic effect and amelioration of chronic nephropathy of corn silk are reported in the dictionary of traditional Chinese medicines [4]. Diuretic and uricosuric properties have traditionally been attributed to corn silk [5]. In Peru, people consume a typical drink made from purple corn called "Chicha Morada" which is believed to improve health [6]. Corn silk is recognized and used both in traditional and official medicine, as a mild diuretic, urinary demulcent, to pass stone and gravel from kidney and urinary bladder, against benign prostatic hyperplasia, cystitis, gout, chronic nephritis and similar ailments [7-12]. Corn silk is used to treat pathological swelling and asthma in Korea based on the folk remedies [13]. Corn silk is reported to prevent the growth of *Aspergillus flavus* [14], inhibits tumor necrosis factor (TNF)-α and lipopolysaccharide (LPS)-induced cell adhesion [15]. The style of *Zea mays* is used in the Chinese traditional medicine for the treatment of dropsy and hypertension [16].

A number of phenolics and flavonoids have been reported from the corn silk [16]. Phytochemical screening of the methanolic extract of corn silk has revealed the presence of a number of phenolics and flavonoids, known for their anti-oxidant and free radical scavenging activity. Free radicals are the reactive species, responsible for cellular damage including hepatotoxicity. *In vitro* anti-oxidant activity due to the polyphenols content of the *Maydis stigma* extract has been reported [17-18]. The present study was carried out to standardize the methanolic extract for the total polyphenols content, evaluation of *in vivo* free radical scavenging and hepatoprotective activities of the extract and also to correlate the role of free radicals in the liver damage.

Methods

Plant material, Extraction and Isolation

The plant material *Maydis stigma* (style of female flower of *Zea mays* L. also known as corn silk) was collected from Varanasi, Uttar Pradesh, India in the month of July and was identified by Prof. K.N. Dubey at the Department of Botany, Faculty of Science, Banaras Hindu University, Varanasi, India. A voucher specimen (Specimen No. PCRL-48) has been deposited in the Pharm. Chemistry Research Laboratory, Department of Pharmaceutics, Institute of Technology, Banaras Hindu University, Varanasi, India for future reference. The plant material was shade-dried and powdered. The dried and powdered corn silk (3.5 kg) was macerated in methanol (10 L) for 7 days with occasional shaking and was filtered and concentrated *in vacuo*. Methanolic extract of the plant kept in the decicator for several days for the complete removal of solvent afforded the dried extract (217 g).

Animals

Albino rats of either sex (100-150 g) were purchased from Central animal house, Institute of Medical Sciences, Banaras Hindu University, Varanasi. The animals were left for 7 days to acclimatize to animal house conditions and allowed free access to Lipton rat feed (Mumbai, India) and water *ad libitum*. The Animals were maintained on twelve hour dark/light cycle at an ambient temperature and were fasted overnight before the experiment.

Experiments were performed in accordance with the current guidelines for the care of laboratory animals and the investigation of experimental pain in conscious animals [19]. Before the commencement of animal experimental study permission was taken from the Institutional Ethical Committee.

Reagents and Chemicals

Nitro-blue tetrazolium (NBT), Hydroxylamine hydrochloride, 5, 5-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Aldrich, India. Vitamin-E capsule, EDTA, Trichloroacetic acid, Hydrogen peroxide were purchased from Merck India Ltd. Silymarin was obtained from Ranbaxy Laboratories Limited, India. Span diagnostic reagent kits were used for the assays of Bilirubin and Alkaline Phosphatase. Crest Biosystem, Goa, India reagent kits were used for the estimation of AST and ALT levels. Bovine serum albumin was purchased from Loba Chemie Indo-Austranal Co., Bombay. All other chemicals used were of analytical grade.

Acute toxicity study

The acute toxicity study for methanolic extract of corn silk was performed using albino rats weighing 100-150 g. The animals were fasted overnight prior to the experiment and maintained under standard conditions. Forty two albino rats were divided into seven groups of six rats in each group and were given graded doses (50, 100. 200, 500, 1000 and 2000 mg/kg body weight) of the extract by gastric tube. The seventh group received single dose of 2 mL normal saline through the same route.The animals were observed for 24 h for any signs of acute toxicity such as increased/decreased motor activity, tremors, convulsion, sedation and lacrimation.

Phytochemical studies

Methanolic extract of the corn silk was subjected to phytochemical screening for the isolation of active constituents.

Determination of total Polyphenol contents

Total polyphenols were determined by Folin-Ciocalteu procedure [20]. Of the methanolic extract of corn silk, 100 mg was taken and dissolved in 100 mL of triple distilled water. Of the above solution, 1mL was transferred in test tube, 8 mL of triple distilled water, 0.5 mL of Folin-Ciocalteu reagent and 1.5 mL of 20% Na_2CO_3 solution was added. Test tubes were vortexed and absorbance of blue colored mixtures was recorded after 40 min at wavelength 725 nm against a blank. The amount of total polyphenols was calculated as a gallic acid equivalent from the calibration curve of gallic acid standard solutions and expressed as mg gallic acid/g of dry mass of extract. All measurements were done in triplicate.

Determination of tannins

Total tannin content was determined by Folin-Ciocalteu procedure. Methanolic extract of corn silk, 100 mg was taken and dissolved in 100 mL of triple distilled water. Of the above solution,1 mL was transferred in test tube, 8 mL of triple distilled water, 0.5 mL of Folin-Ciocalteu reagent and 1.5 mL of 20% Na_2CO_3 solution was added. The absorbance was recorded at 775 nm against blank. Total tannin contents were calculated as tannic acid equivalent from the standard curve of tannic acid standard solutions and expressed as mg tannic acid/g of dry mass of extract. All measurements were done in triplicates.

Determination of flavonoids

The total flavonoids content was determined by a state pharmacopeia of USSR (1989) method with few modifications using rutin as standard. Of the methanolic extract of corn silk in methanol

(10 mg/mL), 1 mL was mixed with 1 mL of aluminium trichloride in methanol (20mg/mL) and a drop of acetic acid, and then diluted with methanol to 25 mL. The absorbance was recorded at 415 nm after 40 min. Blank samples were prepared from 1 mL of plant extract and a drop of acetic acid, and then diluted to 25 mL with methanol. The absorbance of standard rutin solution (0.5 mg/mL) in methanol was measured under the same conditions. All determinations were carried out in triplicate. The amount of total flavonoids in methanolic extract of corn silk expressed as mg rutin /g of dry mass of extract was calculated by the following formula:

$X = (A.m_0) / (A_0.m)$,

where X is the flavonoids content, mg/ g of dry mass of extract in rutin equivalent, A is the absorbance of plant extract solution, A_0 is the absorbance of standard rutin solution, m is the weight of plant extract in mg, m_0 is the weight of rutin in the solution in mg.

Determination of flavonols

The total flavonols content was determined by Yermakov, Arasimov & Yarosh method [21]. The rutin calibration curve was prepared by mixing 2 mL of various concentrations of methanolic solutions of rutin with 2 mL (20 mg/mL) aluminium trichloride and 6 mL (50 mg/mL) sodium acetate. The absorbance at 440 nm was recorded after 2.5 h. the same procedure was used for 2 mL of plant extract (10 mg/mL) instead of rutin solution. All determinations were carried out in triplicate. The content of flavonols mg/g of dry mass of extract equivalent to rutin was obtained from various concentration of rutin.

Animal model for free radical scavenging activity

Animals were divided into five groups A, B, C, D and E with 6 animals in each group. All the drugs at the mentioned dose were administered orally in Tween-20: Normal saline (5:95) suspension. Group-A marked as control group received vehicle only 1.0 mL/kg p.o. Group B received a single dose of CCl_4 (50% v/v dilution with arachis oil) 1.0 mL/kg p.o. only on the sixth day. Group C received standard drug Vitamin-E in a dose 50 mg/kg p.o. Group D & group E received methanolic extract of the silk of *Z. mays* in a dose of 100 & 200 mg/kg p.o. respectively. On the sixth day all the groups except group A were administered CCl_4 (50% v/v dilution with arachis oil) 1.0 mL/kg p.o. and group A was administered equivalent amount of arachis oil. On the eighth day all the animals were sacrificed by cervical dislocation. Brain was taken out immediately and brain homogenate was prepared.

Estimation of free radical scavenging enzymes and lipid peroxidation

Brain homogenates were used for the estimation of LPO (Lipid peroxidation) [22], SOD (Superoxide dismutase) [23], Reduced glutathione [24], Catalase [25] and Peroxidase [26]. The protein content was determined by the method of Lowry et al. [27].

CCl_4-induced hepatotoxicity

Animals were divided into five groups A, B, C, D and E with 6 animals in each group. All the drugs at the mentioned dose were administered orally in Tween 20: Normal saline (5:95) suspension. Group A received vehicle only 1.0 mL/kg p.o. Group B received a single dose of CCl_4 (50% v/v dilution with arachis oil) 1.0 mL/kg p.o. only on the sixth day. Group C received standard drug silymarin in a dose 50 mg/kg p.o. Group D & group E received methanolic extract of the corn silk in a dose of 100 & 200 mg/kg p.o. respectively. On the sixth day all the groups except group A were administered CCl_4 (50% v/v dilution with arachis oil) 1.0 mL/kg p.o. and group A was administered equivalent amount of arachis oil. On the eighth day blood was collected by cardiac puncture in a heparinised 1 mL tuberculin syringe and all the animals were sacrificed by cervical dislocation. The blood so collected was centrifuged immediately at 1500 rpm for 20 min. when serum clearly separated out.

Estimation of biochemical parameters
The serum was used for the estimation of ALT (alanine aminotransferase), AST (aspartate aminotransferase) [28], ALP (alkaline phosphatase) [29] and bilirubin [30] levels.

Histopathology
The animals used were sacrificed, liver tissue was collected and immediately fixed in 10%, formalin, dehydrated in gradual ethanol (50–100%), cleared in xylene and embedded in paraffin. Sections (4–5 μm) were prepared and then stained with hematoxylin and eosin (H–E) dye for photomicroscopic observations [31].

Statistical analysis
All Data were expressed as means ± SEM, and analyzed with one-way analysis of variance (ANOVA), followed by Dunnett's *t*-test. Values less than 0.05 ($p < 0.05$) were considered statistically significant.

Results

Acute toxicity study
Methanolic extracts of corn silk did not show any sign and symptoms of toxicity and mortality up to 2000 mg/kg dose.

Phytochemical study
Phytochemical study of methanolic extract of corn silk showed the presence of phytosterols, glycosides of phytosterols, terpenoids, flavonoids and phenolic compounds. We isolate a few common phytosterols, β-sitosterol glycoside and terpenoids from the methanolic extract of corn silk.

Total polyphenols, tannins, flavonoids, flavonols content
Total polyphenols, tannins, flavonoids and flavonols content of methanolic extract of corn silk were found to be 175.8 ± 12.5 mg/g, 65.58 ± 6.2 mg/g, 24.31 ± 2.8 mg/g and 16.82 ± 1.6 mg/g of dry weight of extract respectively.

Effect of methanolic extract of Corn Silk on SOD, Catalase, GSH, LPO and Peroxidase
The results of the effect of methanolic extract of corn silk on free radical scavenging enzymes and lipid peroxidation are shown in Table-1. CCl_4 in a dose of 1.0 mL/kg p.o. significantly decreased the level of enzymes SOD, catalase, GSH, peroxidase and induced the lipid peroxidation ($p < 0.001$). Methanolic extract of corn silk in a dose of 200 mg/kg p.o. significantly reversed the level of enzymes SOD, catalase, GSH, peroxidase and reduced the lipid peroxidation significantly ($p < 0.001$), which was found to be comparable with the standard drug Vit-E in a dose of 50 mg/kg p.o. ($p < 0.01$). Methanolic extract of corn silk in a dose of 100 mg/kg p.o. also significantly reversed the level of enzymes SOD, catalase, GSH, peroxidase and reduced the lipid peroxidation significantly. But the effect was less than that found in a dose of 200 mg/kg p.o. ($p < 0.05$ for SOD and GSH, $p < 0.01$ for Catalase).

Table1. Effect of the methanolic extract of corn silk on the level of SOD, GSH, LPO, Catalase and Peroxidase in brain homogenate.

Group	SOD (U/mg of Protein)	Catalase (U/mg of Protein)	GSH (µg/g of Protein)	LPO (nMDA/g of Protein)	Peroxidase (µM/10g of tissue)
A	14.83 ± 0.35	176.54 ±1.36	18.74 ± 0.05	7.59 ± 0.29	175.73 ± 4.30
B	8.24 ± 0.21^{c}	80.05±1.85^{c}	8.131 ± 0.03^{c}	23.56 ± 0.43^{c}	93.43 ± 0.53^{c}
C	13.92 ± 0.18^{b,f}	169.85 ±1.41^{b,f}	18.41 ± 0.13^{b,f}	10.1 ± 0.37^{c,f}	164.45 ± 0.64^{c,f}
D	8.96 ± 0.13^{c,d}	87.51 ± 1.42^{c,e}	8.42 ± 0.03^{c,d}	21.1 ± 0.47^{c,f}	104.31 ± 0.51^{c,f}
E	12.96 ± 0.14^{c,f}	168.35 ± 1.68^{b,f}	18.12 ± 0.06^{c,f}	11.86 ± 0.40^{c,f}	152.83 ± 0.47^{c,f}

Values are expressed as mean ± SEM, (n=6)
b = $p < 0.01$, c = $p < 0.001$ when compared with control group.
d = $p < 0.05$, e = $p < 0.01$, f = $p < 0.001$ when compared with toxic control group.

Effect of methanolic extract of corn Silk on the level of ALT, AST, ALP and total Bilirubin

The levels of ALT, AST, ALP, and bilirubin in different animal groups are shown in Table 2.The animal group treated with CCl_4 in a dose of 1.0 mL/mg p.o. showed significant increase in the level of ALT, AST, ALP, and bilirubin ($p < 0.001$) in the serum. Methanolic extract of corn silk significantly reduced the level of ALT, AST, ALP, and bilirubin ($p < 0.001$) in a dose of 200 mg/kg p.o. and was found comparable with the standard drug silymarin in a dose of 50 mg/kg p.o. ($p < 0.001$). Methanolic extract of corn silk in a lower dose of 100 mg/kg p.o. also significantly reduced the level of AST ($p < 0.01$), ALP ($p < 0.001$) and bilirubin ($p < 0.01$).

Table 2. Effect of methanolic extract of corn silk against CCl_4 induced elevation of ALT, AST, ALP and total bilirubin level in serum.

Group	ALT (IU/mL)	AST (IU/mL)	ALP (KAU)	Total Bilirubin (mg/dL)
A	118.95 ± 1.76	346.7 ± 3.47	35.30 ± 1.74	0.256 ± 0.016
B	395.16 ± 3.89^{c}	682.12 ± 5.13^{c}	158.68 ± 3.65^{c}	1.81 ± 0.049^{c}
C	129.97 ± 2.14^{a,f}	365.12 ± 4.31^{b,f}	48.32 ± 2.22^{b,f}	0.426 ± 0.059^{b,f}
D	387.18 ± 3.51^{c,ns}	665.34 ± 4.31^{c,e}	143.58 ± 3.45^{c,f}	1.624 ± 0.054^{c,e}
E	142.76 ± 2.84^{c,f}	381.25 ± 4.20^{c,f}	61.54 ± 1.72^{c,f}	0.583 ± 0.038^{c,f}

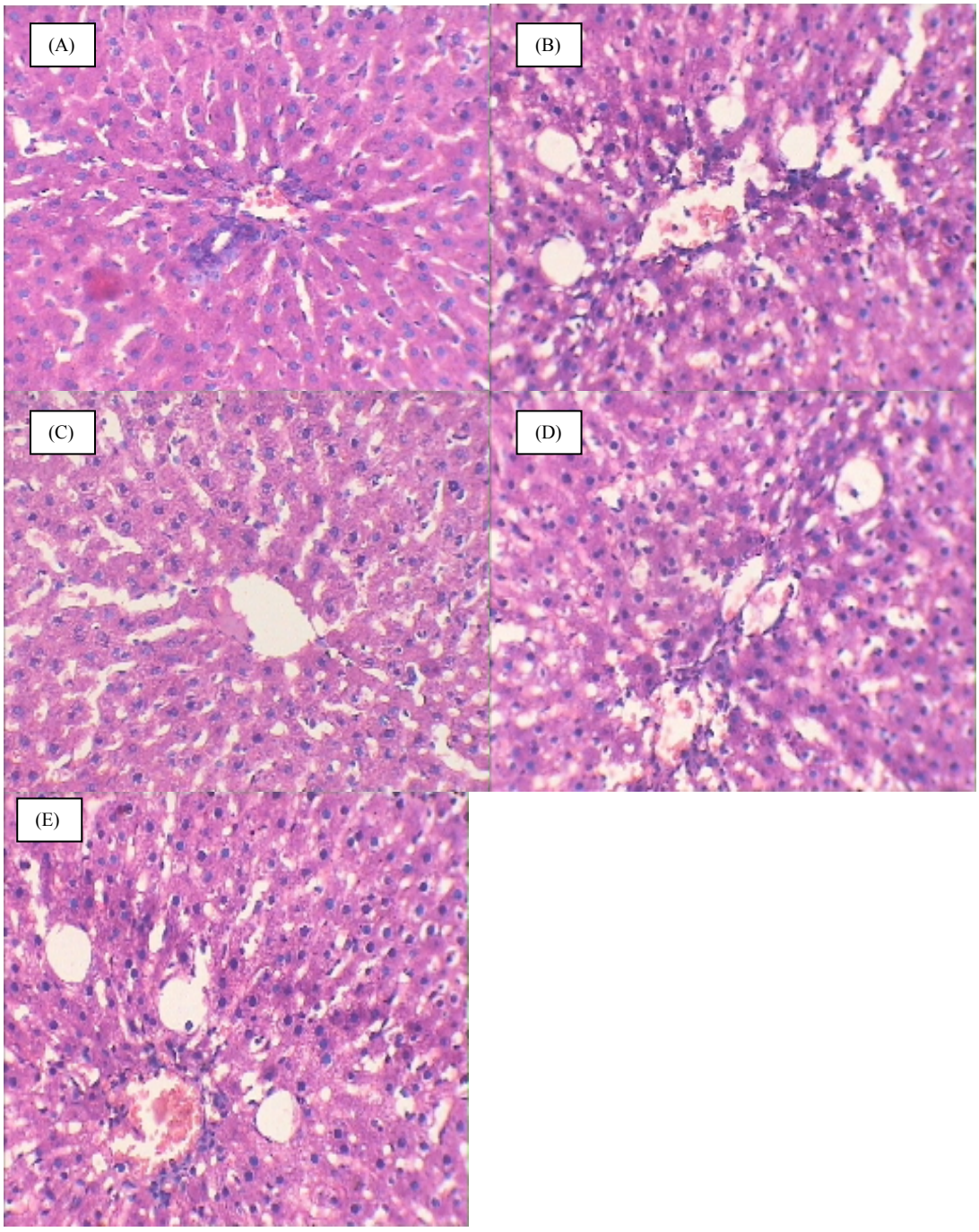
Values are expressed as mean ± SEM, (n=6)
a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$ when compared with control group.
d = $p < 0.05$, e = $p < 0.01$, f = $p < 0.001$, ns = non-significant when compared with toxic control group.

Histopathological observations

Histopathological observations of the H-E stained liver section of animal group treated with the vehicle only showed the normal cellular architecture with distinct hepatic cells, nucleus and central vein (Fig. 1A). Liver section of animal group treated with CCl_4 showed severe histopathological changes such as enlargement of the central vein, fatty changes, ballooning degeneration and

infiltration of lymphocyte (Fig. 1B). Methanolic extract of corn silk in a dose of 100 and 200 mg/kg p.o. (Fig. 1D and Fig. 1E) reversed these histopathological changes toward normal significantly and was found to be comparable with the standard drug silymarin in a dose of 50 mg/kg p.o. (Fig. 1C).

Figure1. Histopathological examination of hematoxylin and eosin-stained liver section of normal and experimental rats with magnification X400. (A)-Vehicle treated control group. (B)-CCl_4 treated group. (C)-CCl_4+Silymarin treated group. (D)-CCl_4+Methanolic extract (100 mg/kg) treated group, (E)-CCl_4+Methanolic extract (200 mg/kg) treated group.



Discussion

In the present study methanolic extract of corn silk was evaluated for the free radical scavenging and hepatoprotective activities. CCl_4 was used to induce the hepatotoxicity and to decrease the level of free radical scavenging enzymes. An attempt was made to find out the role of free radicals in the hepatotoxicity.

CCl_4 is commonly used to induce the hepatotoxicity in various experimental animals [32]. It produces an experimental damage that histologically resembles viral hepatitis [33]. Toxicity starts with the changes in the endoplasmic reticulum, which result in the loss of metabolic enzymes located in the intracellular structures [34-35]. Cytochrom P450 2E1 is the enzyme responsible for the conversion of CCl_4 into reactive metabolite $^{\cdot}CCl_3$ radical, which binds covalently to the macromolecule and causes peroxidative damage of lipid membrane of the adipose tissue [36]. Peroxidative degradation of hepatocyte cell membrane results in the elevation of hepatic enzymes AST, ALT and ALP in the blood. CCl_4 administration also causes hyperbilirubinaemia [37].

Phytochemical analysis of methanolic extract of corn silk has shown the presence of phenolic compounds. Phenolic compounds are well known for their free radical scavenging and hepatoprotective activities [38]. Lipid peroxidation is one of the principal cause of CCl_4-induced liver injury and is mediated by the production of free radical derivative ($^{\cdot}CCl_3$) from CCl_4. Therefore, the inhibition of free radicals generation is important in terms of protection of liver from CCl_4-induced damage [39]. The enzyme GSH forms a conjugate with the trichloromethyl free radical, a P450 2E1 mediated CCl_4 metabolite, and play a key role in detoxifying the reactive toxic metabolite of CCl_4. The liver necrosis begins when the GSH stores are markedly depleted [40]. Methanolic extract of the corn silk in a dose of 200 mg/kg p.o. significantly increase the level of GSH ($p < 0.001$) and thus significantly inhibited the lipid peroxidation ($p < 0.001$).

During hepatic injury, superoxide and hydroxyl radicals are generated at the site of damage, and SOD is exhausted as a result of oxidative stress caused by CCl_4 that further leads to accumulation of free radicals. SOD is a ubiquitous cellular enzyme that dismutates superoxide radical to H_2O_2 and oxygen and is one of the chief cellular defense mechanisms. The H_2O_2 formed by SOD and other processes is scavenged by catalase that catalyses the dismutation of H_2O_2 into water and molecular oxygen. Administration of CCl_4 results in the reduction in the level of these enzymes in the tissue homogenate. Methanolic extract of corn silk returns the level of these enzymes by its anti-oxidant mechanism.

Histopathological observation under the light microscope confirm the efficacy of methanolic extract of corn silk against CCl_4-induced hepatic injury. Methanolic extract of corn silk in a dose of 200 mg/kg p.o. significantly protected the liver cell from the toxicity of CCl_4 and were comparable with standard drug silymarin.

Conclusions

The above results indicate that methanolic extract of corn silk in a dose of 200 mg/kg p.o. has significant hepatoprotective effect on liver damage caused by CCl_4. The extract in a dose of 100 mg/kg p.o. has hepatoprotective activity but less than what was found with 200 mg/kg p.o. It might be postulated that its hepatoprotective activity may be due to its inhibitory effect on the free radical formation as evident by the recovery of GSH, SOD, catalase, peroxidase and decreased lipid peroxidation.

Histopathological parameters indicate the structural and functional integrity of the cells and provide further support to the proposed mechanism of action. Thus methanolic extract of corn silk produced hepatoprotective activity due to its free radical scavenging nature. The plant drug *Maydis stigma* can be used as anti-oxidant and hepatoprotective agent.

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