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Evaluation of Antioxidant and Hepatoprotective Effects of *Dendrophthoe pentranda* Leaves on CCl_4 -Induced Hepatotoxic Rat

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Abstract

The aim of the study was to evaluate the phytochemical profile, antioxidant and hepatoprotective effects of aqueous (ADPL), ethanol (EDPL), chloroform (CDPL) and petroleum ether (PDPL) extracts of *Dendrophthoe pentandra* leaves. The total phenolic (TPC) and flavonoid contents (TFC) were determined by using Folin Ciocalteu method and aluminium chloride assay, respectively. Antioxidant effect was assessed by using DPPH radical scavenging, ferric reducing power (FRP) and total antioxidant content (TAC) assay. Hepatoprotective effect was evaluated against CCl_4-induced liver toxicity in long evans rats. Among the extracts, EDPL and PDPL had the highest TAC and TFC. EDPL and PDPL showed potent antioxidant effect that exhibited IC_{50} 31.62 ± 4.10 and 24.30 ± 3.45 µg/ml (DPPH), EC_{50} 28.18 ± 4.24 and 39.25 ± 5.20 µg/ml (FRP) and 373.83 ± 9.10, 352.67 ± 10.23, mg/g equivalent of ascorbic acid (TAC), respectively. The CCl_4 significantly induced elevation of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total protein (TP) and bilirubin as well as liver damage (cirrosis, ballooning and necrosis). However, treatment with *D. pentandra* extracts (200 mg/kg, p.o) significantly counter balanced the toxicity towards the normal animals. The research may direct further advanced studies to find out hepatoprotective compounds.

Key words: *Dendrophthoe pentandra,* antioxidant, hepatoprotective, carbon tetrachloride

Introduction

The liver performs a wide range of functions like metabolism of carbohydrate, protein, lipid and xenobiotics. Production of plasma proteins, blood clotting factors, bile and cholesterol is also associated with it (Barsha *et al.,* 2018). However, as the liver has a high concentration of toxin-metabolizing enzymes, it can convert xenobiotics to nontoxic or less toxic metabolites that are suitable for proper elimination from the body. But sometimes toxic substances are converted to active metabolites which can exacerbate liver damage and cause changes in the macroscopic structure of specific molecules such as bile acid transporters, families of nuclear receptors, intracellular lipids, proteins and nucleic acids. These molecules induce some secondary paths which finally lead to apoptosis, necrosis, autophagy, mitochondrial defects and ballooning of liver tissue (Yavar *et al.,* 2017). Despite intensive ongoing research, drug-induced lives injury remains a serious issue for healthcare providers and patients and has been a major cause of drug withdrawal and non-approval by regulatory authorities in the past 50 years (Regev, 2014). Oxidative stress, the imbalance between endogenous generation of free radicals and activity of antioxidant systems, has recently been recognized as a key factor in the pathophysiological changes observed in a wide range of liver diseases (Zhang *et al.* 2011). It is a causative factor in the etiology of several human degenerative diseases and the aging process. The reactive oxygen species (ROS), provokes the oxidative stress and is counterbalanced by some enzymatic antioxidants (Brito *et al.* 2012). But in case of liver disorders the ability of natural antioxidant system is impaired and the level of ROS elevates than its threshold level. In this condition, it is

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necessary to take antioxidant drug or to eat antioxidant rich foods. Moreover, conventional drugs have profound side effects and cannot offer proper hepatoprotection by stimulating liver function or regenerating hepatic cells (Islam *et al.* 2012). However, herbal extracts not only have shown a good source of antioxidant but also have proved their ability to enhance activities of the antioxidant enzymes (Brito *et al.* 2012). These traditional products may have the hepatoprotective potential and therefore can be effectively used to treat acute and chronic liver diseases (Aguirre et al. 2014).

Dendrophthoe pentandra is a mistletoe that belongs to the Loranthaceace family. It is an evergreen semi parasitic shrub which grows on the branches of various deciduous trees and around 1500 species of mistletoe have been found worldwide (Lau *et al.,* 2017). It is widely distributed in China, Cambodia, India, Indonesia, Laos, Malaysia, Myanmar, Philippines, Thailand and Vietnam (Nik *et al.,* 2015). It is used traditionally for the treatment of diabetes, hypertension, cancer, diuretic, smallpox, ulcer and skin infection. The phytochemical screening revealed the presence of flavonoids, saponins, tannins and terpenoids . Quercetin from the flavonoid's group is one of the compounds found in *D. pentandra* that poses high antioxidant activity (Afiqah *et al.,* 2016). The present study was designed to evaluate antioxidant and hepatoprotective effects of aqueous, ethanol, chloroform and petrolium ether extracts of its leaves.

Materials and Methods

Plant materials: For the investigation leaves of *D. Pentandra* Linn. were collected from Joypurhat, Bangladesh in December, 2016 and identified by experts of the Bangladesh National Herbarium, Dhaka, where a voucher specimen has also been retained with accession no. DACB 394322. The collected leaves were cleaned, dried for one week and pulverized into a coarse powder using a suitable grinder. The powder was stored in an airtight container and kept in a cool, dark and dry place until further analysis.

Extract preparation: Approximately 500 g powder was kept in a clean, flat-bottomed glass container and soaked in ethanol. Similarly, powder of the leaves was soaked in distilled water, chloroform and petroleum ether. All the containers were sealed and kept for 5 days. Then extraction was carried out by using ultrasonic sound bath. The entire mixture then underwent a coarse filtration by a piece of clean, white cotton material and the extract was filtered through Whatman filter paper. After that gummy extracts were obtained by heating the filtrates in an electric oven. The gummy extracts were then stored in air tight containers. Extraction yield was calculated by using the following formula:

$$\text{Yield} = \frac{\text{Weight of dried extract (g)}}{\text{Weight of leaves powder (g)}} \times 100$$

Drugs and chemicals: Silymarin, diagnostic kits for serum alanine aminotransferase (ALT) and aspartate amino transferase (AST), alkaline phosphatase (ALP), total proteins (TP) and bilirubin were purchased from Human, Germany. DPPH, quercetin, gallic acid, ascorbic acid were purchased from Sigma Aldrich, USA. Other chemicals and solvents were of the highest analytical grade commercially available.

Animals: Long Evans rat of either sex weighing approximately 100-130 g were used for this experiment. The rats were purchased from the animal research branch of the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B). After their purchase, the rats were kept in standard environmental conditions ($24.0 \pm 0°C$ & 55-65% relative humidity and 12 h light/dark cycle) for one week to acclimate and fed ICDDR, B formulated rodent food and water *ad libitum.* The experimental procedures involving animals were followed according to the Medical Ethics, Biosafety and Biosecurity Standards (Zimmermann, 1983).

Preliminary phytochemical screening: Phyto-chemical screening tests were performed to ascertain the presence or absence of phytoconstituents in the extracts by using standard procedure described by Harborne, 1973; Sofowara, 1993; Somkuwar, 2013.

Determination of phytoconstituents

Determination of total phenol content (TPC): Total phenol contents were determined using Folin-Ciocalteu reagent as described by Yang et al, 2007 with slight modifications. Total phenol assay was conducted by mixing 2.7 ml of deionised water, 0.01 ml of extracts, 0.3 ml 20% Na_2CO_3 and 0.10 ml Folin-Ciocalteu reagent. Absorbance of mixture was measured at 725 nm. A standard curve was prepared with gallic acid (r^2 = 0.965) and final results were given as mg/g gallic acid equivalent.

Determination of total flavonoid content (TFC): 1 ml of plant extract in methanol was mixed with 1 ml aluminium trichloride in ethanol (20 mg/ ml) and a drop of acetic acid was added in it and then diluted with ethanol to 25 ml. The absorption at 415 nm was read after 40 min. Blank samples were prepared using all the reagents with equal volume used in the sample except extract. The total flavonoid content was determined by using a standard curve (r^2 = 0.942) of quercetin (12.5-200 µg/ml) where quercetin was used as standard sample. Total flavonoid content was expressed as mg/g of quercetin equivalent (Kumaran *et al,* 2007).

Antioxidant ability assay

DPPH$^•$ radical scavenging activity: The DPPH free radical scavenging activity of the extracts were measured by using DPPH (Braca *et al,* 2002). Briefly, 0.004% w/v of DPPH radical solution was prepared in methanol and then 900 µl of this solution was mixed with 100 µl of extract solution (12.5-200 µg/ml) and kept in a dark place for thirty minutes. Then absorbance was measured at 517 nm where methanol (98%), DPPH solution and ascorbic acid were used as blank, control and standard antioxidant, respectively. Scavenging capacity of DPPH radicals (% Inhibition) was measured by the following formula and finally calculated 50% inhibition concentration (IC_{50}) using software.

$$\text{Inhibition (\%)} = (A_0 - A_s)/A_0 \times 100$$

Where A_0 = Absorbance of control group, A_s= Absorbance of sample

Ferric-reducing power (FRP) assay: The Fe^{3+} reducing power of the extracts was determined by the method of Oyaizu, 1986 with slight modifications. Different concentrations of the extracts and standard ascorbic acid (12.5, 25, 50, 100, 200 µg/ml) were prepared. 1 ml of both the extracts and standard ascorbic acid of all concentrations were taken in separate test tubes and were mixed with 2.5 ml of phosphate buffer solution (0.2 M, pH 6.6). 2.5 ml of potassium ferricyanide (1%) was added in each test tube, and incubated at 50°C for 30 min. Then, 2.5 ml of trichloroacetic acid (10%) was added to the mixture which was then centrifuged at 4000 rpm for 10 minutes. Finally, 2.5 ml of supernatant was mixed with 2.5 ml of distilled water and 0.1 ml of $FeCl_3$ (0.1%) solution followed by incubation at 35ºC for 10 minutes. The absorbance was measured at 700 nm and the reducing powers of the extracts were compared with the standared ascorbic acid.

Determination of total antioxidan content (TAC): The total antioxidant capacity of the extracts was evaluated by phosphomolybdenum complex according to the method of Prieto et al, 1999. In brief, 0.3 ml extracts (100 and 200 µg/ml) solution was combined with 3ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 minutes in water bath. Then, after cooling to room temperature, the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank solution (methanol). Ascorbic acid was used as standard antioxidant (r^2 = 0.974) and total antioxidant capacities of the extracts were expressed as mg/g equivalents of ascorbic acid.

Hepatoprotective studies: Hepatoprotective activity of extracts was evaluated by intraperitonial administration of CCl_4 in Long Evans rats which was previously described by Gerhard, 2002. Briefly, thirty animals of both sexes were divided into 5 groups of six animals in each group and subjected to the following experiment. Group-I served as the control and received 1ml olive oil daily for 7 days. Group-II served as the toxic group (CCl_4 control) and received

25% CCl_4 in olive oil (1 ml/kg i.p) daily for 7 days. Group-III served as positive control and received the standard drug silymarin (50 mg/kg p.o.) for 7 days. Group-IV, V, VI and VII were treated with ADPL, EDPL, CDPL and PDPL at 200 mg /kg.p.o for 7 days. On the 7th day animals of each group were sacrificed after anaesthesia carried out by chloroform. Blood sample was collected from anticubital vain and serum was separated by centrifugation for estimation of hepatic enzymes. Then the liver markers such as ALT, AST, ALP, TP and bilirubin were estimated from the serum by blood chemistry analyzer Olympus AU-400 (USA).

Histopathological examination: Liver of each animal was separated and washed with normal saline (0.9%) solution and preserved with 10% formalin for histopathological examination. Sections (4-5 mm thick) were prepared from each liver and stained with Hemotoxylin and Eosin dye for photomicorscopic observation (Suvarna, et al. 2013). The microscopic slides of the liver cells were photographed at a magnification of x 40.

Statistical analysis: All the data are expressed as mean ± S.E.M.. Statistical significance calculated by ANOVA done in SPSS, Version 15.0, followed by Dunnett ´s Test. $P < 0.01$ and 0.001 were considered to be statistically significant. All the graphs are prepared using Graph Pad Prism software, version 5.0.

Results

Phytochemical screening: The phytochemical screening of the extracts showed the presence of different types of secondary metabolites such as saponins, tannins, flavonoids, alkaloids, terpenoids and steroids.

Phytoconstituents

Total phenolic content (TPC): Among the four extracts, PDPL showed the highest TPC (371.12 ± 6.61 mg/g Eq of gallic acid), while ADPL showed the lowest TPC. The order of TPC is PDPL> EDPL> CDPL> ADPL (Table 1).

Total flavonoid content (TFC): PDPL presented the highest TFC (342.04 ± 8.20 mg/g Eq of quercitine) while ADPL have shown the lowest TFC (168.71 ± 8.92 mg/g Eq of quercitine). The order of TFC is PDPL> EDPL> CDPL> ADPL (Table 1).

Table 1. Total phenol and flavonoid content as well as yield value of the extracts

Samples	Total phenol (mg/g Eq of gallic acid)	Total flavanoid (mg/g Eq of quercitine)	Yield (%)
ADPL	192.50 ± 8.92	168.71 ± 8.92	16.25
EDPL	312.50 ± 8.92	321.72 ± 8.90	12.26
CDPL	218.38 ± 6.60	175.33 ± 7.71	8.23
PDPL	371.12 ± 6.61	342.04 ± 8.20	6.25

All the data are presented as mean ± SEM.

In vitro antioxidant activity

DPPH radical scavenging assay: All the extracts have inhibited DPPH radicals and the potent inhibitory was shown by PDPL and EDPL, where median inhibitory concentration (IC_{50}) value of these were 31.62 ± 4.10 and 24.30 ± 3.45 µg/ml, respectively. IC_{50} value of CDPL and ADPL were 57.11 ± 6.23 and 45.11 ± 5.36 µg/ml, respectively (Figure 1).

Feric reducing power (FRP) assay: All the extracts have shown feric reducing power and the potent FRP was shown by PDPL and EDPL, where as median effective concentration (EC_{50}) value of these were 28.18 ± 4.24 and 39.25 ± 5.20 µg/ml, respectively. EC_{50} value of CDPL and ADPL are 53.28 ± 6.52 and 51.27 ± 6.23 µg/ml, respectively (Figure 1).

Total antioxidant content (TAC): This method is based on the reduction of molybdenum (VI) to

molybdenum (V) which takes place in the presence of antioxidant. In this assay the TAC of PDPL, EDPL, CDPL and ADPL was 373.83 ± 9.10, 352.67 ± 10.23, 198.72 ± 8.36 and 173.83 ± 7.26 mg/g equivalent of ascorbic acid, respectively (Figure 2).

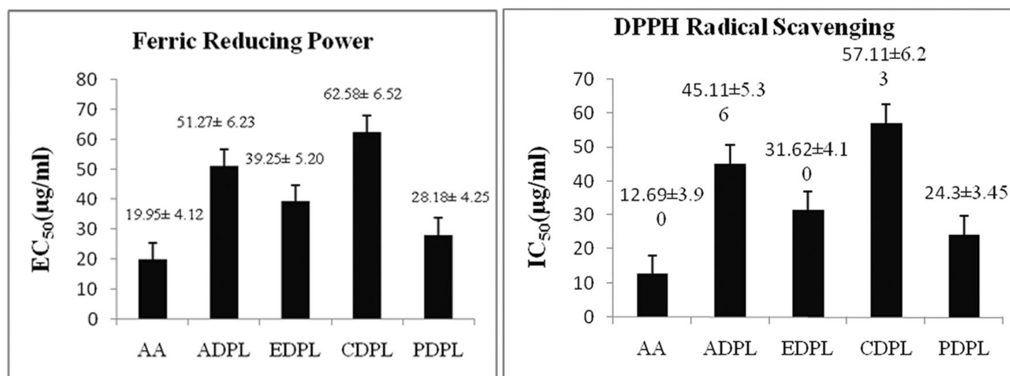

Figure 1. DPPH Radical scavenging and FRP assay of *D. pentandra* leaves extracts. All the data are presented as mean ± SEM.

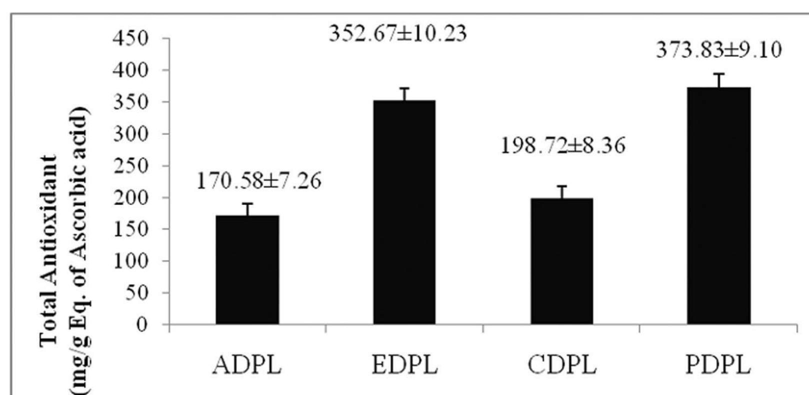

Figure 2. Total antioxidant content (TAC) of the *D. pentandra* leaves extracts.

Hepatoprotective studies

Specific liver enzymes, total protein and bilirubin: The plasma level of specific liver enzymes and protein profile were assessed to determine the liver function of each rat. The liver damage, induced by CCl_4, significantly (P<0.001) elevated the plasma level of specific liver enzymes (ALT, AST, ALP and bilirubin) and lowered total protein (TP) levels in the hepatotoxic rats compared with the normal group. However co-treatment with the extracts (200 mg/kg bw, p.o.) and the reference drug silymarin (50 mg/kg bw, p.o) causes significant reduction of liver markers such as ALT, AST, ALP and bilirubin, as well as elevation of protein level against the CCl_4 group. These data demonstrated that the effects of toxicity induced by CCl_4 could be effectively counter balanced by the treatment of the extracts (Table 2).

Histopathological evaluation: The microscopic assessment (H&E staining) of liver sections in the experimental Groups I–VII is shown in Figure 3. Normal group showed no pathological abnormality. The control group rats showed CCl_4-induced severe necrosis and substantial changes in liver section such as ballooning, microvesicular steatosis, fatty degeneration and increase in sinusoidal space dilation as compared to normal group. On the other hand, livers of rats in all treatment groups (extracts and silymarin) showed noticeable recovery from CCl_4 - induced liver damages with less microvesicular steatosis and hepatocytes necrosis features compared to CCl_4 control group (Figure 3).

Table 2. Effects of *D. pentandra* leaf extract and silymarin on liver markers.

Sample	ALT (U/ml	AST (U/ml)	ALP (U/ml	TP (g/dl)	Bilirubin (mg/dl)	Cholesterol (mg/dl)
Normal	65.80 ± 2.55	46.28 ± 4.33	37.12 ± 2.47	11.24 ± 0.25	0.65±0.1	117.26 ± 5.26
CCl4	125.36 ± 4.7^{a}	108.25 ± 5.1^{a}	95.36 ± 3.78^{a}	2.78 ± 0.01^{a}	8.25 ± 0.23^{a}	235.45 ± 6.25^{a}
Silymarin	69.12 ± 3.45^{a}	51.20 ± 3.58^{a}	43.25 ± 3.69^{a}	9.36 ± 0.10^{a}	0.95 ± 0.20^{a}	120.36 ± 6.25^{a}
ADPL	99.28 ± 5.12^{b}	87.12 ± 5.47^{b}	81.36 ± 4.25^{b}	4.71 ± 0.12^{b}	4.33 ± 0.20^{a}	182.33 ± 7.39^{b}
EDPL	81.30 ± 4.23^{a}	69.23 ± 5.36^{a}	56.47 ± 2.89^{a}	7.56 ± 0.20^{a}	2.36 ± 0.16^{a}	146.10 ± 4.37^{a}
CDPL	92.90 ± 3.95^{b}	78.69 ± 3.47^{a}	70.25 ± 3.10^{a}	5.24 ± 0.10^{a}	3.11 ± 0.11^{a}	165.23 ± 3.68^{a}
PDPL	75.36 ± 3.12^{a}	60.13 ± 4.33^{a}	48.36 ± 2.22^{a}	8.36 ± 0.25^{a}	1.55 ± 0.10^{a}	135.20 ± 5.24^{a}

All the data are presented as mean±SEM (n=6). $P^{a}<0.001$ and $P^{b}<0.01$ were considered significant as compared to the control group

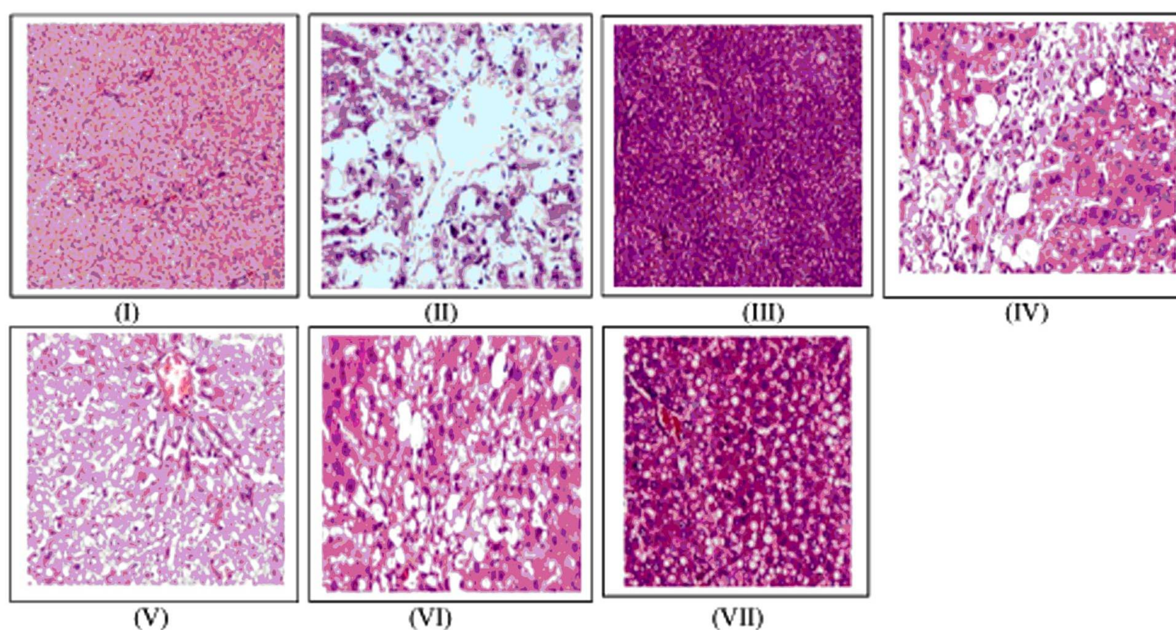

Figure 3. Photomicrographs of histological changes of rat liver by CCl_4 and extract treatment. I= Saline + Olive oil, II= CCl_4 +Olive oil, III= CCl_4 +Olive oil+ Silymarin, IV= CCl_4 +Olive oil+ ADPL, V= CCl_4 +Olive oil+ EDPL, VI= CCl_4 +Olive oil+ CDPL, VII= CCl_4 +Olive oil+ PDPL.

Discussion

Carbon tetrachloride (CCl4) is a lipophilic potent hepatotoxin which can easily cross the cell membrane and distribute throughout tissues soon after its exposure (Barsha et al., 2018). Then it is biotransformed to the trichloromethyl free radical ($NCCl_3^*$) and further converted to a peroxy radical ($CCl_3O_2N^*$). This conversion takes place in the liver by the help of cytochrome P450 (CYP2E1) of endoplasmic reticulum (Sahil *et al,* 2013; Wang *et al,* 2011). These radicals lead to auto-oxidation of cellular lipids and proteins, and thereby induce cellular damage and generate some endogenous toxicants. This toxic substances cause liver cell necrosis, apoptosis, fibrosis and cirrhosis (Rašković *et al.,* 2014, Gupta et al, 2014; Sahreen *et al,* 2011). The change causes significant reduction of antioxidant enzymes such as catalase (CAT), peroxidase (POD), superoxide dismutase (SOD),

glutathione peroxidase (GPx), glutathione reductase (GSR), glutathione-S-transferase (GST) and quinone reductase (QR) (Sahreen *et al.,* 2011). As a consequence, the cell membranes of hepatocytes become more permeable to ALT, AST and ALP that are generated by hepatic cell damage (Althnaian *et al,* 2013). So the level of ALT, AST and ALP is elevated in the blood circulation (Xia *et al,* 2013). On the other hand, liver is known to play a significant role in the serum protein synthesis. So reduced level of total protein (TP) is the consequence of defective biosynthesis of protein in it (Sharma et al, 2012). Thus the extent of liver damage can be effectively assessed by estimating the activities of the liver markers: ALT, AST, ALP, TP and bilirubin.

Any antioxidant acts against free radicals like $NCCl_3^*$ and $CCl_3O_2N^*$ and protect liver cell from damage (Showkat *et al,* 2013). In addition, the harmful effects of reactive oxygen species (ROS) are counterbalanced by antioxidant defense system involving a variety of enzymatic and non-enzymatic mechanisms. GSH is the main non-enzymatic endogenous antioxidant in the hepatic cells and reduced form of it is abundantly available in the liver. Its function is to remove ROS such as H_2O_2, superoxide anions and alkoxy radicals. Besides, it maintains integrity of membrane proteins and acts as a substrate for GPx and GST (Panda *et al,* 2012). Antioxidant enzymes GPx and CAT catalyze the reduction of peroxides to alcohols or water (Rašković *et al,* 2014). Studies have reported that CCl_4 reduces the activities of antioxidant enzymes and causes hepatopathy (Gupta *et al,* 2014).

Phytochemical screening of the extracts revealed major bioactive compounds such as saponins, tannins, flavonoids, alkaloids, terpenoids and steroids. They also revealed significant amount of TPC and TFC (Table 1). Among the extracts, PDPL and EDPL showed potent antioxidant potential (Figures 1-2). Co-administration of the extracts (PDPL, EDPL, CDPL and ADPL) prevented the toxic effects of CCl_4 on the tested liver. They may ameliorate the liver injuries by restoring the activities of antioxidant enzymes, scavenging of free radicals, or by preventing cytochrom P450 enzyme which is further confirmed by the reduced amount of histopathological injuries (Gupta et al, 2014, Farah *et al,* 2014). Among the four extracts PDPL and EDPL showed better protection as it possesses more antioxidant effect than the others.

The results of this study demonstrated that *D. pentanda* extracts (PDPL, EDPL, CDPL and ADPL) have significant antioxidant potential and considerable action on CCl_4-induced hepatotoxicity. Further study on the plant extracts can be extended for the isolation of active constituents, structural determination and mode of action of the extracts.

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Conflict of Interest

Authors have no conflict of interest.

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