

Effect of chloroform extract of traditional *Dicranopteris linearis* leaves against paracetamol- and CCl₄-induced liver toxicity in rats

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ABSTRACT

The present study aimed to determine the hepatoprotective activity of the chloroform extract of *D. linearis* leaves (CEDL) using the paracetamol (PCM)- and carbon tetrachloride (CCl₄)-induced liver injury models in rats. The rats received dH₂O (negative control), 200 mg/kg of silymarin (positive control) or CEDL (50, 250 and 500 mg/kg) orally once daily for 7 days and then were subjected to the hepatotoxic induction on the 7th day. The samples (i.e. blood and liver) were collected and underwent biochemical and microscopical analysis, respectively. From the data obtained, both inducers caused significant ($p < 0.05$) increase in the levels of AST and ALT when compared to the control group, which were significantly ($p < 0.05$) reduced by CEDL in a generally dose-dependent manner. These biochemical findings were supported by the histopathological analysis and histological scoring. In conclusion, CEDL possesses potential hepatoprotective activity, which could be associated with its flavonoid and tannin contents with the mechanisms of hepatoprotection linked to either its antioxidant or anti-inflammatory/immunomodulating activities. Further in-depth studies are required to identify the responsible bioactive compound.

Keywords *Dicranopteris linearis*, Gleicheniaceae, *in vivo*, hepatoprotective activity, chloroform extract, leaves

INTRODUCTION

The liver plays a vital role in maintaining the homeostasis of the body via the metabolism, detoxification and elimination of endogenous and exogenous molecules (Guegenrich, 1994; Lee, 1995). These activities are attributed to the presence of various types of metabolic enzymes involved in phase I oxidation-reduction reactions (Mroueh et al., 2004). The liver function can be impaired upon exposure to alcohol, drugs, infections etc. Prolonged injury to hepatocytes by those agents will lead to liver injury due to oxidative damage mediated by those agents (Adewusi and Afolayan, 2010). Approximately 20,000 deaths are reported every year due to liver diseases. This could be related to the limited efficacy of the presently available pharmacotherapy-based medicine in the protection against or treatment of liver diseases.

Plants have been used traditionally to treat various types of ailments including liver diseases (Chaudhary et al., 2010) and recent research related to hepatoprotective activity has shifted towards medicinal plants as new sources of gastroprotective agents (Mroueh et al., 2004; Zakaria et al., 2012). This is in corroboration with various claims related to numerous botanical based preparations in Unani, Ayurvedic, Arab and

Chinese traditional medicines that are effective as hepatoprotective agents (Mathew and Babu, 2011). Malaysia is among the most biologically diverse countries in the world with approximately 12,000 species of flowering plants found growing within its territory (Jamal et al., 2010). However, only 10% of the higher plants available in Peninsular Malaysia were reported to possess medicinal values, with only about a hundred of them subjected to scientific studies to validate their medicinal claims (Jamal et al., 2010). There are several plant species that are found to grow wild in Malaysia but are at present considered to be underutilized and neglected due to the lack of traditional claims on their medicinal uses among the Malaysian people, particularly the Malays.

One of these plants that is being studied for its hepatoprotective activity in our laboratory is *Dicranopteris linearis* (L.). *D. linearis*, belonging to the family Gleicheniaceae, which is known to the Malays as '*Resam*'. Despite its limited usages (i.e. to reduce body temperature and to control fever) within Malay traditional medicine (Zakaria et al., 2010), the plant has been traditionally used to treat asthma and woman's sterility by the tribes in the Indian mountains, to treat external wounds, ulcers and boils by the people of Papua New Guinea and to get rid of intestinal worms by the people of Indochina (Zakaria et al., 2011a). Interestingly, we have scientifically reported the presence of antinociceptive, anti-inflammatory, antipyretic, antiproliferative, and antioxidant activities in the various extracts of *D. linearis* leaves. Despite reports on links between the mechanisms of anti-inflammatory, antioxidant and antiproliferative activities

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with the hepatoprotective activity, no scientific study has been carried out to evaluate the hepatoprotective potential of *D. linearis* leaves. Therefore, the present study aimed to determine the hepatoprotective activity of the chloroform extract of *D. linearis* leaves (CEDL) using a rat model.

MATERIALS AND METHODS

Plant material and preparation of the extract

The leaves of *D. linearis* were collected between July and August, 2010 from its natural habitat around Serdang, Selangor, Malaysia. A new voucher specimen (IR 0128/11) was deposited at the Herbarium of the Institute of Bioscience (IBS), Universiti Putra Malaysia.

The preparation of CEDL was carried out according to the method of Zakaria et al. (2011b). Forty grams of coarse powdered dried leaves was soaked three times, each for 72 h, in chloroform (1:20; w/v) at room temperature. The chloroform supernatant was collected and evaporated under reduced pressure at 40°C to yield approximately 2.8 g of dried CEDL (percentage yielded was $\approx 7.1\%$).

Animals used

Adult male Sprague-Dawley rats (weight 180 - 200 g) were adopted in the present study and the animal ethics approval was obtained from the Animal Ethics Committee, UPM (reference no: UPM/FPSK/PADS/BR-UUH/00383). The animals were obtained from the Veterinary Animal Unit, Universiti Putra Malaysia (UPM) and housed at the Animal House, Faculty of Medicine and Health Science, UPM. The animals were kept in polypropylene cages with wood shavings, fed with standard pellets and water *ad libitum* and maintained in a 12 h light/dark cycle at $27 \pm 2^\circ\text{C}$. At all times the rats were cared for in accordance with current UPM principles and guidelines for the care of laboratory animals and the UPM ethical guidelines for investigations of experimental pain in conscious animals as adopted from Zimmermann (1983). Animals were fasted for 48 h prior to all assays, and the standard drug (200 mg/kg silymarin) and extract were administered orally (by gavage) with 10% dimethyl sulfoxide (DMSO; 10 ml/kg) as the vehicle.

Pharmacological study

Acute toxicity study in rats

The extract was subjected to an acute toxicity study using a single dose administration of 5000 mg/kg (p.o.) prior to the hepatoprotective study (Mohamed et al., 2011).

Hepatoprotective assay

In the hepatoprotective study, 50, 250 and 500 mg/kg CEDL were assayed against the paracetamol (PCM; 3 g/kg)- and carbon tetrachloride (CCl_4 ; 1.5 ml/kg)-induced liver toxicity models (Zakaria et al., 2011c). The test solutions were administered for 7 consecutive days and followed 24 h after the last test solution administration by a single oral administration of 100% glycerol (vehicle for diluting the inducers), PCM or CCl_4 . After the administration of the respective inducer, the rats were fasted for 48 h and later anaesthetized. The blood was collected for biochemical parameters analyses and then the rats were sacrificed and their livers were excised immediately and fixed in 10% formalin for histopathology studies.

Statistical analysis

The results are presented as Mean \pm Standard Error of Mean (S.E.M), and analyzed using the one-way analysis of variance (ANOVA) test with a Dunnett post-hoc test with $p < 0.05$ as the limit of significance.

RESULTS

Acute toxicity study

At 5000 mg/kg, orally administered CEDL did not show any signs and symptoms of toxicity or mortality. Moreover, the rats also exhibited a normal behavior pattern.

Effects of CEDL on the blood liver enzymes level

Table 1 shows the effects of CEDL on the levels of two liver enzymes (aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) in rats induced with PCM or CCl_4 . Both inducers caused significant ($p < 0.05$) increases in the levels of AST and ALT when compared to the control group. On the other hand, the CEDL significantly ($p < 0.05$) reduced the levels of AST and ALT in PCM and CCl_4 -induced hepatotoxic rats in a generally dose-dependent manner. These findings were supported by the histological scoring (Table 1).

Histopathological study

PCM (Fig. 1B) and CCl_4 (Fig. 1C) induced severe damage to the architecture of the livers of 10% DMSO-pretreated rats as indicated by the presence of necrosis, infiltration of leukocytes, hemorrhaging and steatosis observed throughout the tissues when compared to the normal untreated tissues (Fig. 1A). Pre-treatment with 200 mg/kg silymarin attenuated the PCM-induced hepatotoxic effect with the absence of necrosis, very mild steatosis and infiltration of leukocytes seen

Table 1. Effects of CEDL on the levels of serum's liver enzymes of PCM- and CCl_4 -induced hepatotoxicity in rats

Hepatotoxicity models	Treatment	Dose (mg/kg)	ALT	AST	Histological Scoring
PCM-induced	Control	-	58.4 \pm 16.1	107.5 \pm 8.3	-
	DMSO	-	589.8 \pm 87.9	1395.6 \pm 199.0 ^a	8.3 \pm 0.4
	Silymarin	200	359.1 \pm 75.8 ^a	612.1 \pm 155.0 ^{ab}	4.5 \pm 0.5 ^x
		50	558.1 \pm 43.0 ^a	1125.4 \pm 138.2 ^a	6.0 \pm 0.6
	CEDL	250	548.1 \pm 81.0 ^a	987.0 \pm 141.3 ^a	7.2 \pm 0.2
		500	356.4 \pm 28.6 ^{ab}	605.30 \pm 73.1 ^{ab}	3.7 \pm 1.3 ^x
CCl_4 -induced	DMSO	-	804.4 \pm 147.1 ^a	1738.4 \pm 322.8 ^a	7.5 \pm 0.2
	Silymarin	200	467.7 \pm 56.2 ^{ac}	720.3 \pm 92.0 ^{ac}	4.0 \pm 0.0 ^y
		50	574.3 \pm 58.1 ^a	1113.5 \pm 149.2 ^a	7.3 \pm 0.2
	CEDL	250	390.1 \pm 71.5 ^{ac}	943.9 \pm 129.3 ^{ac}	7.2 \pm 0.8
		500	296.7 \pm 32.3 ^{ac}	574.3 \pm 58.1 ^{ac}	2.7 \pm 0.7 ^y

Values are expressed as means \pm S.E.M; $n = 6$. ^aData differed significantly ($p < 0.05$) when compared to the normal group; ^bData differed significantly ($p < 0.05$) when compared to the DMSO-treated group induced with PCM; ^cData differed significantly ($p < 0.05$) when compared to the DMSO-treated group induced with CCl_4 ; ^xData differed significantly ($p < 0.05$) when compared to the DMSO-treated group induced with PCM; ^yData differed significantly ($p < 0.05$) when compared to the DMSO-treated group induced with CCl_4 .

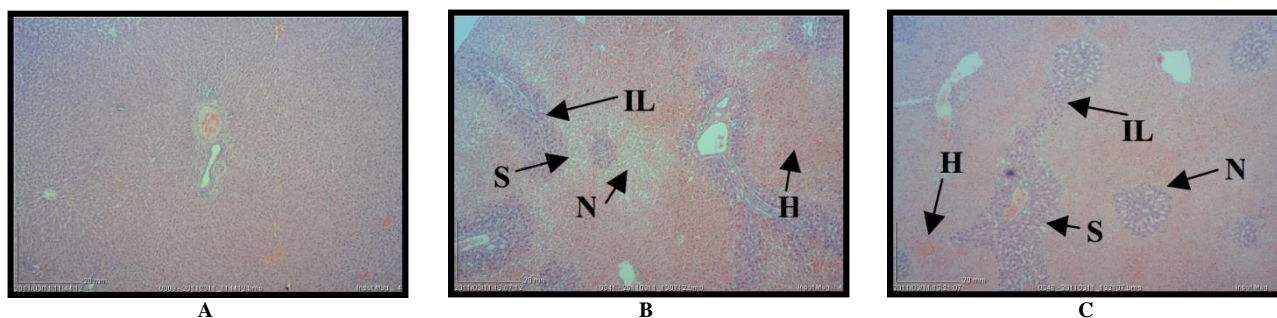


Fig. 1. Photomicrograph (H&E staining, 100 × magnification) showed the architecture of a normal or toxicant-induced rat's liver after pre-treatment with 10% DMSO. (A) Normal liver architecture showing a portal vein (PV), hepatic artery (HA), and bile duct (BD), along with normal hepatic cells with well-preserved cytoplasm and well-defined nucleus around the perivascular area. The sinusoids line is well defined; (B) Hepatotoxic liver after induction with PCM showing severe necrosis (N) of hepatocytes in the parenchyma region, infiltration of leukocytes (IL), hemorrhages (H) within the necrotic parenchymal region of the liver and diffuse steatosis (S); (C) Hepatotoxic liver after induction with CCl₄ showing extensive hepatitis with severe multiple foci necrosis (N) and steatosis (S), infiltration of leukocytes (IL) and hemorrhage (H) within the parenchyma region of the liver.

throughout the tissue (Fig. 2A). In the CCl₄-induced group, pretreatment with silymarin also attenuated the former's hepatotoxic effect wherein only mild necrosis, steatosis as well as infiltration of leukocytes were seen (Fig. 3A). Generally, pretreatment with CEDL exerted dose-dependent hepatoprotection against the toxic effects of PCM and CCl₄. From the microscopic analysis of the livers, the effectiveness of CEDL as an hepatoprotective agent can be suggested based on the reduction in necrotic surface area after treatment against either PCM (Fig. 2B-D) or CCl₄ (Fig. 3B-D). The normal hepatocytes architecture can be seen in PCM- and CCl₄-induced groups pretreated with 500 mg/kg CEDL. Therefore, those biochemical findings were supported by the microscopical observations and the histopathological scoring (Table 1).

DISCUSSION

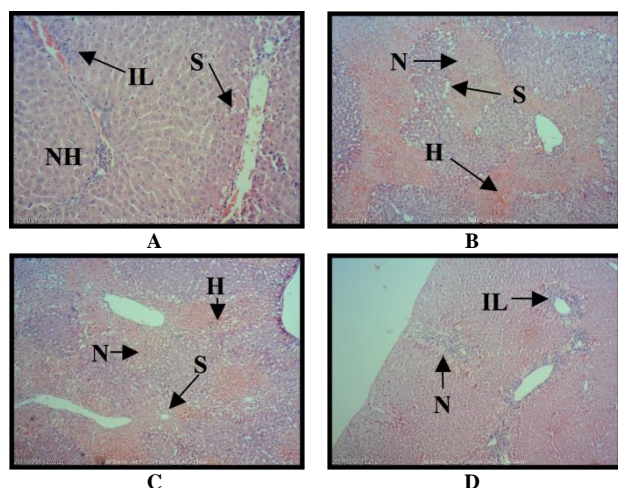


Fig. 2. Photomicrograph (H&E staining, 100x magnification) showed the architecture of a PCM-induced toxic rat's liver after pre-treatment with test solutions. (A) 200 mg/kg silymarin attenuated PCM-induced hepatotoxic effect with normal hepatocytes architecture (NH) seen in between areas, wherein focal infiltration of leukocytes (IL) seen in the perivascular area. No signs of hemorrhage and necrosis with mild steatosis (S) were observed; (B) 50 mg/kg CEDL slightly attenuated PCM-induced hepatotoxic effect with moderate necrosis (N), mild steatosis (S) and hemorrhages (H) observed; (C) 250 mg/kg CEDL also slightly attenuated PCM-induced hepatotoxic effect causing only moderate necrosis (N), mild steatosis (S) and hemorrhages (H) with normal hepatocytes architecture seen in between them; (D) 500 mg/kg CEDL almost completely reversed PCM-induced hepatotoxic effect indicated by the presence of normal hepatocytes architecture (NH) in between areas filled with mild infiltrations of leukocytes (IL) and necrosis (N).

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In our attempt to establish the pharmacological profile for *D. linearis* leaves, we have successfully demonstrated that the various extracts of *D. linearis* leaves possess various pharmacological activities (i.e. antinociceptive, anti-inflammatory and antipyretic, and cytotoxic and antioxidant activities (Zakaria et al., 2006, 2008, 2011a)). Despite the association in mechanisms of action between some of those pharmacological activities (i.e. anti-inflammatory and antioxidant) with the hepatoprotective activity as described in the Introduction section, the latter activity has yet to be determined. Therefore, in the present study we report for the first time the hepatoprotective potential of CEDL against the PCM- and CCl₄-induced rats' liver toxicity models. The PCM-induced mechanisms of hepatotoxicity is completely different from the one induced by CCl₄ wherein the former induced hepatotoxicity via its toxic metabolite, N-acetyl-p-benzoquinone-imine (NAPQI), action on DNA, proteins, and cellular proteins to produce protein adducts (Somchit et al., 2005). On the other hand, the latter caused liver toxicity via the action of its active metabolite, trichloromethyl radicals, on polyunsaturated fatty acid (PUFA) leading to the formation of alkoxy and peroxy radicals. The action of free radicals resulting from the metabolism of PCM leads to the dysfunction and death of hepatocytes, which results in liver necrosis (Zakaria et al., 2012). In contrast, the free radicals produced through CCl₄ metabolism caused damage to the cell membrane and disruption of the normal enzymes' activities via a process known as lipid peroxidation (Feroz Khan et al., 2009; Weber and Boll, 2003), which will also result in hepatic necrosis (Popovic et al., 2006).

As described above, two of the factors important in the development of hepatotoxicity are the presence of free radicals and oxidative processes. Therefore, it is postulated that compounds/extracts with potential antioxidant and free radical scavenging activities may also exert liver protection abilities. Interestingly, CEDL has been reported to exert antioxidant and free radical scavenging activities and is considered to have high total phenolics content (Zakaria et al., 2011b). Moreover, the ability of the extract to prevent leukocytes infiltration could be associated with its anti-inflammatory activities (Zakaria et al., 2006).

Furthermore, the CEDL-demonstrated hepatoprotective effect could also be attributed to its phytochemical constituents, which consist of flavonoids, tannins and steroids (Zakaria et al., 2011b). In addition, the extract has also been reported to contain high phenolic content after the methanol and aqueous extracts of *D. linearis* (Zakaria et al., 2011b). According to Wang et al. (2006), it is not clear whether one, several or all of these compounds are active ingredients for liver protection, but it is possible that some specific components of this extract play

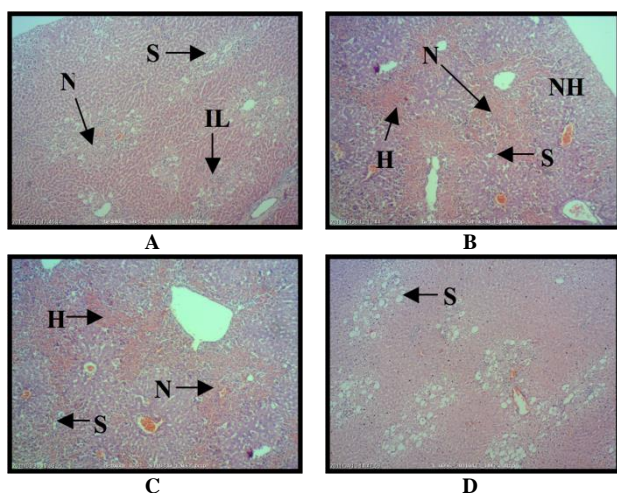


Fig. 3. Photomicrograph (H&E staining, 100x magnification) showed the architecture of a CCl₄-induced toxic rat's liver after pre-treatment with test solutions. (A) 200 mg/kg silymarin attenuated CCl₄-induced hepatotoxic effect indicated by the presence of normal hepatocytes architecture (NH) in between areas filled with mild necrotic hepatocytes (N) and steatosis (S). Mild infiltration of leukocytes (IL) are also seen in certain areas; (B) 50 mg/kg CEDL attenuated CCl₄-induced hepatotoxic effect indicated by the presence of normal hepatocytes architecture (NH) in between areas filled with mild infiltrations of leukocytes (I) and steatosis (S); (C) 250 mg/kg CEDL almost completely reversed the CCl₄-induced hepatotoxic effect with mild necrosis (N) and steatosis (S), and hemorrhages (H) seen in certain areas. (D) 500 mg/kg CEDL also almost completely reversed the CCl₄-induced hepatotoxic effect indicated by the presence of normal hepatocytes architecture (NH) in between areas filled with mild steatosis (S).

a vital role in liver protection/treatment, by this way contributing to the overall therapeutic effects. This statement was supported by findings that flavonoid- (Kim et al., 2011), tannin- (Shimoda et al., 2008) and phenolic-based (Akanitapichat et al., 2010) compounds exhibited hepatoprotective activities. Therefore, these types of compounds are believed to additively/synergistically contribute to the hepatoprotective effect of CEDL.

In another study, Wang et al. (2007) earlier claimed that the mechanisms of hepatoprotective activity can be influenced by the electron affinity of the compounds (E_{lumo}). According to the researchers, the E_{lumo} values can be divided into three subsets ranging from -1.12 ~ -0.10, and 0.03 ~ 1.07, to 1.10 ~ 2.12 and these positive and negative ranges may indicate different mechanisms of actions in hepatoprotection. Extracts containing the main constituents with negative E_{lumo} values may exert their hepatoprotective effects via an antioxidative mechanism, while those containing constituents with positive E_{lumo} values may act through an anti-inflammatory and/or immunomodulating mechanisms. Since the E_{lumo} values for flavonoids and their glucosides fall within the negative value range (-1.03 to -0.83), their hepatoprotective effects may be due to their antioxidative actions, while saponins, whose E_{lumo} values fall within the positive value range (1.07 to 6.12), may exert their liver protection through the anti-inflammatory and/or immunomodulating actions (Wang et al., 2006). Interestingly, CEDL has been reported earlier to possess antioxidant and anti-inflammatory activities and, therefore, supported the above claims. As discussed earlier, the flavonoids and saponins present in the CEDL are suggested to act additively /synergistically to exhibit the hepatoprotective activity.

CONCLUSION

The present study demonstrated that the leaf of *D. linearis*

possesses hepatoprotective activity against PCM- and CCl₄-induced liver toxicity, which could be attributed, partly, to the free radical scavenging and antioxidant activities, and high phenolics and flavonoids contents. Thus, further extensive studies are warranted.

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CONFLICT OF INTEREST

All authors declare that there is no conflict of interest.

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