Hepatoprotective effect of *Drynaria quercifolia* fronds hydroalcoholic extract and isolated constituent against CCl_4-induced hepatocellular damage

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ABSTRACT

Aims: The present study was conducted to evaluate the hepatoprotective effect of hydroalcoholic extract of *Drynaria quercifolia* fronds (Dq), its fractions and isolated compound (Dq-4) from ethyl acetate (EA) fraction.
Place and Duration of Study: Department of Pharmacognosy and Department of Herbal Drug Research, ISF College of Pharmacy, Moga, between June 2010 and May 2012.
Methodology: The toxicant CCl_4 (1ml/kg) was administered on 4^{th} and 5^{th} day to induce hepatotoxicity in Wistar rats (*in-vivo*) and the *in-vitro* hepatoprotection was evaluated against CCl_4 (1%) induced toxicity in HepG2 cellines.
Results: The pre-treatment of rats with Dq extract, EA fraction and Dq-4 for 7 days produced a significant dose dependent hepatoprotective action by decreased levels of hepatic enzymes, total bilirubin and TBARS and increased levels of total proteins, albumin, and reduced glutathione. The histological examination provided the supportive evidences. Additionally, Dq extract, EA fraction and Dq-4 significantly decreased the CCl_4-induced *in-vitro* toxicity in HepG2 cellines evident by MTT reduction assay and trypan blue method.
Conclusion: The study scientifically validated the traditional use of *D. quercifolia* for liver disorders and strongly demonstrates antioxidative effect on hepatocytes in restoring their normal architecture and functional ability.

Keywords: Hepatoprotective, HepG2, Drynaria quercifolia, Naringin

1. INTRODUCTION

The liver as a vital organ in the body is primarily responsible for the metabolism of endogenous and exogenous agents [1]. The pathogenesis of liver injury is initiated by the participation of toxic agents or by their bio-activation to chemically reactive metabolites [2, 3]. These metabolites can be electrophilic chemicals or free radicals, that either elicits an immune response or directly affects the biochemistry of the cells by interacting with cellular macromolecules *viz.* proteins, lipids and nucleic acids leading to protein dysfunction, lipid peroxidation, DNA damage, oxidative stress and depletion of natural antioxidants [4, 5]. Hepatocellular damage is known to be associated with impaired hepatic drug metabolizing capacity and impaired activity of hepatic enzymes [6, 7]. In absence of reliable conventional and synthetic drugs for alleviation of hepatic diseases, traditional medicines are

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recommended for the treatment of liver diseases in India [8, 9]. Therefore, many folk remedies from plant origin are scientifically evaluated for their possible hepatoprotective potential against experimental induced hepatotoxicity.

Drynaria quercifolia J. Smith (Polypodiaceae) is locally known as Attukalkizhangu and Gurar [10, 11]. Traditionally, the fronds of plant are reported to be used by tribal communities of Tamil Nadu and Kerala in treatment of diverse ailments including typhoid fever [12], chronic jaundice, anti-inflammatory agent [13], as a poultice and antifertility agent [14, 15], and antipyretic agent [16]. The whole plant is used to treat chest and skin diseases, and is also anthelmintic, expectorant and tonic [17]. Various phytoconstituents like 3,4-dihydroxybenzoic acid friedelin, epifriedelinol, *β*-amyrin, *β*-sitosterol and *β*-sitosterol 3-*β*-D-glucopyranoside has been isolated from the plant [16]. Although the plant is widely used for remission of several ailments related to liver disorders, there are no systematic scientific reports in the modern literature regarding the usefulness of the plant and its phytoconstituents as a hepatoprotective agent. Hence, to scientifically validate this ethnopharmacological relevance, hepatoprotective potential of *Drynaria quercifolia* fronds was studied in CCl_4-intoxicated both *in-vivo* and *in-vitro* experimental models of hepatocellular damage.

2. MATERIAL AND METHODS

2.1. Plant material

The fronds of *Drynaria quercifolia* were collected from the forest of Mudumalai National Park, district Udhagamandalam, Tamil Nadu (India) in the month of December 2009. Botanical identification and authentication was done by Dr. H.B. Singh, Scientist F & Head, Raw Material Herbarium & Museum, National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India under references and authoritative voucher specimen number: NISCAIR/RHMD/Consult/-2010-11/1646/244.

2.2. Extraction and fractionation procedures

The fronds were cleaned and air dried for a week and pulverized in electric grinder. The dried and powdered fronds (1200 g) were extracted to exhaustion by triple maceration with 50% hydroalcohol (2000 ml×3) at ambient temperature with constant stirring. The combined filtrate was concentrated under reduced pressure below 40^0C to afford hydroalcoholic extract of *Drynaria quercifolia* fronds (Dq extract: 9.76% w/w). Dq extract was further suspended in distilled water and fractionated through successive extractions with chloroform (1000 ml×6), ethyl acetate (1000 ml×9) and *n*-butanol (1000 ml×12). Each fraction was concentrated to dryness under reduced pressure to give $CHCl_3$ (18.10% w/w), EA (18.64% w/w) and *n*-BuOH (11.35% w/w) fractions, respectively. The extract and fractions were preserved under refrigeration till further use.

2.3. Phytochemical screening

The extract and fractions obtained were screened phytochemically for the presence of alkaloids, steroids, terpenoids, glycosides, flavonoids, saponins, proteins, tannins and phenolic compounds, as previously described by [18, 19].

2.4. Chemicals and reagents

Carbon tetrachloride (CCl_4) and silymarin were purchased from S.D. Fine-Chem. Ltd., Mumbai and Micro Labs Ltd., Baddi, respectively. All other chemicals and solvents used were of analytical grade and obtained from Sigma Chemicals Co., USA and Merck India Ltd., Mumbai. Biochemical enzymatic kits were procured from ERBA, Diagnostics Mannheim Gmbh, Germany.

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2.5. Experimental animals

Wistar albino rats of either sex weighing between 180-220g were employed in the present study *vide* approval no ISF/CPCSEA/IAEC/2010/39. The experiments were conducted according to the ethical norms approved by Institutional Animal Ethics Committee (IAEC) guide lines for animal care and were adhered to as recommended by CPCSEA guidelines for the use and care of experimental animals. Animals were housed in environmentally controlled (25±2 ^{0}C, 12 h light & dark cycle) small cages, with free access to standard laboratory chow diet and water *ad-libitum*.

2.6. Acute toxicity study

Acute oral toxicity studies were performed as per revised OECD guideline No. 423 in the albino mice [20]. The animals were fasted overnight with water *ad-libitum* and administered with single dose of 2000mg/kg test drug. Animals were observed individually at predetermined time intervals during the first 24 h, with special attention given during the first 4 h followed by daily observation for a total of 14 days. The animals were observed for toxic symptoms such as behavioral changes, locomotion and mortality [21].

2.7. *In-vivo* experimental design

The rats were divided into eight groups comprising of six animals each (n=6). The test and standard drug silymarin were suspended in 0.5% w/v carboxymethyl cellulose (CMC) for oral administration. The toxicant 50% CCl_4 in olive oil (1 ml/kg, *s.c.*) was given on 4th and 5th day, 2 hrs after the test and standard drug administration [22]. The doses of the fractions ($CHCl_3$, EA and *n*-BuOH) were calculated according to their percentage yields. The treatment protocol is summarized and given below.

Group 1: Normal control; rats received 0.5% CMC for 7 days
Group 2: CCl_4 control; rats administered with 0.5% CMC for 7 days and received toxicant CCl_4 on 4th and 5th day
Group 3: Silymarin; rats treated with silymarin suspension 50 mg/kg for 7 days and received toxicant CCl_4 on 4th and 5th day
Group 4 & 5: Dq extract; rats treated with Dq extract 200 & 400 mg/kg, respectively for 7 days and received toxicant CCl_4 on 4th and 5th day
Group 6: $CHCl_3$ fraction; rats treated with $CHCl_3$ fraction 72.40 mg/kg for 7 days and received toxicant CCl_4 on 4th and 5th day
Group 7: EA fraction; rats treated with EA fraction 74.55 mg/kg for 7 days and received toxicant CCl_4 on 4th and 5th day
Group 8: *n*-BuOH fraction; rats treated with *n*-BuOH fraction 45.40 mg/kg for 7 days and received toxicant CCl_4 on 4th and 5th day

2.8. Analysis of hepatic injury

The blood samples were withdrawn from the orbital sinus on 7th day to obtain haemolysis free serum for biochemical estimations. The serum ALT- alanine transaminase, AST- aspartate transaminase [23], ALP- alkaline phosphatase [24], TB- total bilirubin [25], TP- total proteins [26] and ALB- albumin [27] were estimated using commercial enzymatic biochemical diagnostic kits.

2.9. Analysis of *in-vivo* GSH level and oxidative stress

All the animals were sacrificed by an overdose of ketamine and xylazine mixture and liver was quickly excised, free from any adhering tissues, washed and perfused with chilled normal saline and blotted dry. Perfused liver samples were minced and homogenized in chilled 10mM Tris–HCl buffer (pH 7.4) to obtain 10% whole liver homogenate for the estimation of GSH [28]. The malondialdyhyde (MDA) content, a measure of lipid

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peroxidation, was assayed in the form of thiobarbituric acid reacting substances (TBARS) [29].

2.10. Histopathological studies

The livers were immediately removed and the tissues were fixed in 10% formalin, dehydrated in ethanol (50–100%), cleared in xylene and embedded in paraffin wax. These were then cut into 4–5µm thick sections in rotary microtome and stained with haematoxylin-eosin for photomicroscopic assessment [30, 31].

2.11. Isolation of compounds

The EA fraction showed significant *in-vivo* hepatoprotective activity and 5.02g of fraction was charged in column, silica gel as stationary phase. The column was initially eluted using chloroform and ethyl acetate of increasing polarity. 220 fractions, each of 45-50 m1 were collected and fractions with similar TLC profile were pooled. The pooled fractions A1(6-24), A2(40-55) and A3(69-86) resulted in the isolation of compounds Dq-1 (15.24mg), Dq-2 (13.87mg) and Dq-3 (14.43mg), respectively (data not shown). The pooled fraction (198-220), obtained through elution with 100% ethyl acetate, when concentrated and left overnight in deep freezer resulted in a pale yellow coloured compound, which on repeated crystallization yielded pure compound Dq-4 (17.91mg).

2.12. Characterization of compounds

The identification of compounds was done by physical, chemical and spectral analysis. The melting points were determined in open-glass capillaries on Stuart SMP10 melting point apparatus. The IR spectra (υ, cm^{-1}) were obtained with a Nicolet 380 FTIR spectrometer (Thermo Scientific) in KBr pellets. ^{1}H-NMR spectra (δ, ppm) were recorded in DMSO-d_6 solutions on a Varian-Mercury 400 MHz spectrometer using tetramethylsilane (TMS) as the internal reference. Mass spectra were recorded on a Shimadzu GCMS-QP 1000 EX.

2.13. *In-vitro* hepatoprotective activity

The *in-vitro* hepatoprotective activity was performed on human HepG2 cellines, obtained from the National Centre for Cell Science (NCCS) Pune, India. The cells were cultured in 96-well plates at density of 1.0×10^5 cells/well over night in DMEM containing 10% FBS maintained at 5% CO_2 at 37 ^{0}C [32]. After 24 hours, when partial monolayer was formed, the supernatant was flicked off and the monolayer was washed once. The hepatocytes were exposed to fresh medium containing CCl_4 (1%) along with various concentrations of Dq extract, fractions ($CHCl_3$, EA & *n*-BuOH) and isolated compound Dq4. 60 min after the CCl_4 intoxification, cytotoxicity was assessed by estimating the percentage viability of HepG2 cells by MTT reduction assay [33]. In addition, morphological changes in HepG2 cells and loss of membrane integrity during the later stages of cell death were determined by trypan blue dye membrane integrity assay [34].

2.14. Statistical analysis

The data of *in-vivo* and *in-vitro* studies were expressed as mean ± SD and mean ± SEM of triplicate experiments, respectively. The data was analysed by one-way ANOVA followed by Tukey's multiple comparison analysis as *post-hoc* test using GraphPad Prism 4 (GraphPad Software Inc., CA, USA). The $p<0.05$ was considered to be statistically significant.

3. RESULTS

3.1. Phytochemical study

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Preliminary phytochemical screening revealed presence of carbohydrates, proteins, saponins, steroids, triterpenes, phenolic compounds and flavonoids in Dq extract. The EA fraction showed the presence of terpenoids, phenolic compounds and flavonoids.

3.2. Acute toxicity study

The Dq extract and fractions ($CHCl_3$, EA & *n*-BuOH) did not show any sign and symptoms of behavioral changes, toxicity and mortality up to 2000 mg/kg; b.w.

3.3. Effect of Dq extract and fractions ($CHCl_3$, EA & n-BuOH) on serum biochemical parameters

Compared to the vehicle (normal control) group, CCl_4 at sub lethal dose caused increases in serum AST (280.80%), ALT (300.57%), ALP (70.61%) and TB (151.72%) levels. CCl_4 also caused marked reductions in serum TP (51.32%) and ALB (53.07%) levels. The pre-treatment of groups with Dq extract and EA fraction for 7 days reversed the toxicity affect and exhibited hepatocellular protection as manifested by the reduction in serum ALT, AST, ALP, TB and decreased levels of serum TP and ALB. The EA fraction exhibited the highest percent of protection among all the tested samples. The EA fraction (74.55 mg/kg) markedly reduced the levels of serum AST (40.88%), ALT (41.49%), ALP (19.72%) and TB (28.76%) along with increased levels of serum TP (60.38%) and ALB (28.57%) as compared to CCl_4 control group. However, $CHCl_3$ (72.40 mg/kg) and *n*-BuOH (45.40 mg/kg) fractions did not show any protection against CCl_4-induced hepatocellular injury (Tables 1).

3.4. Effect of Dq extract and fractions ($CHCl_3$, EA & n-BuOH) on tissue GSH and TBARS levels

As shown in Table 1, the GSH level was decreased and TBARS contents were increased in the liver homogenate in CCl_4 control group by 51.34% and 163.67%, respectively as compared to normal control group. The pre-treatment of rats for 7 days with Dq extract and EA fraction markedly reversed these toxic effects and restored the altered levels of GSH and TBARS. The Dq extract (400 mg/kg) and EA fraction (74.55 mg/kg) increased the level of reduced GSH by 22.09% and 22.46% and inhibited the levels of TBARS by 26.23% and 22.38%, respectively as compared to CCl_4 control group.

3.5. Histopathological studies

The histological examination of CCl_4-toxicated liver section showed various degree of architecture damage with de-arrangement of normal hepatic cells, centrilobular necrosis and fibrosis, ventral vein enlargement, sinusoidal dilation, fatty vacuolization, ballooning degeneration and broad infiltration of lymphocytes. The Dq extract and EA pre-treated rats caused degenerative changes and retained the structural integrity of hepatic cells, which closely resembles to the liver histology of the normal control group with less vacuole formation, absence of necrosis and overall less visible parenchymal injury (Figure 1).

3.6. Characterization of compound Dq-4

The isolated compound Dq-4 was characterized as 7-[[2-O-(6-Deoxy-*α*-L-monopyanoxyl)-*β*-D-glucopysanoxyl] oxy)-2,3-dehydro-5,7-dihydroxy-2-(4 hydroxyphenyl]-4H-1-benzopyran-4-one (flavanone glycoside; naringin). The yield was 17.91mg; m.p. 169-172 ^{0}C; IR (KBr; cm^{-1}): 3468 (OH strech), 2935 (CH strech), 2865, 2843 (CH_2 strech), 1685 (C=O), 1606 (C=C Ar), 1235 (C-O). ^{1}H-NMR (400 MHz, DMSO-d_6, δ ppm): 11.99 (*s*, 1H, OH, C-5, chromone), 9.38 (*s*, 1H, OH, chromone, C-7), 7.93 (*s*, 1H, OH, C-4', phenyl), 7.29 (*d*, 2H, C-2'-C-6', phenyl, *J* = 8.4 Hz), 6.38 (*d*, 2H, C-3'-C-5', phenyl, *J* = 8.4 Hz), 6.11 (*s*, 1H, C-8, chromone), 5.39 (*m*, 1H, C-2, chromone), 5.19 (*m*, 3H, OH, C-3'',C-4'',C-5'', pyranoside), 4.99 (*t*, 1H, C-3''), 4.23 (*m*, 1H, C-2''), 3.83 (*m*, 2H, C$\underline{H_2}$-OH, pyranoside), 3.63 (*m*, 1H, C-

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220 **Table 1. Effect of Dq extract and fractions ($CHCl_3$, EA & *n*-BuOH) on serum biochemical parameters, tissue GSH and TBARS**
221 **levels in CCl_4-induced hepatic injury in rats**

Groups	Serum parameters						Tissue parameters	
	AST (U/L)	ALT (U/L)	ALP (U/L)	TB (mg/dl)	TP (g/dl)	ALB (g/dl)	TBARS (nM/mg protein)	GSH (µM/mg protein)
Normal control	47.78±7.06	41.66±9.12	160.31±14.94	0.29±0.06	6.43±0.53	3.58±0.41	21.31± 5.41	108.96± 14.92
CCl_4 control (1 ml/kg; *s.c.*)	177.17±14.92 (270.80%)[a]↑	166.88±13.92 (300.57%)[a]↑	273.51±21.10 (70.61%) [a]↑	0.98±0.14 (237.93%)[a]↑	3.13±0.51 (51.32%)[a]↓	1.68±0.25 (53.07 %)[a]↓	56.19± 8.63 (163.67%)[a]↑	53.01± 9.41 (51.34 %)[a]↓
Silymarin (50 mg/kg; *p.o.*)	65.51±9.83 (63.02%)[b]↓	54.69±11.63 (67.22%)[b]↓	190.74±18.35 (30.26%)[b]↓	0.39±0.09 (60.20%)[b]↓	6.18±0.55 (97.44%)[b]↑	3.14±0.27 (86.90%) [b]↑	31.59± 6.19 (43.78%)[b]↓	84.83± 10.29 (60.02%) [b]↑
Dq extract (200 mg/kg; *p.o.*)	127.98±13.78 (27.76%)[b]↓	123.43±13.66 (26.03%)[b]↓	245.23±16.73 (10.33%) ↓	0.57±0.08 (41.83%)[b]↓	4.75±0.41 (51.75%) [b]↑	2.17±0.30 (29.16%)↑	47.68± 8.42 (15.14%) ↓	67.14± 8.26 (26.65%) ↑
Dq extract (400 mg/kg; *p.o.*)	96.59±11.73 (45.48%)[b]↓	90.24±13.28 (45.92%)[b] ↓	217.40±16.60 (20.51%)[b]↓	0.48±0.08 (51.02%)[b]↓	5.61±0.43 (79.23%)[b]↑	2.66±0.38 (58.33%)[b]↑	41.45±8.28 (26.23%)[b]↓	74.72±7.38 (40.95%)[b]↑
$CHCl_3$ fraction (72.40 mg/kg; *p.o.*)	167.18±14.18 (5.63%)↓	153.41±14.62 (8.07%)↓	255.23±17.71 (6.68%) ↓	0.87±0.09 (11.22%) ↓	3.55±0.31 (13.41%)↑	1.79±0.23 (6.54%)↑	52.63± 8.13 (6.33%) ↓	56.11± 7.29 (5.84%) ↑
EA fraction (74.55 mg/kg; *p.o.*)	104.73±12.13 (40.88%)[b,c,d]↓	97.64±14.17 (41.49%)[b,c,d] ↓	201.57±17.61 (26.30%)[b,c,d]↓	0.52±0.07 (46.93%)[b,c,d]↓	5.42±0.45 (73.16%)[b,c,d]↑	2.66±0.27 (58.33%)[b,c]↑	43.61±7.76 (22.38%)[b,c,d]↓	64.92±6.12 (22.46%)[b,c,d]↑
n-BuOH fraction (45.40mg/kg; *p.o.*)	175.89±13.21 (0.72%)↓	165.28±14.93 (0.95%) ↓	270.47±16.92 (1.12%) ↓	0.90±0.09 (8.16%) ↓	3.24±0.54 (3.51%) ↑	1.72±0.31 (2.38%) ↑	55.23± 7.75 (1.70%) ↓	54.25± 6.94 (2.33%) ↑

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223 **The results are expressed as the Mean ± SD of six rats/group; One way ANOVA followed by Tukey's multiple test*
224 *a = p< 0.05 vs normal group; b = p< 0.05 vs CCl_4 control; c = p< 0.05 vs $CHCl_3$ (72.40 mg/kg); d = p< 0.05 vs n-BuOH (45.40 mg/kg)*
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4''), 3.53 (*m*, 2H, C-3'-chromone), a doublelet at 1.157, *J* = 6 Hz of 3H for CH_3 of rhamnose . MS (ESI) m/z = 580.53 (M+) analysed for $C_{27}H_{32}O_{14}$.

The glycoside was hydrolysed with 5% HCl, which resulted into a crystalline product and was separated by filtration. The solid was crystallized from methanol to give a crystalline product (m.p. 250-251 ^{0}C, λmax 226 & 292 nm) and was therefore identified as a glycone (naringenin). The aqueous part was concentrated after neutralization and subjected to paper chromatography using isoprapanol: 5% boric acid (7:3), which resulted in the identification of two sugars as rhamnose and glucose at Rf 0.43 and 0.18, respectively. Therefore the compound Dq-4 was confirmed as naringin.

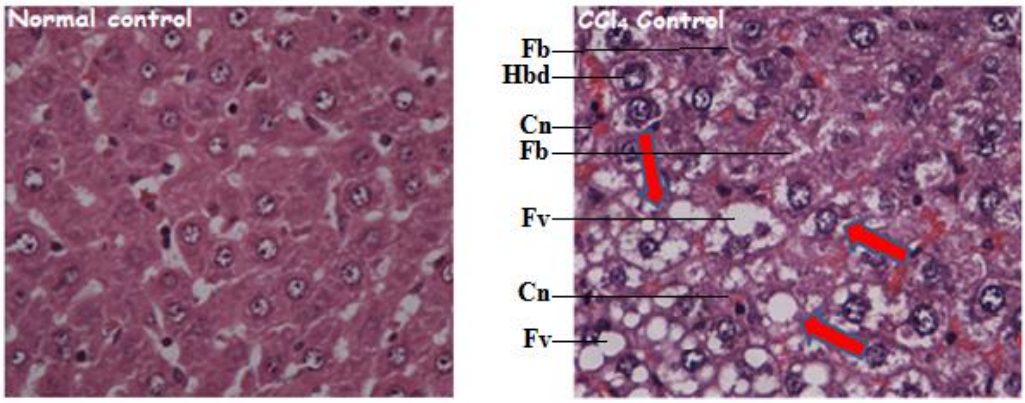
3.7. *In-vitro* hepatoprotective effect in HepG2 cell line

The *in-vitro* hepatoprotective activity of Dq extract, fractions ($CHCl_3$, EA & *n*-BuOH) and isolated compound (Dq-4) at dose levels 3, 6, 12.5, 25, 50 & 100 µg/ml were evaluated. The hepatocytes exposed to CCl_4 (1%) showed a decrease percentage of cells viability (41.25%) as compared to normal control, indicating the HepG2 cells injury caused by CCl_4 toxicant. The isolated compounds Dq-4 at dose level 50µg/ml markedly protected the viability of HepG2 cells against CCl_4-induced cytotoxicity by 91.66%. The hepatocyte protecting effect of Dq-4 was better than that of standard drug silymarin at dose levels 50 and 100µg/ml; similar kinds of results were observed in case of Dq extract and EA fraction. The percentage protection of Dq extract and EA fraction at dose level 100µg/ml was 71.62% and 84.33%, respectively. However, $CHCl_3$ and *n*-BuOH fractions did not show any hepatoprotection in both the assays (Figures 2, 3 & 4).

3.8. Effect on morphology of the HepG2 cells

Normal HepG2 cells (in clumps adherent to the walls) were of spindle shape, clear cell borders and nuclei with darker cytoplasm. When exposed to toxicant CCl_4 (1%), morphology of HepG2 cells changed to round shape (swollen), showed irregular and bleeding plasma membrane, the ability of cells to adhere to walls was decreased and debris emitted increased around the cells. The changes in the cells were largely prevented with the increasing concentration of Dq extract, EA fraction and isolated compound (Dq-4) (Figures 2, 3 & 4).

Figure 1. Effect of Dq extract & EA fraction on histological characteristics in CCl_4-induced hepatic injury in rats



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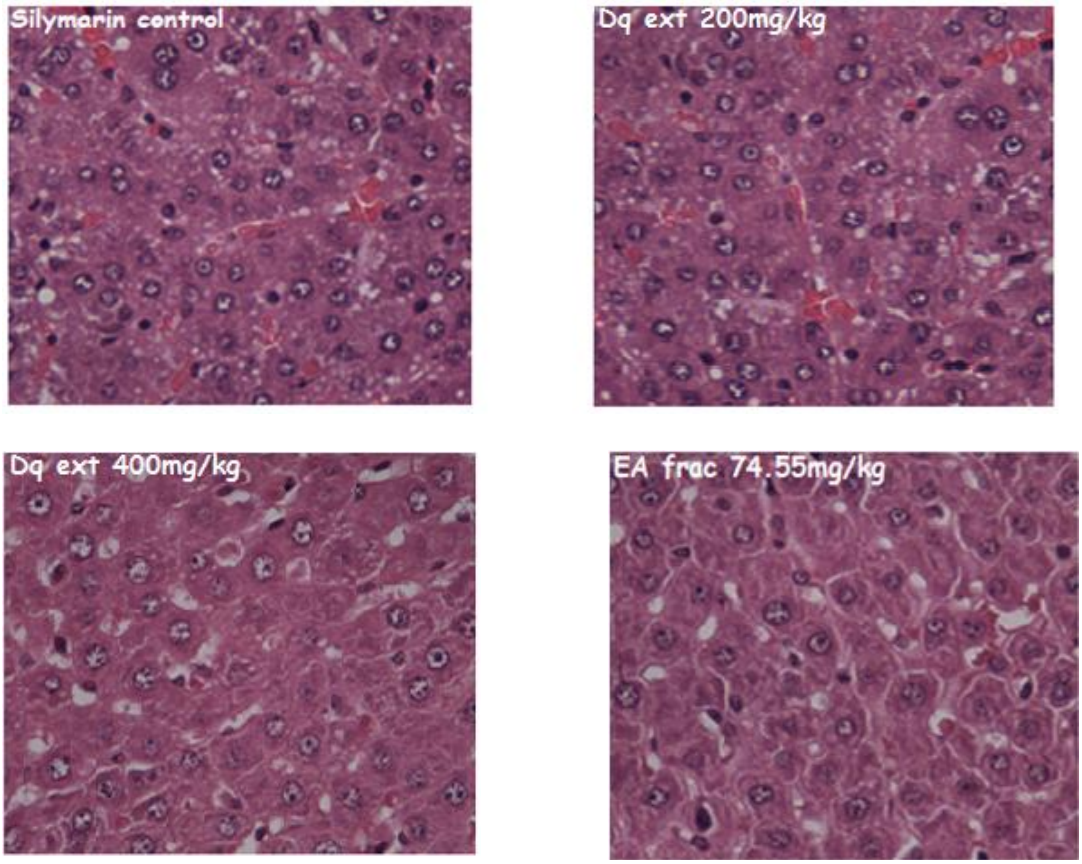
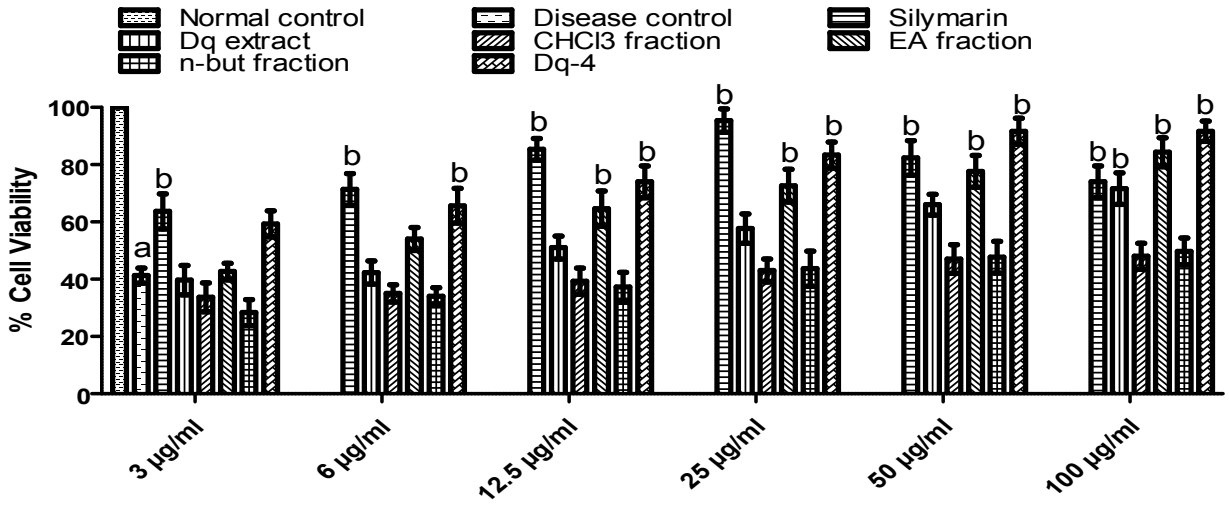


***Fv**- fatty vacuoles, **Cn**- centrilobular necrosis, **Fb**- hepatic fibrosis, **Hbd**- hepatocyte ballooning degeneration and broad infiltration of lymphocytes. Arrows shows the loss of cellular boundaries*

Figure 2. MTT assay and cytoprotection of Dq extract, fractions ($CHCl_3$, EA & *n*-BuOH) and Dq-4 in HepG2 cells



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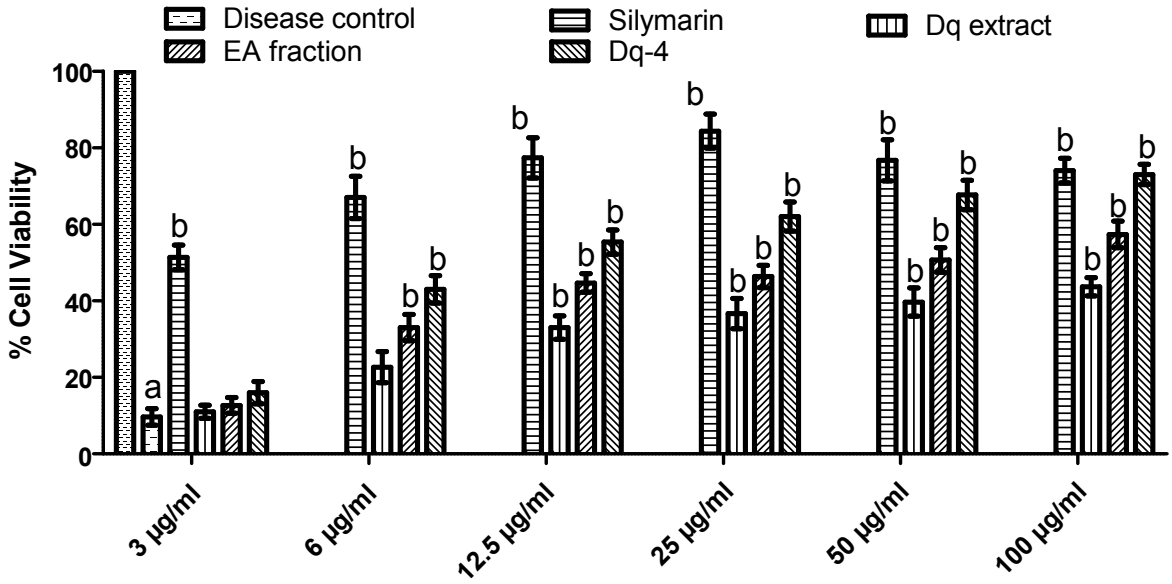
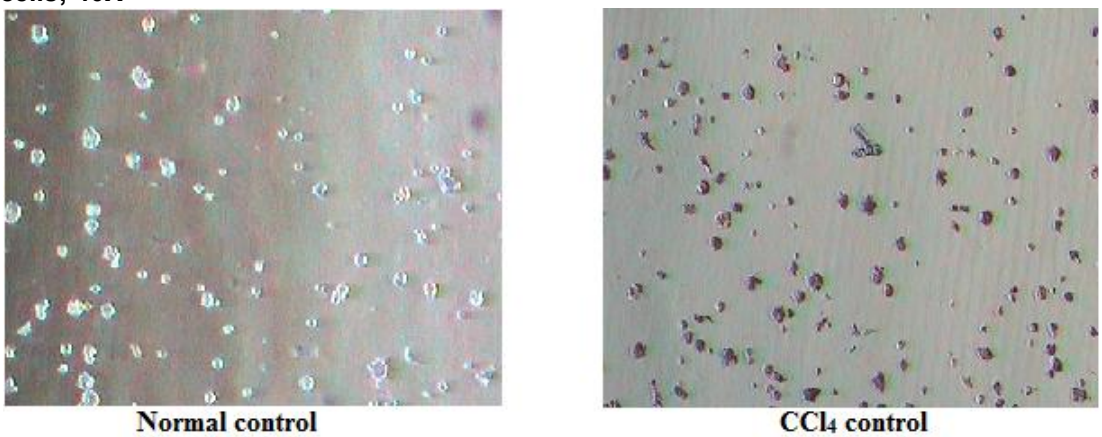
Values are Mean ± SEM of three separate experiments; a = p< 0.05 vs normal control; b = p< 0.05 vs CCl_4 control

Figure 3. Trypan blue assay; showing the viability of treated and untreated HepG2 cells



Values are Mean ± SEM of three separate experiments; a = p< 0.05 vs normal control; b = p< 0.05 vs CCl_4 control

Figure 4. Face contrast images of trypan blue assay: treated and untreated HepG2 cells; 40X



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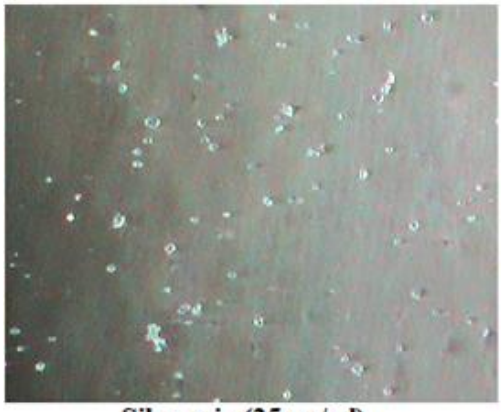
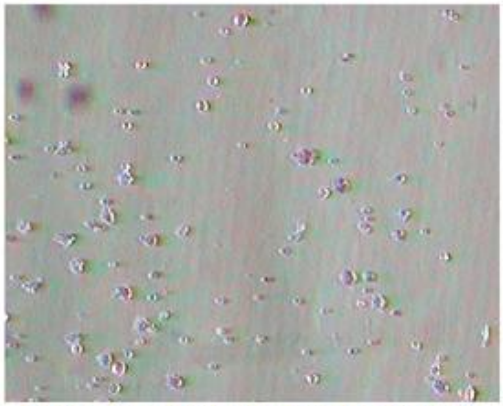
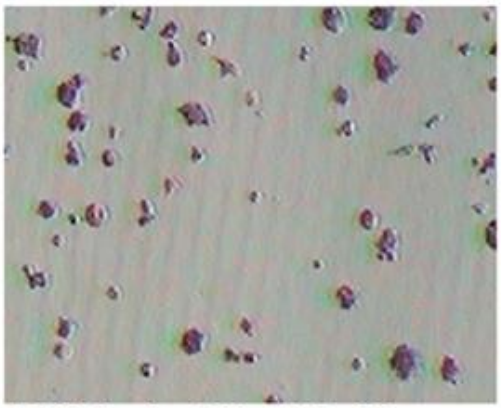


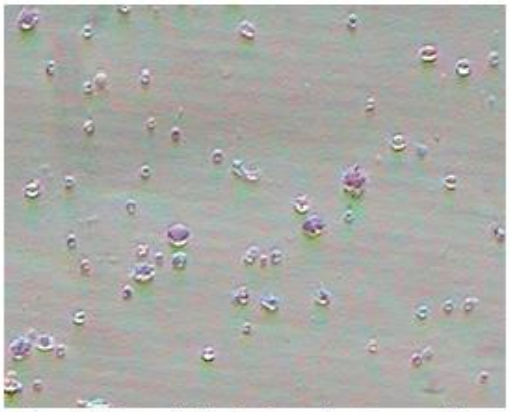
Silymarin (25 µg/ml)



EA fraction (100 µg/ml) of Dq extract



$CHCl_3$ fraction (100 µg/ml) of Dq extract



Dq-4 (50 µg/ml); isolated compound

4. DISCUSSION

The present study was undertaken to study the possible hepatoprotective role of *Drynaria quercifolia* fronds, a hepatoprotective agent, used by tribals in India, against CCl_4-induced hepatocellular damage.

Hepatic damage induced by CCl_4 results from its metabolic bioactivation, primarily through the activity of CYP2E1, to the free radicals $CCl_3^{\bullet}$ and $CCl_3OO^{\bullet}$ [2]. The free radicals bind covalently to macromolecules by abstracting a hydrogen atom from the polyunsaturated fatty acids of phospholipids and induce peroxidative degradation of the membrane lipids of endoplasmic reticulum [35, 36]. This initiate the formation of lipid peroxides followed by pathological changes such as depression of protein synthesis [37], elevated levels of serum marker enzymes, alkaline phosphatase, total bilirubin, lipid peroxidation [38] and depletion of glutathione content [39]. The antioxidant activity or the inhibition of the generation of free radicals is important in providing protection against hepatic damage [40].

Serum transaminases and alkaline phosphatase have long been considered as sensitive indicator of hepatic injury [41]. Injury to the hepatocytes alters enzymes transport function and membrane permeability, leading to leakage of enzymes from the cells; this leakage causes increased levels of AST and ALT in the blood [42]. ALP activity is related to the functioning of hepatocytes, increase in its activity being due to increased synthesis in the

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presence of increased biliary pressure [43]. The induced elevation of this enzymatic activity in the blood is associated with high level of bilirubin content [44]. This may explain the increased levels of serum AST, ALT, ALP and bilirubin in CCl_4-intoxicated rats in the present study. The pre-treated groups, Dq extract & EA fraction, induced significant ($p<0.05$) suppression of the increased serum AST, ALT and ALP activities with the significant ($p<0.05$) depletion of raised serum bilirubin. The reduction in the levels of AST and ALT suggests the ability of the test drugs to stabilize the plasma membrane as well as repair of hepatic tissue during CCl_4-induced liver injury. The depletion of increased ALP activity with simultaneous suppression of raised bilirubin level indicates the stabilization of biliary dysfunction in rat liver during the hepatic injury [45].

CCl_4 induces fatty liver and cell necrosis, which plays a significant role in diminution of serum protein and albumin, depletion of GSH and increased lipid peroxidation [39, 46]. This expected decline in serum TP, ALB and enhanced lipid peroxidation can be deemed as a useful index for the severity of hepatocellular dysfunction and liver injury [7, 47, 48]. The pre-treated groups, Dq extract & EA fraction, demonstrated hepatoprotective activity by significantly ($p<0.05$) increasing the CCl_4-induced reduction of serum TP and ALB. Scavenging of free radicals is one of the major antioxidation mechanisms to inhibit the chain reaction of lipid peroxidation. A major defense mechanism involves the antioxidant enzymes as well as GSH (non-enzymatic biological antioxidant), which convert active oxygen molecules into non-toxic compounds [46]. Increase in TBARS levels in the CCl_4-intoxicated rats, suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals [49]. These effects were significantly ($p<0.05$) reversed in pre-treated groups, attenuation of these hepatotoxic effects might be either through decreased production of free radical derivatives or due to the abilities of Dq extract and EA fraction to act as radical scavengers that might lessen oxidative damage to the liver tissue [50].

The histopathological observations of liver sample provided the supportive evidence for the biochemical analysis and explain the hepatoprotective potential of tested plant [51, 52]. The liver of intoxicated rats manifested massive fatty changes, gross necrosis and broad infiltration of lymphocytes and kupffer cells around the central vein and loss of cellular boundaries. The Dq extract and EA fraction pre-treated rats showed a more or less normal architecture, having reversed to a large extent, the hepatic lesions produced by the toxin, thus protecting the histostructural integrity of the liver cells.

The human HepG2 cells have proven to be a valuable tool to study *in-vitro* hepatotoxicity of different chemicals or drugs as these cells retain many of the morphological and biochemical characteristics of normal hepatocytes [53, 54]. The percent cell viability, determined using MTT assay and Trypan blue dye exclusion method is useful to predict the cell injury that affects cell attachment or progress to cell death [55]. CCl_4-induced hepatic cell damage causes instability of cell metabolism, inducing triacylglycerol accumulation, increased lipid peroxidation and membrane damage [56]. The Dq extract, EA fraction and Dq-4 exhibited significant ($p<0.05$) restoration of the cell viability and altered morphological changes towards normal in CCl_4 intoxicated HepG2 cells. The reversed hepatotoxic effects is due to the presence of flavonoids in test drugs, which could accelerates the excretion of free radical derivatives and inhibit lipid peroxidation that leads to decrease in severity of oxidative damage in the HepG2 cells [40, 57].

5. CONCLUSION

The present study concludes that the plant *Drynaria quercifolia* exhibited hepatoprotective potential due to the presence of compounds of Dq-4 like flavonoids substances. The

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hepatocellular protection might be due to the antioxidant & antifibrotic properties and/or due to the membrane stabilizing cascades for the prevention of progression in liver injury. The results of present study support and justify the traditional and folklore medicinal claims attributed to this plant in the treatment of liver ailment. However, in addition to its free radical scavenging potential, further investigations are in process to ascertain the precise cellular/molecular mechanism(s) of hepatoprotective effect.

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REFERENCES

1. Bacon BR, Bisceglie AD. Liver disease: diagnosis and management. Philadelphia: Churchill Livingstone; 1999.

2. Gonzalez FJ. The molecular biology of cytochrome P450s. Pharmacol Rev. 1998;40(4):243-88.

3. Guengerich FP. Analysis and characterization of enzymes. In: Hayes AW, editor. Principles and methods of toxocology. Raven Press: New York; 1994.

4. Zimmerman HJ. Drug-induced liver disease. In: Zimmerman HJ, editor. Hepatotoxicity: the adverse effects of drugs and other chemicals on the liver. Lippincott Williams & Wilkins: Philadelphia, PA; 1999.

5. DeLeve LD, et al. Characterization of a reproducible rat model of hepatic veno-occlusive disease. Hepatology. 1999;29(6):1779-91.

6. Amin A, Hamza AA. Hepatoprotective effects of Hibiscus, Rosmarinus and Salvia on azathioprine-induced toxicity in rats. Life Sci. 2005;77(3):266-78.

7. Navarro VJ, Senior JR. Drug-related hepatotoxicity. New Engl J Med. 2006;354(7):731-39.

8. Handa SS, Sharma A, Chakraborthi KK. Natural products and plants as liver protecting drugs. Fitoterapia. 1986;57(5):307-51.

9. Chatterjee TK. Medicinal plants with hepatoprotective properties. In: Herbal Options., Books and Allied (P) Ltd., Kolkata; 2000.

10. Kirtikar KR, Basu BD. Indian Medicinal Plants. Dehra Dun Publisher Ltd., Dehra Dun, India; 1994.

11. Bhattacharya S. In: Chrinjib Banoushadi. Anand Publishing Ltd., Calcutta, India; 1990.

12. Dixit RD, Vohra JN. A dictionary of pteridophytes of India. In: Botanical survey of India. Howrah: India; 1984.

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13. Irudayaraj V, Senthamarai R. Pharmacognostical studies on a medicinal fern, *Drynaria quercifolia* (L.) J. Sm. (Polypodiaceae: Pteridophyta). Phytomorphology. 2004;54(3-4):193-200.
14. Shokeen P, et al. Preliminary studies on activity of *Ocimum sanctum*, *Drynaria quercifolia*, and *Annona squamosa* against *Neisseria gonorrhoeae*. Sex Transm Dis. 2005;32(2):106-11.
15. Rajendran A, Rajan S. *Drynaria quercifolia*: an antifertility agent. Ancient Sci Life. 1996;15(4):286-87.
16. Khan A, et al. Isolation of antibacterial constituent from rhizome of *Drynaria quercifolia* and its sub-acute toxicological studies. DARU. 2007;15(4):205-11.
17. Koushik P, Dhiman AK. Common medicinal pteridophytes. Indian Fern Journal. 1995;12(1-2):139-45.
18. Harborne JB. Phytochemical methods: A guide to modern techniques of plant analysis. Chapman and Hall, London; 1984.
19. Farnsworth NR. Biological and phytochemical screening of plants. J Pharmaceut Sci. 1966;55(3):225-76.
20. OECD, In: Guidelines for the testing of chemicals revised draft guideline 423: acute oral toxicity. 2000.
21. Botham PA. Acute systemic toxicity prospects for tiered testing strategies. Toxicol Vitro. 2004;18:227-30.
22. Rosa MPG, Solis RV. Hepatoprotective and inhibition of oxidative stress in liver of *Prostechea michuacana*. Record Nat Prod. 2009;3(1):46-51.
23. Bradley DW, et al. Transaminase activities in serum of long-term hemodialysis patients. Clin Chem. 1972;18:1442.
24. Lieberman D, Phillips D. Isolated elevation of alkaline phosphatase: significance in hospitalized patients. J Clin Gastroenterol. 1990;12(4):415-19.
25. Jendrassik L, Grof P. Determination of total and direct bilirubin in serum or plasma on manual system. Biochemische Zeitschrift. 1938;297:81-9.
26. Doumas BT. Standards for total serum protein assays-a collaborative study. Clin Chem. 1975;21:1159-66.
27. Ingwersen S, Raabo E. Improved and more specific bromcresol green methods for the manual and automatic determination of serum albumin. Clin Chim Acta. 1978;88(3):545-50.
28. Moron MJ, Diperre JW, Mannerv KB. Levels of glutathione, glutathione reductase and glutathione-s-transferase activities in rat lungs and liver. Biochim Biophys Acta. 1979;582:67-71.

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429 29. Wills ED. Mechanisms of lipid peroxide formation in animal tissues. Biochem J.
430 1996;99(3):667.
431 30. Galigher AE, Kozloff EN. Essentials of Practical Microtechniques. Lea and Febiger:
432 Philadelphia; 1971.
433 31. Krajian AA. Tissue cutting and staining. In: Frankel S, Reitman S, editors. Gradwohl's
434 Clinical Laboratory methods and diagnosis. The CV. Mosby Co.: Saint Louis, USA; 1963.
435 32. Mitra SK, et al. Liv.52 regulates ethanol induced PPAR c and TNF a expression in
436 HepG2 cells. Mol Cell Biochem. 2008;315(1-2):9-15.
437 33. Ke H, et al. Antineoplastic agents III: steroidal glycosides from *Solanum nigrum*. Planta
438 Medica. 1999;65(1):35-38.
439 34. Freshney I. Cytotoxicity, culture of animal cells. In: A manual of basic technique. Wiley-
440 Liss: New York; 2000.
441 35. Cesaratto L, et al. The importance of redoxstate in liver damage. Ann Hepatol.
442 2004;3(3):86-92.
443 36. Weber LW, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes:
444 carbon tetrachloride as a toxicological model. Crit Rev Toxicol. 2003;33(2):105-36.
445 37. Faroon O, De Rosa CT, Smith L. Carbon tetrachloride: Health affects toxicokinetics,
446 human exposure and environment fate. Toxicol Ind Health. 1994;10:4-20.
447 38. Zimmerman HJ, Seeff LB. Enzymes in hepatic disease. In: Goodly EE, editor. Diagnostic
448 enzymology. Lea and Febiger: Philadelphia; 1970.
449 39. Kamiyama T, Sato C, Liu J. Role of lipid peroxidation in acetaminophen induced
450 hepatotoxicity; comparision with carbontetrachloride. Toxicol Lett. 1993;66:7-12.
451 40. Ferguson LR. Role of plant polyphenols in genomic stability. Mutat Res. 2001;475(1-
452 2):89-111.
453 41. Kew MC. Serum aminotransferase concentration as evidence of hepatocellular damage.
454 Lancet. 2000;355:591–92.
455 42. Nkosi CZ, Opoku AR, Terblanche SE. Effect of pumpkind seed (*Cucurbita pepo*) protein
456 isolate on the activity levels of certain plasma enzymes in CCl_4-induced liver injury in low
457 protein fed rats. Phytother Res. 2005;19(4):341-45.
458 43. Moss DW, Butterworth PJ. Enzymology and Medicine. Pitman Medical: London; 1974.
459 44. Pimple BP, et al. Protective effect of *Tamarindus indica* Linn against paracetamol
460 induced hepatotoxicity in rats. Indian J Pharmaceut Sci. 2007;69(6):827-31.
461 45. Mukherjee PK. Quality control herbal drugs. In: An approach to evaluation of botanicals.
462 1st ed New Delhi: Business Horizons; 2002.
463 46. Husain K, Somani SM. Interaction of exercise training and chronic ethanol ingestion on
464 hepatic and plasma antioxidant system in rat. J Appl Toxicol. 1997;17(3):189-94.

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E-mail address: dr.pradeepkamboj@gmail.com.

47. Hampel B, et al. Differential regulation of apoptotic cell death in senescent human cells. Exp Gerontol. 2004;39(11-12):1713-21.
48. Brattin WJ, Glende Jr EA, Recknagel RO. Pathological mechanisms in carbon tetrachloride hepatotoxicity. Free Radic Biol Med. 1985;1(1):27-38.
49. Souza MF, Rao VSN, Silveira ER. Inhibition of lipid peroxidation by ternatin, a tetramethoxyflavone from *Egletes viscosa* L. Phytomedicine. 1997;4(1):27-31.
50. Blair IA. Endogenous glutathione adducts. Curr Drug Metabol. 2006;7:853-72.
51. Plaa GL, Charbonneau M. Detection and evaluation of chemically induced liver injury. In: Hayes AW, editor. Principles and methods of toxicology. Raven Press: New York; 1989.
52. Junnela M, et al. Reduction of carbon tetrachloride induced hepatotoxic effects by oral administration of betaine in male Hans-Wistar rats: A morphometric histological study. Veterinary Pathology. 2000;37:231-38.
53. Gomez-Lechon MJ, et al. The use of cultured hepatocytes to investigate the metabolism of drugs and mechanisms of drug hepatotoxicity. Alternatives to Laboratory Animals. 2001;29(3):225-31.
54. Guillouzo A. Liver cell models in *in-vitro* toxicology. Environ Health Perspect. 1998;106(2):511-32.
55. Knowles BB, Aden DP. Human hepatoma derived cell line, process for preparation thereof, and uses therefor. US Patent 4393133; 1983.
56. Recknagel RO, et al. Mechanisms of carbon tetrachloride toxicity. Pharmacol Therapeut. 1989;43(43):139-54.
57. Tapas AR, Sakarkar DM, Kakde RB. Flavonoids as nutraceuticals: a review. Trop J Pharmaceut Res. 2008;7(3):1089-99.

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