

Hepatoprotective and Antioxidant Activities of Hepacare[®], a Herbal Formulation Against Carbon Tetrachloride-Induced Liver Injury

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Key words

- antioxidant systems
- DPPH
- flavonoid
- hepatotoxicity
- liver tonic
- oxidative stress
- reducing power capacity
- serum transaminases

Abstract



Background: Hepacare[®] is a herbal formulation used to treat patients with sickle-cell anaemia complicated with jaundice, also recommended as a protective agent against liver damage due to chronic ingestion of alcohol.

Methods: In vitro antioxidant properties of Hepacare[®] was determined using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), total antioxidant capacity, reducing power ability, and nitric oxide assays. Hepatoprotective effect of Hepacare[®] (50–400 mg/kg/day for 7 days, p.o.) was investigated in male Sprague Dawley rats against carbon tetrachloride (CCl₄/olive oil, 1:1, 0.7 ml/kg, i.p.)-induced liver damage. At the end of the study, blood samples and liver tissue were assayed for biochemical and antioxidant parameters.

Results: Hepacare produced concentration dependent inhibition of DPPH and nitric oxide activity with IC₅₀ of 48.50 and 55.00 µg/ml, respectively, it suppressed the absorbance of

ABTS⁺ with total antioxidant capacity of 423.47 ± 8.37 mg QUE/g. CCl₄ administration induced significant (P<0.001) elevation of serum aspartate transaminase (1.70 fold), alanine transaminase (1.60 fold), alkaline phosphatase (2.90 fold) and bilirubin (2.00 fold) in comparison to control. The increase in serum biomarker were dose-dependently reversed by Hepacare[®] pretreatment. Moreover, CCl₄ pretreatment increased (P<0.001) malondialdehyde (MDA) (73.98%) and decreased (P<0.001) antioxidant enzymes level but Hepacare pretreatment produced dose-dependent attenuation of the increased MDA (3.84 fold) with enhancement of glutathione (3.08 fold), superoxide dismutase (2.08 fold), and catalase (3.14 folds) levels in comparison to CCl₄ treated group, similar to those of silymarin reference standard.

Conclusion: Hepacare was beneficial in the prevention of CCl₄-induced hepatocellular injury, possibly by scavenging reactive free radicals, and boosting endogenous antioxidant systems.

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Bibliography

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Introduction



The liver is the largest organ in the human body, it has the enormous task of maintaining the body's metabolic homeostasis, necessary for metabolism of drugs and exogenous toxins. Liver damage is a prevalent pathology that involves a variety of disorders including oxidative stress, steatosis, hepatitis, fibrosis, cirrhosis, apoptosis and hepatocellular carcinoma [1]. Liver diseases are one of the major causes of mortality and morbidity worldwide [2]. They are mainly caused by toxic chemicals, excessive consumption of alcohol, infections and autoimmune disorders. Most of the hepatotoxic chemicals damage liver cells by inducing lipid peroxidation and oxidative stress [3]. Cell damage results from the increased generation of reactive species from oxygen and nitrogen or deficiency of antioxidants [4]. Carbon

tetrachloride (CCl₄) is a hepatotoxin widely used in animal models, biotransformed by hepatic microsomal cytochrome P₄₅₀ to produce the trichloromethyl free radicals [5] which can react with sulphhydryl groups, such as reduced glutathione (GSH) and protein thiols. Covalent binding of trichloromethyl free radicals to cell protein is considered to be the initial step in a chain of events that eventually lead to membrane lipid peroxidation and finally to cell apoptosis and necrosis [6]. There is a paradigm shift to the use of complementary and alternative therapies for the management of liver diseases as a result of limited efficacy and serious adverse effects of the conventional therapies [7]. Moreover, the search for new drugs capable of alleviating liver injury with fewer side effects and satisfactory therapeutic outcome has increased over the years.

Hepacare[®] (liver tonic), a herbal formulation prepared from the milled dried leaves of *Calliandra portoricensis* (Leguminosae), barks of *Canarium schweinfurthii* (Burseraceae), and roots of *Uvaria chamae* (Annonaceae). Hepacare is used as a natural liver cleanser and is thought to be helpful for patients suffering from sickle cell anaemia who present with jaundice and also for those who consume excessive alcoholic beverages. Hence, this study sought to scientifically investigate the veracity of the claimed hepatoprotective effect of Hepacare[®] in carbon tetrachloride induced liver damage model in rats. Silymarin, a mixture of bioactive flavonolignans from the milk thistle (*Silybum marianum*), which is traditionally used in herbal medicine to defend against various hepatotoxic agents was used as positive control. Hepacare in vivo antioxidant activities were also evaluated in CCl₄-induced liver injury and oxidative stress in rats.

Materials and Methods

Drugs and chemicals

Hepacare[®] capsules were purchased from Health Forever Product Limited, Lagos, Nigeria, and silymarin from Micro Laboratory Limited, Hosur-635126, India. Other drugs and chemicals including 1,1-diphenyl-2-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), ferric chloride, gallic acid, sodium chloride (NaCl), sodium nitrate (NaNO₂), sulphanilamide, naphthylamine diamine dihydrochloric, bovine serum albumin (BSA), 5, 5-dithiobis(2-nitro-benzoic acid) (DTNB), Folin-Ciocalteu's, hydrochloric acid, 2-thiobabutaric acid (TBA) and quercetin were purchased from Sigma Aldrich, St. Louis MO, USA. Ascorbic acid and aluminium chloride were obtained from SD Fine Chem. Ltd., Biosar, India. Ammonium molybdate, methanol, sodium phosphate, concentrated H₂SO₄, sodium carbonate, potassium acetate, mono-sodium phosphate, bi-sodium phosphate, and potassium ferricyanide were purchased from Merck, Germany.

In vitro evaluation of antioxidant activities

DPPH radical scavenging activity assay

The free radical scavenging capacity of Hepacare[®] was determined using the stable free radical DPPH [8]. Hepacare was mixed with 95% ethanol to prepare a stock solution (5 mg/ml). DPPH solution (0.004%, w/v) was prepared in 95% ethanol. A freshly prepared DPPH solution (0.004%, w/v) was placed in test tubes and Hepacare[®] was added followed by serial dilution (25 µg to 150 µg) in every test tube so that the final volume was 3 ml. After 10 min, the absorbance was read at 515 nm using a spectrophotometer (HACH 4000 DR UV-visible spectrophotometer, USA). Ascorbic acid (5 mg/ml in distilled water) was used as a reference standard. A control sample of the same volume was prepared without any extract and reference ascorbic acid. A solution of 95% ethanol served as a blank. The percentage (%) scavenging of the DPPH free radical was calculated using the following equation:

$$\% \text{ scavenging activity} = \frac{\text{Absorbance of the control} - \text{Absorbance of the test sample}}{\text{Absorbance of the control}} \times 100$$

The IC₅₀ value is the concentration of the sample required to inhibit 50% of the radical [9].

Total antioxidant capacity assay

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method in accordance with the procedure described by Prieto et al. [10]. The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at an acidic pH. 0.3 ml Hepacare[®] was combined with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Test tubes containing the reaction solution were incubated at 95 °C for 90 min. The absorbance of the solution was then measured against a blank at 695 nm using a spectrophotometer (HACH 4000 DU UV-visible spectrophotometer) after the solution cooled to room temperature. Ethanol (0.3 ml) in the place of extract/sample served as the blank. Antioxidant activity was expressed as the number of gram equivalents of ascorbic acid.

Determination of total phenol content

Total phenol content in the Hepacare[®] was determined with Folin-Ciocalteu reagent. Hepacare[®] (200 µg/ml) was mixed with 400 µl of the Folin-Ciocalteu reagent and 1.5 ml of 20% sodium carbonate. The mixture was shaken thoroughly and made up to 10 ml using distilled water. The mixture was allowed to stand for 2 h. The absorbance at 765 nm was then determined. The total phenol content in Hepacare[®] was then determined as the mg of gallic acid equivalent using equations that were obtained from a standard gallic acid graph [11].

Determination of total flavonoid content

The total flavonoid content was determined using a method previously described by Kumaran and Karunakaran [12]. 1 ml of Hepacare[®] in ethanol (200 µg/ml) was mixed with 1 ml of aluminium trichloride in ethanol (20 mg/ml) and a drop of acetic acid and then diluted with ethanol to 25 ml. The absorption at 415 nm was read after 40 min. Blank samples were prepared from 1 ml of Hepacare and a drop of acetic acid and then diluted to 25 ml with ethanol. The total flavonoid content was determined using a standard curve for quercetin (12.5–100 µg/ml) and was expressed as mg of quercetin equivalent (QUE/g of extract).

Determination of total tannins content

A total of 500 mg of Hepacare was extracted with 300 ml of diethyl ether for 20 h at room temperature. The residue was boiled for 2 h with 100 ml of distilled water, and then allowed to cool, and was filtered. The extract was adjusted to a volume of 100 ml in a volumetric flask. The content of tannins in the extract was determined colorimetrically using Folin-Denis reagent, by measuring absorbance of the blue complex at 760 nm, using tannic acid solution as a standard solution [13].

Reducing power assay

The reducing capacity of a compound may serve as a good indicator of its potential antioxidant property. The reducing capacity of Hepacare was investigated using Fe³⁺-Fe²⁺ transformation. The presence of reductones causes the reduction of Fe³⁺/ferricyanide complex to the Fe²⁺. Reducing power of the Hepacare[®] was evaluated using the Oyaizu method [14]. Different concentrations of Hepacare[®] (25, 50, 75, and 100 µg/ml) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (2.5 ml, 1% w/v). The mixture was incubated at 50 °C for 20 min. After incubation,

2.5 ml of 10% trichloroacetic acid solution was added to each test tube and the mixture was centrifuged at 3000 rpm for 10 min, subsequently, 5 ml of the upper layer solution was mixed with 5 ml of distilled water and 1 ml of ferric chloride solution (0.1%, w/v) and the absorbance was measured at 700 nm. The reducing power of the extract was linearly proportional to the concentration of the sample. Ascorbic acid served as the reference standard. Phosphate buffer (pH 6.6) served as the blank solution.

Nitric oxide radical scavenging

At physiological pH, nitric oxide generated from aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions, which may be quantified by the Griess Illosvoy reaction [15]. The reaction mixture contained 10 mM SNP, phosphate buffered saline (pH 7.4) and various doses (0–70 µg/ml) of the test solution in a final volume of 3 ml. After incubation for 150 min at 25 °C, 1 ml sulfanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 ml of the incubated solution and allowed to stand for 5 min. Then 1 ml of naphthylethylenediamine dihydrochloride (NED) (0.1% w/v) was added and the mixture was incubated for 30 min at 25 °C. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED was measured spectrophotometrically at 540 nm against a blank sample. All tests were performed 6 times. Ascorbic acid was used as a standard.

Animals

Male Sprague Dawley rats (150–200 g) and female Swiss albino mice (20–25 g) used in this study were obtained from the Laboratory Animal Centre of the College of Medicine, University of Lagos, Lagos, Nigeria. The animals were maintained in a 12 h light/dark cycle, at constant temperature (23 ± 2 °C) and humidity (50 ± 10%). After 1 week of acclimatization, animals had free access to food and water. The experimental procedures adopted in this study was approved by College of Medicine, Research grant and experimentation committee and in strict compliance with the United States National Institutes of Health Guidelines for Care and Use of Laboratory Animals in Biomedical Research [16].

Acute toxicity study

Mice were generally fasted for 12 h before the test. Mice (n=6) received either distilled water 10 ml/kg or Hepacare (500, 1000 and 2000 mg/kg, p.o.). Mice were observed for toxic symptoms and behavioural changes such as (sedation, hyperactivity, diarrhoea, writhing, piloerection, and restlessness) for 2 h post-administration and 14 days for signs of delayed toxicity or mortality.

Hepatoprotective and in vivo antioxidant activity

The method of Habbu et al. [17] was used in this study. Rats were divided into 9 groups of 5 animals each. Treatment was then carried out according to the group allotment below:

Group I (control): Distilled water (10 ml/kg, p.o.)

Group II (control): Olive oil (10 ml/kg, p.o.)

Group III: CCl₄/olive oil, 1:1 (0.7 ml/kg, i.p.)

Group IV (standard reference): Silymarin (200 mg/kg, p.o.) + CCl₄ (0.7 ml/kg, i.p.)

Groups V–VIII: Hepacare (50, 100, 200, or 400 mg/kg, p.o.) + CCl₄ (0.7 ml/kg, i.p.)

Group IX: Hepacare (400 mg/kg, p.o.)

CCl₄ was given on alternate days for a period of 7 days. Control, test and standard agents were administered for 7 successive days. On the 7th day, animals were sacrificed by cervical dislocation and blood was withdrawn into plain bottles by cardiac puncture. The blood was allowed to coagulate for 30 min and serum was separated by centrifugation. Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, total bilirubin, and total protein were estimated using Roche and Cobas commercial kits and Roche/Hitachi 904 automated analyser. The liver tissue was isolated for weight determination (using Mettler-Toledo GmbH digital weighing balance [Type BD202, SNR 06653]), to measure the levels of antioxidant enzymes, malondialdehyde (MDA) and for histopathological studies.

Measurement of antioxidant enzymes and MDA levels

Measurement of antioxidant enzymes activity and MDA level was done according to standard procedures: catalase [18]; superoxide dismutase (SOD) [18]; reduced glutathione [18]; and MDA [18].

Histopathological studies

After rats were sacrificed, the livers of each rat was identified and carefully extracted. Sections were immediately taken from each lobe of the liver. The tissues were fixed in 10% formalin, dehydrated in graded alcohol and embedded in paraffin. These were then cut into 4–5 µm thick sections and stained with haematoxylin-eosin for photomicroscopic assessment [17, 19] using a Model N-400ME photomicroscope (CEL-TECH Diagnostics, Hamburg, Germany).

Statistical analysis

Results are expressed as mean ± SEM (n=5). Statistical significance of differences between groups was determined by ANOVA followed by Tukey post-hoc multiple comparison test. A probability (P) value of less than 0.05 was taken to indicate statistical significance. Linear regression analysis was used to calculate IC₅₀ values.

Results



Total antioxidant activity

The total antioxidant activity of the extract was calculated from the decolourization of ABTS⁺, which was measured spectrophotometrically at 734 nm. Interaction with the Hepacare or standard quercetin suppressed the absorbance of the ABTS⁺ radical cation, the total antioxidant capacity of Hepacare was 423.47 ± 8.37 mg QUE/g (► Fig. 1).

Determination of total phenolic, flavonoid and tannin contents

Phenolic compounds such as flavonoids, phenolic acids, and tannins are considered to be major contributors to the antioxidant capacity of plants. Phenolic compounds may contribute directly to antioxidative action. The total phenolic content of the sample, calculated on the basis of the standard curve for gallic acid, was found to be 342.28 ± 5.35 mg of gallic acid equivalent (GAE)/g of Hepacare. The total flavonoid content of Hepacare was 324.61 ± 14.67 mg of quercetin equivalent (QAE) per gram, while the total tannin content was 448.33 ± 7.93 mg of tannic acid equivalent (TAE)/g of Hepacare (► Fig. 1).

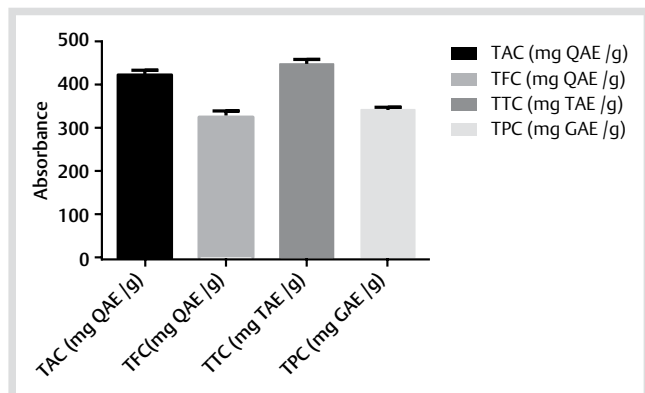


Fig. 1 Anti-oxidant properties of Hepacare. Values are expressed as Mean ± SD (n = 3). [TAC-Total antioxidant capacity; TFC-Total flavonoid content; TTC-Total tannin content; TPC-Total phenolic content; QAE-Quercetin equivalent; TAE-Tannic acid equivalent; GAE-Gallic acid equivalent].

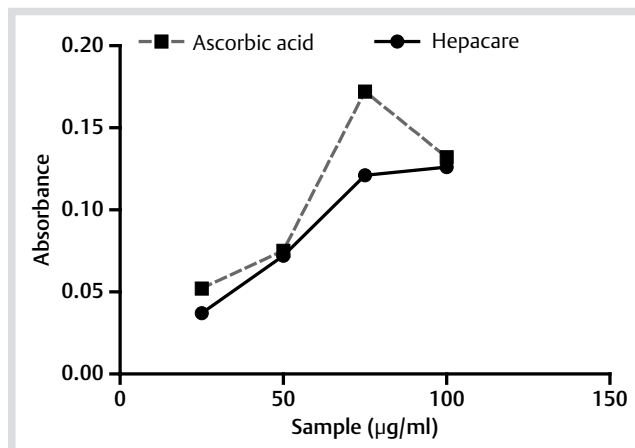


Fig. 3 The reductive abilities of Hepacare and ascorbic acid. The absorbance (A_{700}) was plotted against concentration of sample.

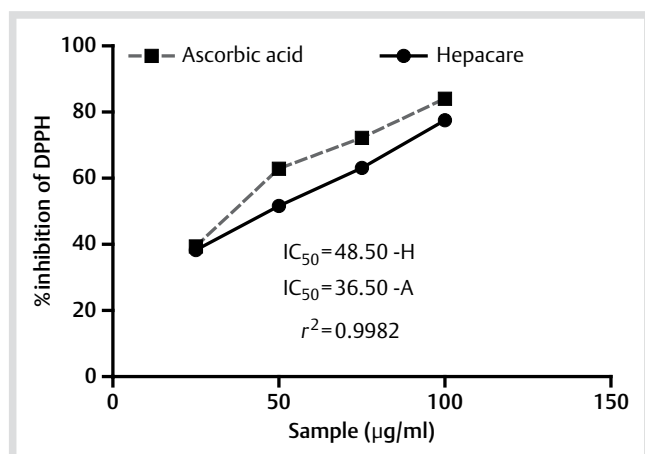


Fig. 2 Effect of Hepacare and ascorbic acid on DPPH radical scavenging activity. H-Hepacare, A-Ascorbic acid.

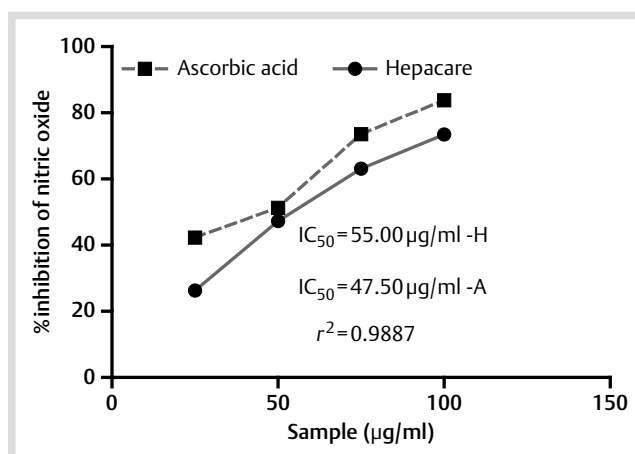


Fig. 4 The nitric oxide scavenging activity of Hepacare and the standard ascorbic acid. Values are expressed as percentage nitric oxide inhibition.

DPPH radical scavenging activity

Comparison of the antioxidant activity of Hepacare and standard antioxidant (ascorbic acid) by DPPH method is shown in **Fig. 2**. Hepacare exhibited dose-dependent inhibition of DPPH activity, and the scavenging activities of the extract and known antioxidants increased with increasing concentration. At higher concentration (100 µg/ml), the extract and known antioxidants gave the highest percentage activities; (77.53%) and (84.00%), respectively. In addition, the IC₅₀ was estimated to be 48.50 µg/ml and 36.50 µg/ml for Hepacare and ascorbic acid, respectively. These results suggest that Hepacare possesses free radical scavenging effect.

Reducing power assay

It depends on the ability of Hepacare to reduce the ferric tripyridyltriazine (Fe(III)TPTZ) complex to ferrous tripyridyltriazine (Fe(II)TPTZ) at a low pH. (Fe(II)TPTZ) has an intensive blue colour which can be read at 593 nm. As illustrated in **Fig. 3**, Fe³⁺ was transformed to Fe²⁺ in the presence of Hepacare and the reference drug ascorbic acid to measure the reductive capability. At 25 µg/ml, the absorbance's of Hepacare and ascorbic acid were 0.037 and 0.052, respectively, while at 100 µg/ml, the absorbance's of both Hepacare and ascorbic acid were 0.126 and 0.132, respectively (**Fig. 3**).

Nitric oxide inhibitory activity

Hepacare caused a moderate dose-dependent inhibition of nitric oxide with an IC₅₀ of 55.00 µg/ml (**Fig. 4**). Ascorbic acid was used as a reference and 47.50 µg/ml was needed for 50% inhibition. The IC₅₀ value of Hepacare was greater than that of the standard. Moreover, there was a strong correlation between nitric oxide inhibitory effect of Hepacare and ascorbic acid.

Effect of Hepacare on serum markers of liver damage

No difference in serum AST, ALT, ALP or bilirubin concentration was detected between distilled water and olive oil (10 ml/kg/day for 7 days) pretreated animals indicating that the vehicles used in these experiments were devoid of hepatotoxic activity (**Fig. 4a-d**). However, significant ($P < 0.001$) increases in serum aspartate aminotransferase (1.7 fold vs. vehicle treated control), alanine aminotransferase (1.6 fold vs. vehicle treated control), alkaline phosphatase (2.9 fold vs. vehicle treated control), and bilirubin (2.0 fold vs. vehicle treated control) were observed following subchronic injection of carbon tetrachloride. In contrast, pretreatment with Hepacare significantly ($P < 0.001$) and dose-dependently reversed the increase in markers of liver injury with peak protective effect at 400 mg/kg/day, serum aspartate aminotransferase (1.5 fold; [F (8, 61) = 23.6, $P < 0.0001$]), alanine aminotransferase (1.8 fold; [F (8, 61) = 8.94, $P < 0.0001$]), alkaline

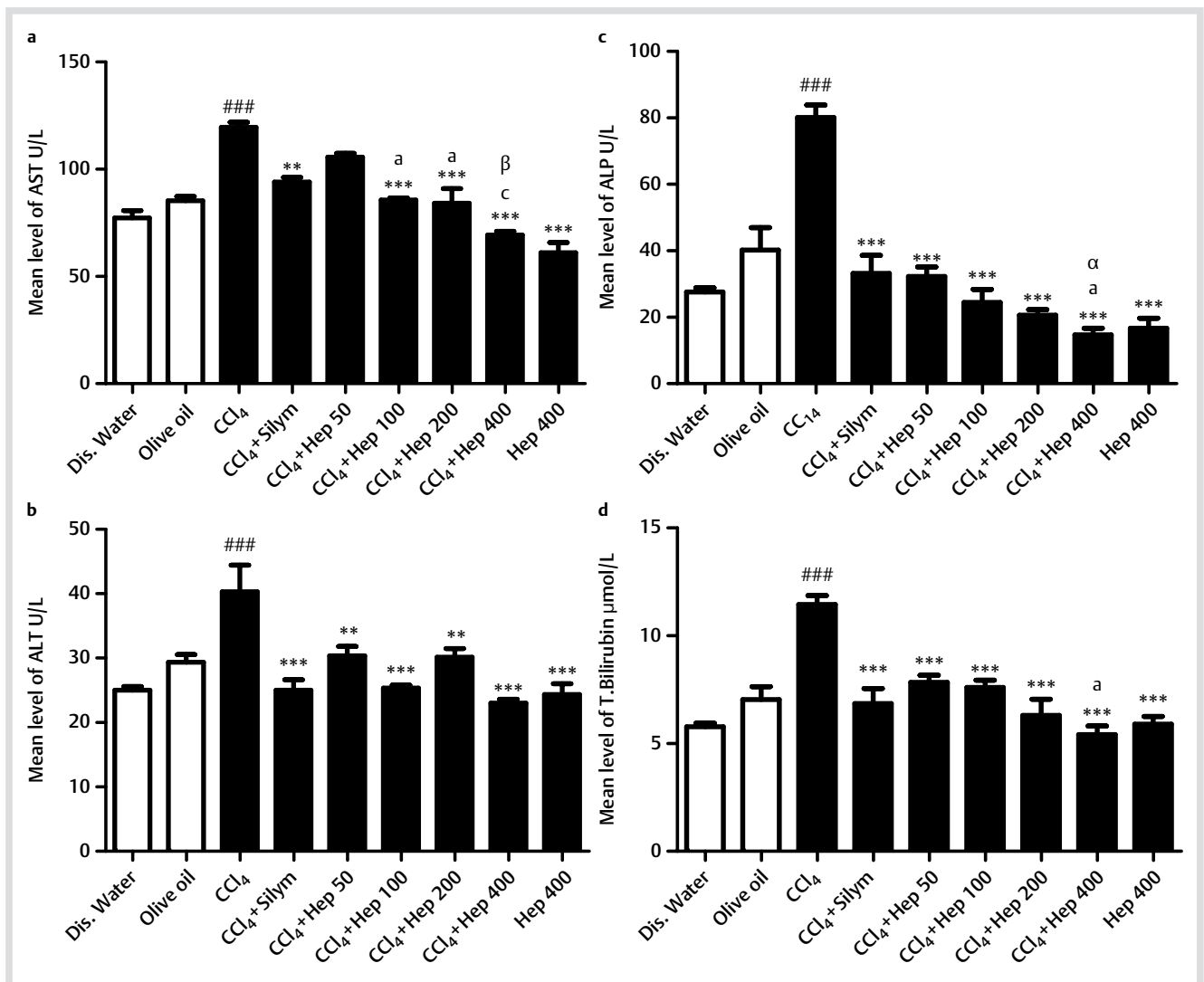


Fig. 5 a–d Hepatoprotective effect of herbal formulation Hepacare® on liver function biomarkers; **a** AST, **b** ALT, **c** ALP, and **d** Total bilirubin in CCl₄-induced liver injury in rats. Values are expressed as mean ± SEM (n=5). ###P<0.001 vs. distilled water 10 ml/kg, or olive oil 10 ml/kg treated normal control; *Significant decrease; **P<0.01, ***P<0.001 vs. CCl₄-induced liver injury treated group, ^aP<0.05, ^βP<0.001 vs. Hepacare® 50 mg/kg treated group; ^αP<0.05, ^βP<0.01 vs. silymarin® 200 mg/kg treated group. Statistical level of significance analyzed by one way ANOVA followed Tukey post hoc multiple comparison test.

phosphatase (5.4 fold; [F (8, 61)=29.5, P<0.0001]), and bilirubin (2.1 fold; [F (8, 61)=14.1, P<0.0001]) in comparison with CCl₄-vehicle treated group. Moreover, the reduction in serum markers of liver injury induced by Hepacare at 400 mg/kg was significantly (P<0.05; p<0.01) higher in comparison to silymarin 200 mg/kg treated group (► Fig. 5a–d).

MDA level

As a parameter of lipid peroxidation, the concentration of malondialdehyde was determined in samples of liver obtained from distilled water, olive oil, CCl₄, and Hepacare pre-treated rats. No significant change in MDA level was observed in distilled water and olive oil non-treated rats. However, CCl₄ pretreatment significantly increased (P<0.001) malondialdehyde level (73.98%) in comparison to control treated. One way ANOVA revealed that oral administration of Hepacare (50–400 mg/kg) significantly [3.84 fold; F (8, 61)=13.13, P<0.0001]) attenuated CCl₄-induced increase in lipid peroxidation with peak effect at 400 mg/kg (► Fig. 6). The ability of silymarin to significantly

reduced MDA generation by 3.84 fold was not significantly (P>0.05) different from protective effect of Hepacare pretreated rats. Similarly, Hepacare (400 mg/kg) only pretreated rats did not induce lipid peroxidation.

GSH level

Intraperitoneal injection of CCl₄ induced significant (P<0.001) deficit in glutathione level (2.90 fold) in comparison to control. However, Hepacare (100–400 mg/kg) pretreatment produced dose dependent and significant [3.08 fold; F (8, 61)=11.67, P<0.0001]) increase in glutathione level following deficit induced by CCl₄ pretreatment. Furthermore, silymarin treated significantly (P<0.001) enhanced glutathione level which was similar to the protective effect of Hepacare (► Fig. 7).

SOD level

Intraperitoneal injection of CCl₄ induced significant (P<0.001) reduction (2.01 fold) in the level of superoxide dismutase in the liver in comparison to vehicle only treated. Interestingly, the

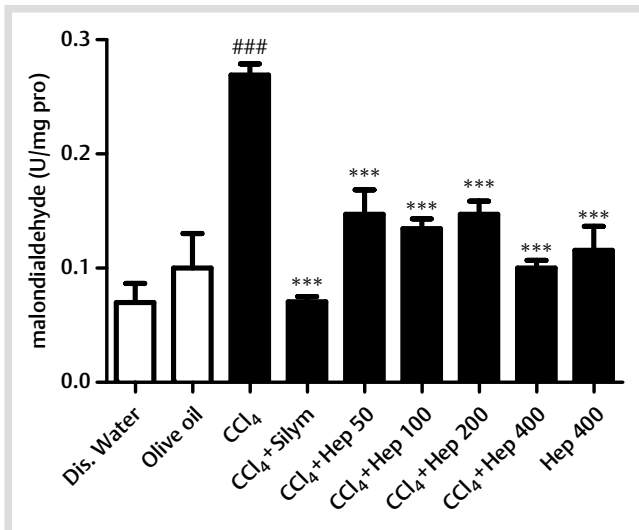


Fig. 6 Hepatoprotective effect of herbal formulation Hepacare® on malondialdehyde (MDA) level in CCl₄-induced liver injury in rats. Values are expressed as mean ± SEM (n = 5). #Significant increase; ###P < 0.001 vs. distilled water 10 ml/kg, or olive oil 10 ml/kg treated normal control; *Significant decrease; ***P < 0.001 vs. CCl₄+vehicle treated group. Statistical level of significance was analyzed by one way ANOVA followed Tukey post hoc multiple comparison test.

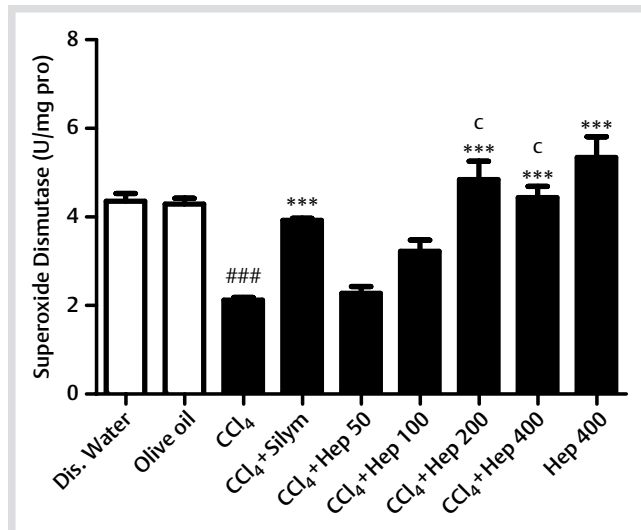


Fig. 8 Hepatoprotective effect of herbal formulation Hepacare® through elimination of reactive oxygen species (ROS) through the activities of superoxide dismutase enzyme in CCl₄-induced hepatic damage. Values are expressed as mean ± SEM (n = 5). #Significant decrease; ###P < 0.001 vs. distilled water 10 ml/kg, or olive oil 10 ml/kg treated normal control; *Significant increase; ***P < 0.001 vs. CCl₄+vehicle treated group; #P < 0.001 vs. Hepacare® 50 mg/kg treated group. Statistical level of significance was analyzed by one way ANOVA followed Tukey post hoc multiple comparison test.

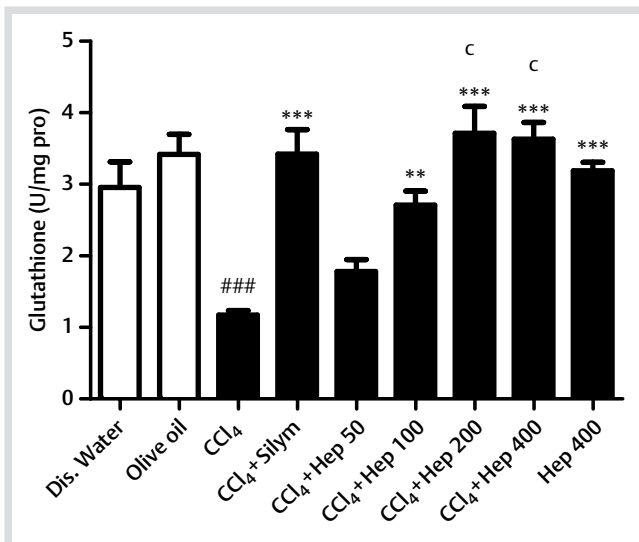


Fig. 7 Hepatoprotective effect of herbal formulation Hepacare® on induction of glutathione in CCl₄-induced liver injury in rats. Values are expressed as mean ± SEM (n = 5). #Significant decrease; ###P < 0.001 vs. distilled water 10 ml/kg, or olive oil 10 ml/kg treated normal control; *Significant increase; **P < 0.01, ***P < 0.001 vs. CCl₄+vehicle treated group; #P < 0.001 vs. Hepacare® 50 mg/kg treated group. Statistical level of significance was analyzed by one way ANOVA followed Tukey post hoc multiple comparison test.

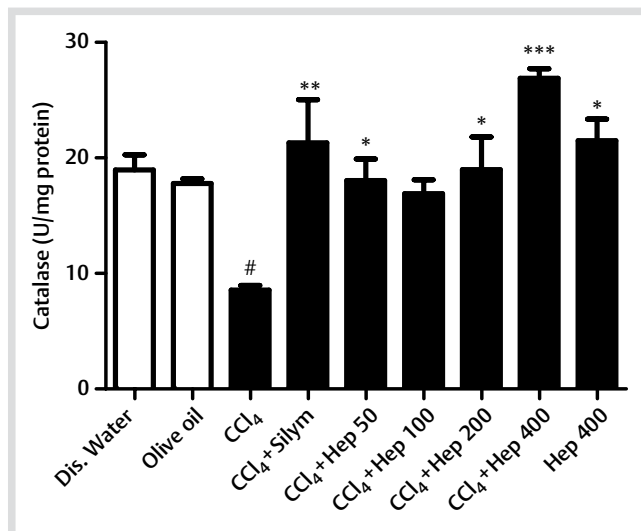


Fig. 9 Hepatoprotective effect of herbal formulation Hepacare® on catalase activities in CCl₄-induced liver injury in rats. Values are expressed as mean ± SEM (n = 5). #Significant decrease; #P < 0.05 vs. distilled water 10 ml/kg, or olive oil 10 ml/kg treated normal control; *Significant increase; #P < 0.05, **P < 0.01, ***P < 0.001 vs. CCl₄+vehicle treated group. Statistical level of significance was analyzed by one way ANOVA followed Tukey post hoc multiple comparison test.

reduction in the level of SOD induced by CCl₄ was reversed by Hepacare pretreatment in a dose related manner, the significant increase [2.08 fold; F (8, 61) = 18.04, P < 0.0001] in the SOD level was similar to the effect of silymarin treated group (► Fig. 8).

Catalase level

Hepacare (100–400 mg/kg) pretreatment produced significant [3.14 fold; F (8, 61) = 6.45, P < 0.01] increase in catalase level in comparison to CCl₄-induced reduction in catalase level indicat-

ing amelioration of CCl₄-induced oxidative stress (► Fig. 9). Moreover, silymarin pretreatment produced significant (P < 0.01) increase in catalase level that was similar to the peak effect of Hepacare 400 mg/kg.

Histology of the liver

Distilled water treatment did not affect architecture of the liver cells (► Fig. 10a), moreover inflammatory cells were seen sur-

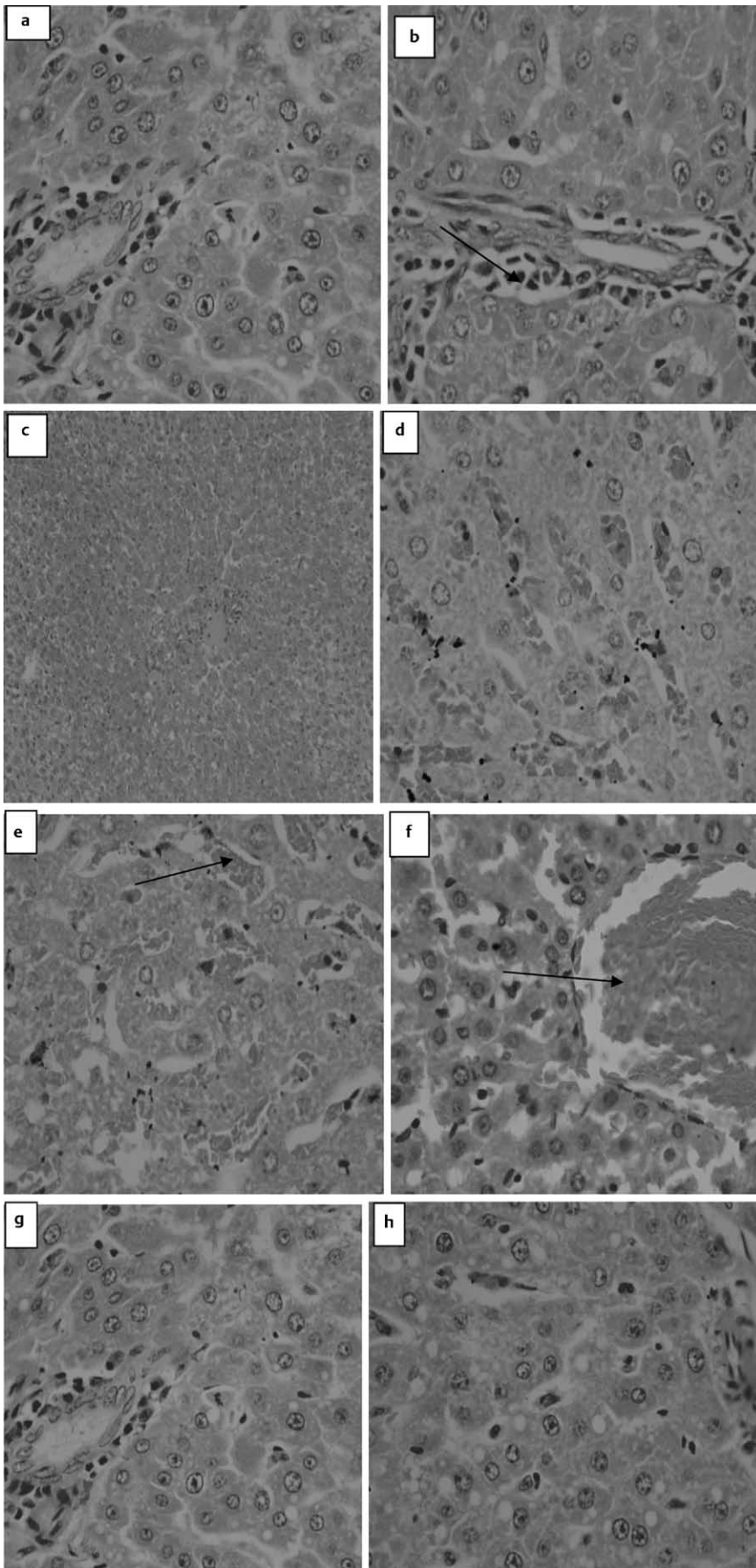


Fig. 10 Effect of Hepacare or silymarin against CCl_4 -induced liver injury **a** distilled water treated control (10 ml/kg), [Hepatocytes appears normal. $\times 40$], **b** olive oil treated control (10 ml/kg), [arrow shows inflammatory cells surrounding portal tract. $\times 40$], **c** CCl_4 (0.7 ml/kg) [arrow shows signs of early changes related to chronic congestion (nutmeg appearance). $\times 40$], **d** Hepacare (50 mg/kg) + CCl_4 [Shows severe sinusoidal congestion. $\times 40$], **e** Hepacare (100 mg/kg) + CCl_4 [Shows extensive sinusoidal haemorrhage. $\times 40$], **f** Hepacare (200 mg/kg) + CCl_4 [Shows moderate congestion. $\times 40$], **g** Hepacare (400 mg/kg) + CCl_4 [Shows hepatocytes with microvesicles. $\times 40$], **h** silymarin (200 mg/kg) + CCl_4 [Hepatocytes appear normal. $\times 40$].

rounding the portal tract in olive oil treated group (◻ Fig. 10b). However, injection of hepatotoxin (CCl_4) intraperitoneally induced chronic hepatocyte congestion with nutmeg appearance (◻ Fig. 10c) but pretreatment with Hepacare produced dose dependent reversal of liver injury with; severe sinusoidal congestion at 100 mg/kg (◻ Fig. 10d), extensive sinusoidal haemorrhage at 200 mg/kg (◻ Fig. 10e), and moderate congestion at 400 mg/kg of Hepacare (◻ Fig. 10f). In comparison, Hepacare only treated group (◻ Fig. 10g) did not affect micro-architecture of the liver cells which was similar to the effect of silymarin only treated group (◻ Fig. 10h).

Discussion

Findings from this study revealed significant antioxidant property of Hepacare[®] both from in vitro and in vivo biological investigation as possible mechanism for its hepatoprotective effect against CCl_4 -induced liver injury in rats. It has been hypothesized that one of the principle causes of CCl_4 -induced liver injury is lipid peroxidation, induced by free radical derivatives of CCl_4 [20]. Thus, antioxidant activity, or the inhibition of the generation of free radicals, is important in the protection against CCl_4 -induced liver injury. The antioxidants activity of medicinal plants is well correlated with their polyphenolic constituents [21]. Polyphenols constitute a large and complex category of compounds and flavonoids represent the most studied group of this category for their important biological activities, such as anti-inflammatory, anti-viral and anti-cancer actions [22] which could be linked to their antioxidant and free radical scavenging effect. The use of medicinal plants with high level of antioxidant constituents has been proposed as an effective therapeutic approach for hepatic damages [23]. Antioxidant compounds like phenolic acids, polyphenols, terpenoids and flavonoids scavenge free radicals such as peroxide, or lipid peroxy and thus inhibit the oxidative mechanisms that lead to degenerative diseases [20]. The total antioxidant capacity of Hepacare was measured spectrophotometrically through phosphomolybdenum method, based on the reduction of Mo (VI) to Mo (V) by the test sample and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 765 nm. The present study demonstrated that Hepacare[®] exhibited significant and potent antioxidant capacity for phosphomolybdate reduction. Studies have shown that many flavonoid and related polyphenols contribute significantly to the phosphomolybdate scavenging activity of medicinal plants [24,25]. Hepacare exhibited significant total phenolic, flavonoids and tannin contents. The phenolic compounds of plants are important because their hydroxyl groups confer scavenging ability [26]. The most described among groups of phenolic compounds is flavonoid which have been linked to array of biological activities due to their capacity to act as antioxidants. Based on the results obtained from this study, it could be suggested that phenolic acids and flavonoids may be the major contributors for the antioxidant activity of Hepacare. Also, compounds rich in tannins have been shown to effectively protect the liver against CCl_4 - and D-galactosamine-induced injury [27]. The electron donation ability of natural products can be measured by DPPH purple-coloured solution bleaching [23]. The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolourizes the DPPH solution. The degree of colour change is proportional to the concentration and potency

of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test [8]. Results of this study suggest that Hepacare[®] contain phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage. The antiradical properties of this extract could be linked to the presence of phenolic compounds. The reducing ability of a compound depends on the presence of reductants, which will break the free radical chain by donating a hydrogen atom [28]. Moreover, the reducing power of secondary metabolites from medicinal plants prevents liver injury by inhibiting formation of lipid peroxides [29]. Furthermore, the reducing power of Hepacare increased with increase in concentration, suggesting a potential reductants [30], in addition, the ferrous ion chelating properties of an antioxidant extract could be attributed to their endogenous chelating agents, mainly, tannins, terpenes and phenolics [31]. In reducing power assay, the yellow colour of the test solution changes to green depending on the reducing power of the test specimen. The presence of the reductants in the solution causes the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by absorbance measurement at 700 nm. Increasing absorbance at 700 nm indicates an increase in reducing ability. The antioxidants present in the extract of Hepacare[®] caused the reduction of Fe^{3+} /ferricyanide complex to the ferrous form, and thus proved the reducing power. This showed that Hepacare reacts with free radicals to convert them to more stable products, hence terminate radical chain reaction. Despite much study, the precise role of nitric oxide (NO) in liver damage induced by hepatotoxicants remains unclear (hepatoprotective and hepatotoxic). However, the study of Gardener et al., [32] showed that aminoguanidine (inducible nitric oxide synthase (iNOS) enzyme inhibitor) reduces the resulting hepatic necrosis and associated increases in serum aspartate and alanine aminotransferase activity. It was concluded that NO derived from iNOS is hepatotoxic possibly as a result of the formation of damaging free radical species such as peroxy nitrite. Interestingly, Hepacare and ascorbic acid dose dependently attenuated in vitro nitric oxide generation. In this study, intraperitoneal administration of CCl_4 on alternate days for a period of 7 days induced liver injury in rats. The liver injury induced by CCl_4 is the best characterized system for xenobiotic-induced hepatotoxicity and are commonly used models for screening of the anti-hepatotoxic and/or hepatoprotective activities of drugs [30,31]. This in vivo study showed that serum ALT, AST, ALP and, total bilirubin activities and hepatic MDA increased in parallel with CCl_4 injection, with concomitant GSH, SOD, and catalase deficit indicating the induction of acute hepatotoxicity by CCl_4 [32]. The acute hepatotoxic effects induced by CCl_4 administration were confirmed histopathologically, revealing extensive sinusoidal haemorrhage, and hepatocellular degeneration. The obtained results are in accordance with those of the previous reports [32]. However, Hepacare pretreatment significantly attenuated the CCl_4 -induced increase in serum activities of ALT, AST and ALP, and reduced hepatic lipid peroxidation. Histological observations of the livers also strongly support the hepatoprotective effect of Hepacare. The return of elevated levels of serum enzymes to near normal values in groups treated with Hepacare or standard silymarin is an indication of the stabilization of the plasma membrane and the repair of hepatic tissue damage caused by CCl_4 . In addition, these results indicated the

potential protective effect of Hepacare against CCl₄-induced liver injury.

It has been recognized that oxidative stress and generation of free radicals play a critical role in CCl₄-induced liver injury. CCl₄ toxicity is due to reactive free radical which is generated by its reductive metabolism through hepatic microsomal cytochrome P₄₅₀ to produce the trichloromethyl (CCl₃·), free radicals which can react with sulfhydryl groups, such as reduced glutathione (GSH) and protein thiols [33]. Covalent binding of CCl₃· to cell proteins is considered to be the initial step in a chain of events that eventually lead to membrane lipid peroxidation and breakdown of cellular membranes [34]. High levels of reactive oxygen species (ROS) damage cells and are involved in several human pathologies, including liver cirrhosis and fibrosis. Therefore, the use of compounds with antioxidant properties may prevent or alleviate many diseases associated with ROS. However, Hepacare pretreatment attenuated CCl₄-induced hepatocellular lipid peroxidation. The significant reduction in the level of MDA; end-product of polyunsaturated fatty acid (PUFA) peroxidation, in Hepacare treated rats liver homogenates indicates its antilipid peroxidative effect. Pretreatment with Hepacare before intraperitoneal injection of CCl₄ increased the activity or level of GSH (i.e. attenuated GSH depletion), which scavenge free radicals and simultaneously reduce lipid peroxidation, thus alleviating the oxidative damage caused by CCl₄. Also, SOD and catalase deficit induced by CCl₄, were significantly increased in a dose dependent manner with the administration of Hepacare or silymarin confirming the antioxidant properties of these 2 agents obtained in in vitro models. GSH, SOD and catalase constitute mutually supportive team of defense against ROS with SOD (a metalloproteinase), which is the first enzyme involved in the antioxidant defense by lowering the steady-state level of O₂⁻ [35]. The hepatoprotective effect of Hepacare may at least in part be due to its phenolic and flavonoids constituents found in this preparation. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching single and triplet oxygen, or decomposing peroxides [36]. As stated earlier, flavonoids possessed the ability to scavenge free radicals, inhibit hydrolytic and oxidative enzymes [37], hence, their antiperoxidative action could be attributed to a concomitant ability to chelate iron. Flavonoids such as apigenin-7-glucoside (AP₇Glu), luteolin-7-glucoside (LU₇Glu) and quercetin have been reported to prevent glutathione depletion and lipid peroxidation induced by an acute intoxication with carbon tetrachloride (CCl₄), ethanol, acetaminophen and bromobenzene in the liver and in the rats with biliary obstruction [37]. The best-described property of almost every group of flavonoids is their capacity to act as antioxidants [38]. The flavones and flavonols (apigenin, luteolin, quercetin, rutin and others) seem to be the most powerful flavonoids for protecting a body against reactive oxygen species. These compounds have the potential to scavenge and quench various radicals (oxygen-centred, carbon-centred, alkoxyl, peroxy, or phenoxy radicals) and reactive oxygen species. The flavonoids may have an additive effect to the endogenous scavenging compounds and also increase the function of the endogenous antioxidants. The flavonoids interrupt the lipid peroxidation chain reaction and thereby prevent glutathione depletion that plays a critical role in cellular defense against oxidative stress [39]. In conclusion, findings from this study demonstrated that Hepacare was significantly beneficial in the prevention of CCl₄-induced hepatocellular injury, possibly by

scavenging reactive free radicals, and boosting the endogenous antioxidant systems. Hence this study provided scientific evidence for the use of Hepacare as herbal medicine in the treatment of liver disorders. However, further study to explicate the shielding effects of Hepacare in CCl₄-induced liver damage is important and will provide additional evidence to support its use as a remedy on a broader scale.

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Conflict of Interest

▼ The authors declare that there are no conflicts of interest in this study.

References

- Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Alimentary Pharmacol Ther* 2011; 34: 274–285
- Abbound G, Kaplowitz N. Drug induced liver injury. *Drug Saf* 2007; 30: 277–294
- Kalyani GA, Ramesh CK, Krishna V. Hepatoprotective and antioxidant activities of *Desmodium triquetrum* DC. *Indian J Pharm Sci* 2011; 73: 463–466
- Medina J, Moreno-Otero R. Pathophysiological basis for antioxidant therapy in chronic liver disease. *Drugs* 2005; 65: 2445–2465
- Williams AT, Burk RF. Carbon tetrachloride hepatotoxicity: an example of free radical mediated injury. *Semin Liver Dis* 1990; 10: 279–284
- Recknagel RO, Glende EA Jr, Dolak JA et al. Mechanism of carbon tetrachloride toxicity. *Pharmacol Ther* 1989; 43: 139–154
- Verma S, Thuluvath PJ. Complementary and alternative medicine in hepatology: Review of the evidence of efficacy. *Clin Gastroenterol Hepatol* 2007; 5: 408–416
- Braca A, De Tommasi N, Di Bari L et al. Antioxidant principles from *Bauhinia terapotensis*. *J Nat Prod* 2001; 64: 892–895
- Vituro C, Molina A, Schmida-Hirschmann G. Free radical scavengers from *Mutisia friesiana* (Asteraceae) and *Sanicula graveolens* (Apiaceae). *Phytother Res* 1999; 13: 422–424
- Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. *Anal Biochem* 1999; 269: 337–341
- Chia CC, Ming HY, Hwei MW et al. Estimation of total phenolic content by two complementary colorimetric methods. *J Food Drug Anal* 2002; 10: 163–173
- Karunakaran K. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J Food Drug Anal* 2007; 10: 178–182
- Chanwitheesuk A, Teerawutgulrag A, Rakariyatham N. Screening of antioxidant activity and antioxidant compounds of some edible plants of Thailand. *Food Chem* 2005; 92: 491–497
- Oyaizu M. Studies on product of browning reaction prepared from glucose amine. *Japanese Journal of Nutrition* 1986; 44: 307–315
- Garratt DC. The quantitative analysis of Drugs. Volume 3. Chapman and Hall Ltd, Japan: 1964; 456–458
- NIH. Guide for the Use of Laboratory Animals DHHS, PHS. NIH Publication No. 85-23 (1985 Revised)
- Habbu PV, Shastry RA, Mahadevan KM et al. Hepatoprotective and antioxidant effects of *Argyrea speciosa* in rats. *African Journal of Traditional, Complementary and Alternative Medicine* 2008; 5: 158–164

- 18 Soon YY, Tan BKH. Evaluation of the hypoglycaemic and antioxidant activities of *Morinda officinalis* in streptozocin-induced diabetic rats. *Singapore Med J* 2002; 43: 077–085
- 19 Galigher AE, Kozloff EN. *Essentials of Practical Microtechniques*. 2nd (ed.). Lea and Febiger, Philadelphia: 1971; 77
- 20 Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. *LWT Food Sci Technol* 1995; 28: 25–30
- 21 Conforti F, Marrelli M, Carmela MC *et al*. Bioactive phytonutrients (omega fatty acids, tocopherols, polyphenols), in vitro inhibition of nitric oxide production and free radical scavenging activity of non-cultivated Mediterranean vegetables. *Food Chem* 2011; 129: 1413–1419
- 22 Nijveldt RJ, van Nood E, van Hoorn DEC *et al*. Flavonoids: A review of probable mechanisms of action and potential applications. *Am J Clin Nutr* 2001; 74: 418
- 23 Govind P. Medicinal plants against liver diseases. *IJPR* 2011; 2: 115–121
- 24 Nunes PX, Silva SF, Guedes RJ *et al*. Biological oxidations and antioxidant activity of natural products, phytochemicals as nutraceuticals – Global Approaches to Their Role in Nutrition and Health. 2012
- 25 Shariffar F, Dehghn-Nudeh G, Mirtajaldini M. Major flavonoids with antioxidant activity from *Teucrium polium* L. *Food Chem* 2009; 112: 885–888
- 26 Saeed N, Khan MR, Shabbir M. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla* L. *BMC Complement Altern Med* 2012; 12: 221
- 27 Shimoda H, Tanaka J, Kikuchi M *et al*. Walnut polyphenols prevent liver damage induced by carbon tetrachloride and d-galactosamine: hepatoprotective hydrolyzable tannins in the kernel pellicles of walnut. *J Agric Food Chem* 2008; 56: 4444–4449
- 28 Gordon MH. In: *Food antioxidants*. Hudson BJ (ed.). London: Elsevier Applied Science. The mechanism of antioxidant action in vitro, 1990; 18
- 29 Kimura Y, Okuda H, Mori K *et al*. Studies on the activities of tannins and related compounds from medicinal plants and drugs, IV: Effects of various extracts of *Geranii herba* and *Geraniin* on liver injury and lipid metabolism in rats fed peroxidized oil. *Chem Pharm Bull* 1984; 32: 1866–1871
- 30 Mahadevappa P, Chapeyil KR, Venkatarangaiah K *et al*. Hepatoprotective and in vitro antioxidant effect of *Carthamus tinctorius* L. var *Annigeri-2*-, an oil-yielding crop, against CCl₄-induced liver injury in rats. *Pharmacogn Mag* 2011; 7: 289–297
- 31 Khan RA, Khan MR, Sahreen S *et al*. Assessment of flavonoids contents and in vitro antioxidant activity of *Launaea procumbens*. *Chem Cent J* 2012; 6: 43
- 32 Gardner CR, Heck DE, Yang CS *et al*. Role of nitric oxide in acetaminophen-induced hepatotoxicity in the rat. *Hepatology* 1998; 27: 748–754
- 33 Recknagel RO, Glende EA Jr, Dolak JA *et al*. Mechanisms of carbon tetrachloride toxicity. *Pharmacol Ther* 1989; 43: 139–154
- 34 Manibusan MK, Odin M, Eastmond DA. Postulated carbon tetrachloride mode of action: a review. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 2007; 25: 185–209
- 35 Lin X, Liu X, Huang Q *et al*. Hepatoprotective Effects of the Polysaccharide Isolated from *Tarphochlamys affinis* (Acanthaceae) against CCl₄-Induced Hepatic Injury. *Biol Pharm Bull* 2012; 35: 1574–1580
- 36 Williams AT, Burk RF. Carbon tetrachloride hepatotoxicity: an example of free radical-mediated injury. *Semin Liver Dis* 1990; 10: 279–284
- 37 De Groot H, Sies H. Cytochrome P-450, reductive metabolism, and cell injury. *Drug Metab Rev* 1989; 20: 275–284
- 38 Islam R, Parvin S, Islam E. Antioxidant and hepatoprotective activity of an ethanol extract of *Syzygium jambos* (L.) leaves. *Drug Discoveries Ther* 2012; 6: 205–211
- 39 Zheng QS, Sun X, Xu B *et al*. Mechanisms of apigenin-7-glucoside as a hepatoprotective agent. *Biomed Environ Sci* 2005; 18: 65–70