Hepatoprotective Effect of *Calendula officinalis* Linn (Asteraceae) Flowers Against CCL₄ – Induced Hepatotoxicity in Rats

Maysa M. El Mallah and Reham A. Mohamed

Department of Nutrition and Food Sciences, Faculty of Home Economics, Helwan University, Cairo, Egypt

**Abstract:** The present study was carried out to identify and quantify the phenolic compounds in alcoholic extract of *Calendula officinalis* flowers and to investigate the hepatoprotective effect of oral administration of alcoholic extract of *Calendula officinalis* flowers against CCL₄-induced liver injury in rats. After 4 weeks of treatment, serum levels of total cholesterol (TC), triglycerides (TG), lipoprotein fractions, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (TP), albumin, total bilirubin (TBil) and oxidative stress markers such as glutathione (GSH), malondialdehyde (MDA), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) were determined. Histopathological examination of liver tissue was also performed. Forty two adult male Wistar rats were divided into six equal groups as follows: group1: negative control group, group 2: positive control (CCL₄) group injected subcutaneously by a single dose of CCL₄ (2 ml/kg b.wt) at the last day of the experiment to induce hepatotoxicity, group 3: rats treated with standard drug Silymarin (200mg/kg b.wt) once daily for 4 weeks prior administration of CCL₄ (2 ml/kg b.wt) and groups (4), (5) and (6) were orally administered with alcoholic extract of *Calendula officinalis* at doses of (200, 400 and 600 mg/kg b.wt) once daily for 4 weeks prior administration of CCL₄ (2 ml/kg b.wt). The results showed that oral administration of *Calendula officinalis* flower extract (COFE) at a dose of 600 mg/kg b.wt to rats for 4 weeks prior induction of hepatotoxicity significantly improved TC, TG, lipoprotein fractions, decreased the elevated serum levels of liver enzymes (AST, ALT and ALP), total bilirubin and increased serum total protein when compared to the control positive group. As well, oxidative stress markers (MDA, GSH, GPx, SOD and CAT) were significantly improved as a consequence of treatment with *Calendula officinalis* flower extract versus the control positive group. Histopathological examination of liver tissue section of rats orally given COFE prior inducing hepatotoxicity by CCL₄ showed alleviation of histological degenerative changes as compared to control positive group. It can be concluded that *Calendula officinalis* flower extract has strong hypolipidemic, hepatoprotective and antioxidant effects. Hepatoprotective activity of *Calendula officinalis* flower extract could be due to presence of many phenolic compounds detected in this study.

**Key words:** *Calendula officinalis* · Hepatoprotective activity · Phenolic compounds · Oxidative stress · Carbon tetrachloride (CCL₄)

**INTRODUCTION**

Liver is one of the largest organs in human body and the chief site for intense metabolism and excretion. So it has a key role in the maintenance, performance and regulation of body homeostasis. It is involved with almost all the biochemical pathways of growth, fight against disease, nutrient supply, energy provision and reproduction [1]. Liver regulates the synthesis and secretion of bile. In addition, to the detoxification of various xenobiotic [2]. Toxic injury occurs in the liver more often than other organs, because all ingested substances that are absorbed, first presented to the liver and then the liver is responsible for the metabolism and elimination of many toxic substances [3]. Thus, liver diseases are some of the fatal disease in the world today.
Carbon tetrachloride (CCl₄), a potent environmental hepatotoxin, has been served as a model compound for study of hepatotoxicity and the cellular mechanisms behind oxidative damage. The principle causes of CCl₄-induced liver injury is the oxidative stress induced by free radical derivatives of CCl₄ [4, 5]. Modern medicines have little to offer for alleviation of hepatic diseases and it is chiefly the plant based preparations which are employed for their treatment of liver disorders. However, there is not much drugs available for the treatment of liver disorders Therefore, many folk remedies from plant origin are tested for their potential antioxidant and hepatoprotective liver damage in experimental animal model [6].

Calendula officinalis Linn (Asteraceae), commonly known as “African marigold” related to family Compositae is an aromatic, erect, annual herb that grows up to 60 cm in height with angular and glandular stems leaves 2.5-7.5 cm long flower-heads terminal, heterogamous, light yellow to deep orange cultivated commonly in North America, Balkans, Eastern Europe, Germany, India and Egypt [7,8]. 

Calendula officinalis is a widely used plant in Egypt. Its leaves are very rich in vitamins and minerals; it is used as antiseptic, muscular pain, piles and applied to boils and carbuncles. The fresh petals are chopped and added to salads, however the dried petals have a more concentrated flavor and used as a seasoning in soups, cakes. In addition the petals offers an edible yellow dye used as a saffron substitute to color and flavor rice, soups etc [9].

Calendula officinalis has a long history of usage by the folk systems because of its rich medicinal values that have been reported to possess potent anti-inflammatory, antitumour, antioxidant, antibacterial, anti-ulcer and chemo protective properties [10, 11]. This plant is rich in many pharmaceutical active ingredients such as carotenoids, volatile oil, amino acid, calenduline and oleanolic acid glycosides, flavonoids, sterol glycosides, alpha-and beta-amin, taraxasterol, lupeol, brein, faradiol, amidol, erythrodiol, calenduladiol, colloidiol and manilladiol [12-15]. The extract of this plant as well as pure compound isolated from it, have been demonstrated to possess multiple pharmacological activities such as anti-HIV, cytotoxic, anti-inflammatory, hepatoprotective and spasmolytic amongst others [16]. Therefore, the present study aimed to indentify and quantify phenolic compounds of Calendula officinalis flower and to evaluate the hepatoprotective and antioxidant effects of Calendula officinalis flower extract against hepatotoxicity–induced by CCl₄ in rats.

Materials and Methods

Materials

Calendula Officinalis Linn: Dried flower of Calendula officinalis Linn (Asteraceae) was purchased from a local market of Agricultural Herbs and Medicinal Plants, Cairo, Egypt. Flowers were finely grinded using a mechanical grinder into a fine powder till used for both determination of phenolic compounds content and for preparation of alcohol extract.

Rats and Basal Diet: Forty two adult male rats of Sprague Dawley strain weighing 200±5g body weight were obtained from the Laboratory Animals Farm, Helwan, Egypt. Basal diet constituents (Casein, cellulose, vitamin mixture, mineral mixture and choline chloride) were purchased from El-Gomhorya Company for Pharmaceutical and Chemical, Cairo, Egypt.

Chemicals and Drug

Carbon Tetrachloride: Carbon tetrachloride (CCl₄) was purchased from El Gomhorya Co., Egypt in the form of 40% liquid dispensed in 1 liter plastic bottles. All other chemicals used in the study were of the highest analytical grade.

Kits for Biochemical Analysis: All kits were purchased from the Gamma Trade Company for Pharmaceutical and Chemicals, Dokki, Egypt.

Silymarin Tablets: Silymarin (Chinese medication) was obtained from Sedico Co. Egypt (Chinese origin).

Methods

Extraction of Calendula officinalis Linn. Flowers: The fine powder of whole flower of Calendula officinalis (200g) was packed in high quality filter paper and then subjected to successive extraction in a Soxhlet apparatus. The methanol extract was prepared by soaking 200 g of fine powder in 1 liter of 90% ethyl alcohol with daily shaking for 5 days and kept in a refrigerator. The ethanol was evaporated using a Rotatory evaporator apparatus (manufactured in Russia) attached with a vacuum pump. Twenty grams of either extract (semisolid) were suspended in 100 ml distilled water with 2 ml of Tween 80 (suspending agent) to prepare a 20% alcoholic extract [17].
Determination of Phenolic Compounds: The ethanolic extract of polyphenolic compounds were fractionated, identified and determined by HPLC according to Goupy et al. [18].

Preparation of the Basal Diet: Basal diet was prepared according to Reeves et al. [19]. It consists of 20% protein (casein), 10% sucrose, 4% corn oil, 0.2% chlorine chloride, 1% vitamin mixture, 3.5% salt mixture, 5% fibers (cellulose) and the remainder was corn starch up to 100%.

Induction of Hepatotoxicity: All animals, except normal control group, were injected subcutaneously by a single dose of CCl\textsubscript{4} (2 ml/kg b.wt) at the last day of experiment to induce acute hepatotoxicity according to the method described by Sundaresan and Subramanian [20].

Experimental Design: All animals were housed at a controlled room temperature of 23±1°C; 55% humidity and under a 12 h light/12h dark schedule. The animals were fed on basal diet and water was provided ad libitum for one week before starting of the experiment for acclimatization. After one week adaptation period, the rats were randomly distributed into 6 equal groups (7 rats each):

Group 1: Was fed on basal diet and kept as a negative control group (normal rats).

Group 2: (Hepatotoxic group) rats injected subcutaneously by a single dose of CCl\textsubscript{4} (2 ml/kg b.wt) at the last day of experiment to induce acute hepatotoxicity [20].

Group 3: Orally given Silymarin in a dose of 200 mg/kg b.wt daily for 4 weeks followed by injection subcutaneously by a single dose of CCl\textsubscript{4} (2 ml/kg b.wt) at the last day of the experiment (standard group).

Groups 4, 5 and 6: were orally given \textit{calendula officinalis} extract at doses of 200, 400 and 600 mg/kg b.wt, respectively towards the end of the experiment period rats were injected subcutaneously with CCl\textsubscript{4} (2ml/kg b.wt). After 24hrs of CCl\textsubscript{4} injection all animals were sacrificed, blood was collected to separate serum for biochemical analysis. Liver was excised out, washed in ice cold saline and small portion was fixed in 10% formalin for histopathological analysis and the other portion was frozen for preparation of liver hemogenate.

Biochemical Analysis: Triglycerides (TG) was determined according to the method described by Trinder [21], total cholesterol (TC) was determined according to the method described by Allain et al. [22] and high density lipoprotein cholesterol (HDL-C) concentration was determined according to the method described by Lopes-Virella et al.[23]. Low density lipoprotein cholesterol (LDL-C) concentration was calculated by using formula of Friedwald et al. [24]:

\[
\text{LDL-Cholesterol} = \text{Total cholesterol} - (\text{HDL-C} + \frac{\text{TG}}{5})
\]

Activities of serum liver enzymes aspartate and alanine aminotransferases and alkaline phosphatase (AST, ALT and ALP) were chemically estimated according to Bergmeyer et al. [25]. Total protein (TP), albumin, total bilirubin (Tbil) were chemically determined as described by Burtis et al. [26]. Liver homogenate was used for determination of tissue lipid peroxide (MDA), enzymatic (GPx, SOD and CAT) and non enzymatic (GSH) antioxidants. Malondialdehyde was determined according to Ohkawa et al. [27]. The reduced glutathione (GSH) content in liver homogenate was determined colorimetrically by the method modified by Vaziri et al. [28]. Activities of glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) antioxidant enzymes were determined chemically according to Paglia and Valentaine [29], Spitz and Oberley [30] and Sinha [31], respectively.

Histopathological Examination: The fixed liver specimens were washed and dehydrated in ascending grades of alcohol. Specimens were then cleared in xylol, embedded in paraffin, sectioned at 4-6 microns thickness and stained with Haemtoxylin and Eosin stain for histopathological examination as described by Carleton [32].

Statistical Analysis: Statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS), version 20 (SPSS Inc., Chicago, IL, USA). Collected data was presented as mean± standard deviation (SD). Analysis of variance (ANOVA) test was used for determining the significances among different groups according to Armitage et al. [33]. All differences were consider significant if P < 0.05.
RESULTS

Table 1 presents the findings upon types and concentrations of polyphenolic compounds in Calendula officinalis flower extract. Data revealed that Calendula officinalis flower extract has salicylic acid, hydroquinone, resorcinol, phenol, ferulic acid, o-coumaric acid, P-coumaric acid and cinnamonic acid which were at concentrations of 12.036, 8.532, 7.523, 4.221, 3.232, 3.089, 1.328 and 0.934, respectively. Salicylic acid and hydroquinon were the most abundant phenolic compounds, the moderate abundant were ferulic acid and o-coumaric acid, but the lowest abundant was Cinnamic acid.

<table>
<thead>
<tr>
<th>Phenolic compounds (mg/100 g)</th>
<th>Concentrations (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylic acid</td>
<td>12.036</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>8.532</td>
</tr>
<tr>
<td>Resorcinol</td>
<td>7.523</td>
</tr>
<tr>
<td>Phenol</td>
<td>4.221</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>3.232</td>
</tr>
<tr>
<td>o-coumaric acid</td>
<td>3.089</td>
</tr>
<tr>
<td>P-coumaric acid</td>
<td>1.328</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>0.934</td>
</tr>
</tbody>
</table>

Table 2: Hepatoprotective effect of oral administration of calendula officinal flower extract on serum levels of total cholesterol (TC), (TG), HDL-c and LDL-c in rats injected S/C with CCl4 in the last day of the experimental period

<table>
<thead>
<tr>
<th>Groups</th>
<th>TC (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>LDL (mg/dl)</th>
<th>HDL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (-ve)</td>
<td>84.57±4.59</td>
<td>40.25±2.31</td>
<td>28.51±1.2</td>
<td>48.01±2.90</td>
</tr>
<tr>
<td>Positive control (+ve)</td>
<td>183.59±4.14</td>
<td>120.35±2.25</td>
<td>126.85±0.05</td>
<td>32.67±3.64</td>
</tr>
<tr>
<td>Silymarin + CCl4</td>
<td>86.32±1.32</td>
<td>40.98±1.41</td>
<td>30.13±0.02</td>
<td>47.99±1.01</td>
</tr>
<tr>
<td>Calendula officinalis at (200ml/kg b.wt) + CCl4</td>
<td>162.82±3.13</td>
<td>105.64±1.11</td>
<td>105.35±0.06</td>
<td>55.29±5.46</td>
</tr>
<tr>
<td>Calendula officinalis at (400ml/kg b.wt) + CCl4</td>
<td>130.35±1.17</td>
<td>74.91±3.51</td>
<td>75.16±0.06</td>
<td>55.29±5.46</td>
</tr>
<tr>
<td>Calendula officinalis at (600ml/kg b.wt) + CCl4</td>
<td>89.52±1.97</td>
<td>43.15±2.73</td>
<td>47.99±1.01</td>
<td>55.29±5.46</td>
</tr>
</tbody>
</table>

Table 1 presents the findings upon types and concentrations of polyphenolic compounds in Calendula officinalis flower extract. Data revealed that Calendula officinalis flower extract has salicylic acid, hydroquinone, resorcinol, phenol, ferulic acid, o-coumaric acid, P-coumaric acid and cinnamonic acid which were at concentrations of 12.036, 8.532, 7.523, 4.221, 3.232, 3.089, 1.328 and 0.934, respectively. Salicylic acid and hydroquinon were the most abundant phenolic compounds, the moderate abundant were ferulic acid and o-coumaric acid, but the lowest abundant was Cinnamic acid. As shown in Table 2, rats injected subcutaneously with CCl4 induced significant increase (P<0.05) in serum levels of total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL) parallel with significant decrease in HDL-c level (183.59±4.14, 120.35±2.25, 126.85±0.05 and 32.67±3.64 mg/dl, respectively) when compared to the negative control group (84.57±4.59, 40.25±2.31, 28.51±1.22 mg/dl and 48.01±2.90 mg/dl, respectively) or with standard group (8.95±1.32, 4.43±0.02 and 5.53±1.78 g/dl, respectively). Pretreatment with Calendula officinalis (200, 400 and 600 mg/kg) to rats intoxicated with a single subcutaneous injection of CCl4 at the last day of the experimental period caused a significant decrease (P<0.05) in the elevated serum marker levels of AST, ALT, ALP, when compared to the hepatotoxic rats (control +v group). Intoxicated rats pre-treated with the large dose (600 mg/kg b.wt) of Calendula officinalis flower extract caused the highest reduction of hepatotoxicity in the elevated serum liver enzymes AST, ALT and ALP enzymes (60.52±1.78, 55.29±0.46 and 55.29±0.46 U/L, respectively) compared to CCl4 intoxicated group (+ve group).

Results presented in Table 4 explained that a single subcutaneous injection of CCl4 to male rats at the last day of the experimental period (control positive group) induced a significant liver damage which observed from a significant decrease (P<0.05) in both total protein (TP) and albumin and increase in the level of serum total bilirubin (5.31±1.2, 2.99±0.12 and 9.42±1.76 g/dl, respectively) compared with control negative group (8.66 ±1.73, 4.15±0.32 and 6.17±1.94 g/dl, respectively). Rats orally given Calendula officinalis flower extract caused the highest protection was observed in both intoxicated rats pretreated with Silymarin (8.66 ±1.73, 4.15±0.32 and 6.17±1.94 g/dl, respectively) and the group of intoxicated rats pretreated with silymarin and high dose of alcoholic extract of Calendula officinalis flower showed the best effect near to the normal group.

From data presented in Table 3, it could be noticed that rats acutely intoxicated by a single subcutaneous injection of CCl4 at the last day of the experimental period had significant (P<0.05) increase (P<0.05) in the serum activities of AST, ALT and ALP enzymes (140.22±0.21, 120.81±0.22 and 133.53±1.55 U/L, respectively) as compared to control (-ve) group (52.22±1.36, 49.04±0.36 and 59.72±8.90 U/L, respectively) or with standard group (53.45±2.44, 51.01±0.13 and 59.01±1.34). Administration of Calendula officinalis flower extract at (200, 400 and 600 mg/kg b.wt) for four weeks before subcutaneous injection of CCl4 induced significant decrease (P<0.05) in all the elevated serum marker levels of AST, ALT, ALP, when compared to the hepatotoxic rats (control +v group). Intoxicated rats pre-treated with the large dose (600 mg/kg b.wt) of Calendula officinalis flower extract caused the highest reduction of hepatotoxicity in the elevated serum liver enzymes AST, ALT and ALP enzymes (60.52±1.78, 55.29±0.46 and 55.29±0.46 U/L, respectively) compared to CCl4 intoxicated group (+ve group).
Table 3: Effect of oral administration of *Calendula officinalis* flower extract on serum liver enzyme AST, ALT and ALP in rats injected S/C with CCl₄ in the last day of the experimental period

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>ALP (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (-ve)</td>
<td>52.22±1.36e</td>
<td>49.04±0.36e</td>
<td>59.77±8.90e</td>
</tr>
<tr>
<td>Positive control (+ve)</td>
<td>140.22±0.21a</td>
<td>120.81±0.22a</td>
<td>133.53±1.55a</td>
</tr>
<tr>
<td>Silymarin + CCl₄</td>
<td>53.45±2.44e</td>
<td>51.01±0.13de</td>
<td>59.01±1.34e</td>
</tr>
<tr>
<td><em>Calendula officinalis</em> at (200ml/kg b.wt) + CCl₄</td>
<td>104.23±4.76b</td>
<td>100.15±0.48b</td>
<td>102.21±2.78b</td>
</tr>
<tr>
<td><em>Calendula officinalis</em> at (400ml/kg b.wt) + CCl₄</td>
<td>79.58±3.43c</td>
<td>69.37±0.33c</td>
<td>80.45±14.91c</td>
</tr>
<tr>
<td><em>Calendula officinalis</em> (600ml/kg b.wt) + CCl₄</td>
<td>60.52±1.78d</td>
<td>55.29±0.46d</td>
<td>66.64±1.51d</td>
</tr>
</tbody>
</table>

Data are presented as means ± standard deviation, (n = 7 for each group). Values with different superscripts within the column are significantly different at P< 0.05. Values with similar or partially similar superscripts are non-significant.

Table 4: Effect of oral administration of *Calendula officinalis* alcoholic extract on total protein, total bilirubin and albumin in rats injected S/C with CCl₄ in the last day of the experimental period

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total protein (g/dl)</th>
<th>Albumin (g/dl)</th>
<th>Total bilirubin (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (-ve)</td>
<td>8.95±1.32a</td>
<td>4.43±0.02a</td>
<td>5.53±1.78d</td>
</tr>
<tr>
<td>Positive control (+ve)</td>
<td>5.31±1.2d</td>
<td>2.99±0.12c</td>
<td>9.42±1.76a</td>
</tr>
<tr>
<td>Silymarin + CCl₄</td>
<td>8.66±1.73a</td>
<td>4.15±0.32b</td>
<td>6.17±1.94c</td>
</tr>
<tr>
<td><em>Calendula officinalis</em> at (200ml/kg b.wt) + CCl₄</td>
<td>7.12±11.15b</td>
<td>3.17±0.04d</td>
<td>7.99±0.83b</td>
</tr>
<tr>
<td><em>Calendula officinalis</em> at (400ml/kg b.wt) + CCl₄</td>
<td>7.61±10.01b</td>
<td>3.35±0.04b</td>
<td>7.32±0.74b</td>
</tr>
<tr>
<td><em>Calendula officinalis</em> (600ml/kg b.wt) + CCl₄</td>
<td>8.17±15.31a</td>
<td>3.93±0.11b</td>
<td>6.31±1.45c</td>
</tr>
</tbody>
</table>

Data are presented as means ± standard deviation, (n = 7 for each group). Values with different superscripts within the column are significantly different at P< 0.05. Values with similar or partially similar superscripts are non-significant.

Table 5: Effect of oral administration of *Calendula officinalis* flower extracts on GPX, SOD, CAT,GSH and MDA in rats injected S/C with CCl₄ in the last day of the experimental period

<table>
<thead>
<tr>
<th>Groups</th>
<th>GPX (U/mg)</th>
<th>SOD (U/mg)</th>
<th>CAT (U/mg)</th>
<th>MDA (µmol/dl)</th>
<th>GSH (µmol/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (-ve)</td>
<td>53.44±3.33a</td>
<td>85.22±1.25a</td>
<td>74.35±0.97a</td>
<td>10.55±1.02d</td>
<td>4.72±0.02a</td>
</tr>
<tr>
<td>Positive control (+ve)</td>
<td>25.19±1.33d</td>
<td>43.43±1.81e</td>
<td>35.54±1.76c</td>
<td>21.99±1.01a</td>
<td>2.54±0.01d</td>
</tr>
<tr>
<td>Silymarin + CCl₄</td>
<td>50.02±2.31a</td>
<td>85.55±1.32a</td>
<td>73.15±1.01a</td>
<td>13.61±1.02cd</td>
<td>4.32±0.03a</td>
</tr>
<tr>
<td><em>Calendula officinalis</em> at (200ml/kg b.wt) + CCl₄</td>
<td>42.42±1.12c</td>
<td>56.18±1.43d</td>
<td>35.45±2.16d</td>
<td>18.02±1.04b</td>
<td>3.16±0.17bc</td>
</tr>
<tr>
<td><em>Calendula officinalis</em> at (400ml/kg b.wt) + CCl₄</td>
<td>42.33±1.16c</td>
<td>68.25±1.32c</td>
<td>49.01±1.36c</td>
<td>17.92±1.02bc</td>
<td>3.75±0.10b</td>
</tr>
<tr>
<td><em>Calendula officinalis</em> (600ml/kg b.wt) + CCl₄</td>
<td>45.15±4.23ab</td>
<td>80.75±2.11b</td>
<td>71.80±2.31ab</td>
<td>15.42±1.04c</td>
<td>4.34±0.01a</td>
</tr>
</tbody>
</table>

Data are presented as means ± standard deviation, (n = 7 for each group). Values with different superscripts within the column are significantly different at P< 0.05. Values with similar or partially similar superscripts are non-significant.

With *Calendula officinalis* flower extract in a dose of 600 mg/kg b.wt (8.17±15.51, 3.93±0.11and 6.31±1.45 g/dl, respectively) compared with control positive group (+ve) (5.31±1.2, 2.99±0.12 and 9.42±1.76 g/dl, respectively).

Data illustrated in Table 5 showed that rats subcutaneously injected with a single dose of CCl₄ at the last day of the experimental period (+ve) had significant decrease in antioxidant enzymes activity (glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT)) and also in non enzymatic (GSH) antioxidant system in liver tissue and enhanced the end product of lipid peroxidation (MDA) level in liver tissues at the last day of the experimental period (control positive group) (Fig.1b). Examined liver sections of rats orally administrated with different doses of *Calendula officinalis* flower extract showed recovering of hepatocyte. The pre treated with *Calendula officinalis* extract at 200 mg/kg b.wt, with the elevated (MDA) levels were found to be reduced back towards the normal level in pre treated rats given the highest dose of *Calendula officinalis* flower extract as well as intoxicated rats pretreated with Silymarin.

**Histopathological Examination:** Histopathological examination showed no histological change in the liver structure of normal control rats (Fig. 1a). Hepatic intense centrilobular, necrosis, vacuolization and macro vesicular fatty changes were observed in the liver tissue sections of rats subcutaneously injected with a single dose of CCl₄ at the last day of the experimental period (control positive group) (Fig.1b). Examined liver sections of rats orally given standard drug, Silymarin and intoxicated with CCl₄ at the last day of the experimental period showed normal hepatocyte (Fig.1c). The intoxicated animals pretreated with different doses of *Calendula officinalis* flower extract showed recovering of hepatocyte. The pre treated with *Calendula officinalis* extract at 200 mg/kg b.wt,
Fig. 1: (a) Liver tissue of the control animal showing normal histology (control negative group) (b) intoxicated rats injected with CCl₄ at the last day of the experimental period (control positive group) showing necrosis, central vein (V) and fatty vacuole (c) Pre treated group with Silymarin intoxicated with CCl₄ at the last day of the experimental period showing normal hepatocytes (arrow mark) with central vein (d) intoxicated rats pretreated with alcoholic extract in a dose of 200 mg/kg showing a moderate number of recovered hepatocytes with a small amount of necrosis, vacuolization and macrovesicular fatty changes (e) minimal inflammation and near-normal architecture possessing higher hepatoprotective activity were shown in intoxicated rats pretreated with alcoholic extract in a dose of 400 mg/kg (f) Significant liver protection was observed in intoxicated rats pretreated with alcoholic extract in a dose of 600 mg/kg, as evident by the presence of normal hepatic cords, absence of necrosis and fatty infiltration, supplementing the protective effect showed a moderate number of recovered hepatocyte with a small amount of necrosis, vacuolization and macrovesicular fatty changes (Fig. 1d). While, the 400 mg/kg b.wt treated group, showed minimal inflammation and near-normal architecture possessing higher hepatoprotective activity (Fig.1e). The liver sections of the intoxicated animals pretreated with calendula extract at 600 mg/kg b.wt, exhibited significant liver protection against CCl₄, as evident by the presence of normal hepatic cords, absence of necrosis and fatty infiltration, supplementing the protective effect (Fig.1f).
DISCUSSION

The present study achieved to indentify types and concentration of phenolic compounds in Calendula officinalis flower. In addition to investigate the hepatoprotective and the antioxidant effects of Calendula officinalis flower extract against carbon tetrachloride (CCL₄)-induced liver damage in rats. The observed finding revealed that phenolic compounds in Calendula officinalis flower, were salicylic acid, hydroquinone, resorcinol, phenol, ferulic acid, o-coumaric acid, P-coumaric acid and cinnamic acid. These results are in accordance with the results reported by Khalid et al. [34], who demonstrated that salicylic acid was the main phenolic compound identified in Calendula officinalis followed by Resorcinol. The liver is the major site for the synthesis and metabolism of cholesterol, bile acids and phospholipids [3]. Distinct alterations in lipid metabolism have been reported in CCL₄-induced hepatotoxicity in rats [8]. In the current study, the results revealed that intoxicated rats with CCL₄ resulted in significant increase in serum level of total cholesterol (TC), triglycerides (TG), low density lipoprotein cholesterol (LDL-c) accompanied with a significant decrease in high density lipoprotein cholesterol (HDL-c) level as compared to the negative control group. Our results are in agreement with those obtained by El-Habibi et al.[35], who reported that there was an increase in the levels of cholesterol, triglycerides and free fatty acids in plasma and tissues of rats intoxicated with CCL₄. These results might be due to an increase in the synthesis of fatty acids and triglycerides from acetate which is responsible for the transport of acetate into the liver cell, resulting in increased substrate (acetate) availability or it could be due to increase the synthesis of cholesterol [36].

The effect of flavonoids and flavonoid rich extracts on reducing lipid levels effectively has been reported by Anila and Vijayalakshmi [37]. Silymarin, a flavonolignan that has been widely used to treat liver disorders, including acute and chronic viral hepatitis, toxin/drug-induced hepatitis and cirrhosis/alcoholic liver diseases. In the present study intoxicated rats pretreated with Silymarin significantly reduced serum total cholesterol, LDL-cholesterol and triglycerides with elevation of HDL. These results are in agreement with those reported by Vaughu [38]. In our study pretreatment of rats with Calendula officinalis flower extract resulted in significant improvement in the tested lipid profile parameters, that could be attributed to an increase in the inhibition of intestinal absorption of cholesterol, interference with lipoprotein production increased expression of hepatic LDL receptor and their protection, leading to an increased removal of LDL-c from the blood and its increased degradation and catabolism of cholesterol from the body. All these events either individually or in combination lead to decrease in serum LDL-c levels, which reduced serum total cholesterol level during the pretreatment[39,40]. Liver injury induced by CCL₄ is the best characterized system of xenobiotic-induced hepatotoxicity and is commonly used models for the screening of anti-hepatotoxic and/or hepatoprotective activities of drugs. The changes associated with CCL₄ induced liver damage are similar to that of acute viral hepatitis [41]. Carbon tetrachloride accumulates in hepatic parenchymal cells and metabolized by cytochrome P-450 enzyme and its metabolic product; trichloromethyl free radicals (CCl₃). These free radicals are highly reactive; alkylate cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids in the presence of oxygen to produce lipid peroxides, leading to liver damage. Lipid peroxidation will initiate pathological changes such as depression of protein synthesis [42].

Assessment of liver function can be performed by determining the activity of serum enzymes AST, ALT and ALP, originally present in high concentrations in the cytoplasm. When there is hepatic injury, these enzymes leak into the blood stream inconformity with the extent of liver damage [43,44]. Total bilirubin (TBil) and total protein (TP) levels on other hand are related to the function of hepatic cell [45]. In the present study, the hepatotoxicity of CCL₄ in rats was confirmed by a significant elevation of AST, ALT, ALP and total bilirubin. In addition, CC1₄ intoxication produced a significant reduction in serum total protein level. This may be due to release of these enzymes from the cytoplasm into the blood rapidly after cellular damage and a reduction in hepatic protein synthesis. Liu et al. [46] reported that elevation of AST, ALT and ALP in response to CCL₄ could be attributed to hepatic structural damage because these enzymes are normally localized to the cytoplasm and are released into the circulation after cellular damage has occurred. Histopathological observations of the liver of CCL₄-administered rats revealed the presence of hepatic intense centrilobular, necrosis, vacuolization and macro vesicular fatty changes. These results were in harmony with the previous results reported by Zalatnai et al. [47] and Candasamy [48].

The presence of phytoconstituents such as flavonoids like quercetin, protocatechuic acid, triterpinoids like faradiol, oleanolic acid, beta-amyrin
Calendula officinalis flower has been reported to elicit a protective response against the toxic manifestations of chemicals, particularly those involving oxidative stress. Fonseca et al. [62] and Preethi et al. [63] reported that, oral administration of Calendula officinalis alcoholic extract inhibited superoxide generation in macrophages in rats. Calendula officinalis significantly increased both of catalase (CTA) and glutathione reductase levels in blood and liver, whereas glutathione peroxidase was found to be decreased. Butanol and water extracts of Calendula officinalis containing flavonoids, showed antioxidant activity based on analysis of plasma and urine malondialdehyde (MDA) and urine isoprostane inventrations as demonstrated by Popovic et al.[64] and Frankic et al.[65]. The biochemical results of our study were confirmed by histopathological findings, which seen in liver sections. The histological findings of liver tissue sections of pre treated rats with calendula official showed almost normal structure with mild fibroblastic proliferation and sporadic cell necrosis. Oval cell hyperplasia in the portal area was very clear and necrosis was more reduced than CCl4-intoxicated rats this may be explained by antioxidant activity of calendula official is that may be attributed to its constituents of phytochemical. These histological findings agree with the study of Khan and Ahmed [66], who reported that the oxidative damage to tissue and their cellular component can be prevented by certain antioxidant metabolites present in plants.

**CONCLUSION**

Calendula officinalis is effectively improved liver functions and protected against liver tissues damage induced by toxic substances. Calendula officinalis has protective effect against the loss of antioxidant activities as result of oxidative process caused by CCl4 injection due to its phytochemicals compounds (Phenolics and flavonoids). This protective activity of Calendula officinalis suggests that regular consumption of food containing phenolics and flavonoids may protect against...
liver disease and imbalanced antioxidant. Thus, the possibility that Calendula officinalis reduce the risk of liver disease and oxidation process remains open and further longitudinal studies are needed to confirm the importance of it in the prevention of liver disease.

REFERENCES


