AJWA DATES AS A PROTECTIVE AGENT AGAINST LIVER TOXICITY IN RAT

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Abstract
Background: Liver toxicity is a worldwide clinical problem caused by a variety of causes. It mostly ends by hepatic fibrosis and or cirrhosis. Materials and methods: Ajwa date extract was prepared from Almadinah AlNawiah ajwa and was given to the animals by gastric gavage at a dose of 1gm/kg/day. CCl4 was dissolved oil at 1: 1 percent and was injected intraperitonealy at a dose of 1.2 ml/ kg of body weight 3 times a week. Fourty adult male rats were divided into five groups. G1 - control group. G2 received CCL4 for 4 weeks. G3 received CCL4 for 12 weeks. G4 received CCL4 and ADE for 4 weeks. G5 received CCL4 and ADE for 12 weeks. Animals were sacrificed and blood samples were collected for SGPT, SGOT and albumin measurements. Liver samples were stained and subjected to image analysis to assess the area percentage of fibrosis. Results: Biochemical analysis showed that ADE treated groups showed a significant lower level of SGPT and SGOT compared to CCL4 treated groups. Light microscopic examination showed that the ADE treated groups showed a decrease in the histological alterations induced by CCL4. The area occupied by collagen fibers was significantly decreased in ADE treated groups. These effects may be due to the rich vitamins and antioxidants in the extract. Conclusion: This study highlights the interest to change toward the use of natural medicinal plants with antioxidant activity for protection against diseases. This may provide a scientific base for the conventional use of ADE as a nutritional protocol of management.

Keywords: Ajwa, dates, liver toxicity, prophetic medicine, complementary, alternative, fibrosis, cirrhosis, area percentage

Introduction
Liver toxicity is a worldwide clinical problem that may presents with acute or chronic hepatitis, cirrhosis, or fulminant liver failure. The onset is usually insidious with long latent period between disease occurrence and detection; and most patients remain asymptomatic until they develop hepatic decompensation (Brewer et al.,1999). Chronic liver injury of any cause eventually ends in healing by scarring healing response that results in hepatic fibrosis.
Underlying etiology include biliary obstruction, helminthic infection, chronic ethanol consumption, autoimmune disorders, drug-induced, iron or copper overload (Friedman, 2003). Hepatic fibrosis affects millions of patients worldwide and if left untreated, fibrosis can progress to cirrhosis, ultimately leading to liver failure and possible death (Tsukada et al., 2006). To avoid liver fibrosis, eradication of continuing liver injury is required. This process depends on reduction of activated myofibroblasts (reversion of activation and/or apoptosis) and degradation of pathologic extracellular matrix (Murphy et al., 2002). Carbon tetrachloride (CCl4) is one of the most used hepatic toxins for laboratory experimental induction of liver fibrosis and cirrhosis (Zasshi, Y. 2006; Tsuchiya et al., 2007). It is mostly used to study liver injury induced by free radicals in animal models. Liver toxicity caused by it is closely analogue to hepatotoxicity in human (Ko and Lim 2006).

Ajwa date is a special luxury type of dates with black color, soft and delightfully fine taste. It is only cultivated at Almadina AlNabawiah in Saudi Arabia. It is the fruits of the female tree date palm (Phoenix dactylifera L.). Its characteristic contents of high percentage of carbohydrates, dietary fibers, fats, proteins, vitamins and minerals gave it the superiority over other types of dates (Al-Shahib and Marshall, 2003; Abdu, 2011). Experimental studies proofed that Ajwa date extract (ADE) have strong antioxidants (Al-Farsi et al., 2005; Chaira et al., 2009; Ragab et al., 2013), anticancer (Ishurd et al., 2004) and antiviral (Vayalil, 2002) activities. These activities were attributed to the high contents of polyphenols, flavonoids, and flavones present in the ADE which helps in free radical scavenging. However, studies on its protective effect on liver toxicity are scarcely presented (Abdu, 2011).

Main Text

Aim: This study aims to investigate the protective effect of ADE against hepatotoxicity induced by CCl4.

Materials and methods

Chemicals: CCL4 dissolved in corn oil at 1: 1 percent was injected intraperitonealy at a dose of 1.2 ml/ kg of body weight 3 times a week.

Dates: Ajwa date (Phoenix dactylifera L.) fruits were obtained from Almadina AlNabawiah, Saudi Arabia. Flesh of the fruits was left in distilled water (1:3) for 48 hours in 4°C (Al-Qarawi et al., 2005). The whole solution was grinded, then centrifuged at 4°C for 20 min at 4000 rpm. The supernatant was collected and stored at -80°C till use (Vayalil, 2002). Animals were given the extract at a dose of 1g/kg/day by gastric gavages, which is equivalent to 7 dates per person per day (Abdu, 2011). The starting point for administration of both CCL4 and ADE was the same.

Animals: Fourty adult male Wistar rats (Rattus norvegicus) (240 gm to 306 gm body weight) were obtained from the Experimental Animal House Center, King Abdul-Aziz University, Jeddah, Saudi Arabia. Rats were housed in polyethylene cages (5 rats/cage) with stainless steel wire tops and were allowed commercial standard diet and water ad-libitum. Rats were housed under standard laboratory conditions (room temperature 22 ± 2 0C°, humidity 55 ± 5L, 12 hours light/dark cycle). Standard care methods were used to maintain the animals healthy and free of infections.

Experimental groups: Animals were divided randomly into five groups (eight rats each) orally treated by gastric gavage for four weeks (5 days/week) as follows: Rats of the control group (G1) received ordinary diet; rats of the second group (G2) received CCl4 intraperitoneally for four weeks, the third group (G3) received CCl4 for twelve weeks. The fourth group (G4) received CCl4 and ADE for four weeks and the fifth group (G5) received CCl4 and ADE for twelve weeks. Animals were anaesthetized by diethyl ether inhalation and sacrificed and samples were collected at the 4th and 12th weeks of treatment (four animals each time).
Biochemical analysis. Blood samples were collected from the orbital sinus using a fine-walled Pasteur tubes. Serum SGPT, SGOT and albumin were measured using the commercially available kits. Image analysis of the area occupied by collagen fibers. Quantitative assessment of liver fibrosis was performed with morphometry on sections processed with Masson’s trichrome stain, which specifically stains collagen fibers (James et al. 1986). The data were obtained using Leica Qwin 500 image analyser computer system (England). The image analyzer was first calibrated automatically to convert the measurement unites (Pixels) produced by the image analyser program into actual micrometer units. Using the measurement menu (the area, area %) and standard measuring frame of a standard area equal to 763882 µm² were chosen from the parameters. In the chosen field the Masson’s trichrome stained areas enclosed inside the standard measuring frame were measured. These measurements were done using an objective lens of magnification 4. The % of the fibrosis area over the whole observed field was assessed to represent the degree of hepatic fibrosis. Several readings were obtained in each specimen (6 sides per animal and at least ten random fields was measured in each slide) (Muller et al. 1988).

Statistical analysis: The data were analyzed by using the SAS software package (SAS, 1999). ANOVA analyses were used to compare the mean of the studied variables (SGPT, SGOT and albumin) among the studied five groups. The level of statistical significance was defined as (P ≤ 0.05) highly significant at (p< 0.01) and very highly significant at (p< 0.001). Scheffe grouping was used to detect the individual significant difference among the studied groups. Box plot was also depicted to show and compare the central tendency and dispersion measures among all the studied groups.

Light Microscopy: The abdominal wall was dissected. Liver samples were removed from each rat and fixed in 10% neutral buffered formalin solution then processed up to paraffin blocks. Tissue sections were cut at 3-5µm and stained with haematoxylin and eosin (H & E) and Mallory stain (Bancroft, 2007).

Results:

Biochemical analysis: The relations between SGPT, SGOT and Albumin mean values are shown in Table (1) and Figs. 1a, b, and c.

<table>
<thead>
<tr>
<th>Studied factor</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>G5</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGPT</td>
<td>39.5 ± 3.4</td>
<td>84.2 ± 6.7</td>
<td>166.4 ± 7.3</td>
<td>73.2 ± 5.3</td>
<td>56.7 ± 7.1</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>SGOT</td>
<td>68.7 ± 2.7</td>
<td>133.2 ± 13.3</td>
<td>235.6 ± 22.0</td>
<td>112.8 ± 13.9</td>
<td>84.4 ± 6.5</td>
<td>&lt;.0001**</td>
</tr>
<tr>
<td>Albumin</td>
<td>3.7 ± 0.3</td>
<td>3.5 ± 0.4</td>
<td>2.4 ± 0.2</td>
<td>3.02 ± 0.2</td>
<td>2.5 ± 0.4</td>
<td>&lt;.0001***</td>
</tr>
</tbody>
</table>

Table (1): Comparison of the mean of SGPT, SGOT and albumin among the studied group of rats

*Highly significant difference between group 1 and other studied groups, but no significant difference between groups 2 and 4.

**Highly significant difference between group 1 and groups 2, 3 and 4, but no significant difference between group 2 and 4, groups 4 and 5, and groups 5 and 1.

***Highly significant difference between group 1 and groups 3, 4 and 5, but no significant difference between groups 1 and 2, groups 4 and 5, groups 5 and 3 and groups 2 and 4.

Table 1 shows the comparison of the mean of the studied variables (SGPT, SGOT and albumin) among the studied five groups of rats. There have been highly significant differences of the mean of all studied variables in the studied groups with p <.0001. For SGPT, the highest mean level was among group 3 (166.4 ± 7.3) and the lowest level was among group 1 (39.5 ± 3.4). No statistically difference, however, was observed for SGPT between groups 2 and 4. The mean SGOT level was also the highest in group 3 (235.6 ± 22.0) and lowest in group 1 (68.7 ± 2.7). It showed significant difference with groups 2, 3 and 4, but no significant difference between group 2 and 4, groups 4 and 5; and groups 5 and 1. For
albumin, its highest level was in group 1 (3.7 ± 0.3) and lowest level in group 3 (2.4 ± 0.2).

There has been significant difference between group 1 and groups 3 and 4 and 5. No significant differences, however, were observed between groups 1 and 2, groups 4 and 5, groups 5 and 3 and groups 2 and 4. The control group showed the lowest level of both SGPT and SGOT and highest level of albumin. CCl4 treated group for 12 week group (G3) showed the highest level of SGPT and SGOT. It also showed the lowest level of albumin. This changes indicted severe liver affection. ADE received groups (G4 and G5) showed lower changes in the level of both enzymes (SGPT and SGOT) compared to the CCl4 only treated groups (G2 and G3).

Figure 1a: Box plot of SGPT in the studied five groups

Figure 1b: Box plot of SGOT in the studied 5 groups

Figure 1c: Box plot of albumin in the studied 5 groups
Light microscopy

Examination of liver sections from the control group at low magnification revealed normal histological appearance of hepatic tissues with normal hepatocytes arrangement in relation to the central veins and sinusoids (Figs. 2 and 7).

Liver sections from CCL4-alone treated rats (Groups 2 and 3) at four weeks after treatment showed loss of general architecture structure of the hepatic pattern and dilatation of the sinusoids. The cells showed pale cytoplasm and intracellular vacuolations (Ballooning degeneration) (Fig. 3). At the end of the 12th weeks, the liver sections showed increased hepatic cellular swelling as well as cytoplasmic vacuolization and degeneration (ballooning degeneration). Moreover, loss of the general architecture structure of the hepatic pattern was also noticed compared to the controls (Figs.4). Many necrotic cells manifested by the deep eosinophilic staining quality of the cytoplasm and the smaller, condensed and intensely basophilic stained nuclei. Pyknotic nuclei and well as disappearance of the nuclei of other cells were also common.

On the other hand, the groups treated with CCL4 and ADE (Groups 4 and 5) at the end of the 4th week showed almost normal architectural structure of the hepatic tissues with distinct hepatocyte strands in many parts. Many histological alterations including dilatation of the central veins and sinusoids, ballooning degeneration and nuclear pyknosis were enormously reduced when compared to group 2. Meanwhile, some vacuolated hepatocytes and limited degenerative cells were also observed (Fig 5). At the end of the 12th week many parts of the liver sections showed normal architecture structure of the hepatic tissues with distinct hepatocyte strands. Many histological alterations including tissue congestion, dilatation of the central veins and sinusoids, necrosis and nuclear pyknosis were enormously reduced together with obvious decrease in the inflammatory changes and the extent of the lesions of the liver tissues compared to CCL4-only treated groups. Meanwhile, some vacuolated hepatocytes (Fig. 6) and limited degenerative cells were also recorded.

Masson trichrome stained sections showed the bluish stained fibrous tissue of variable amounts at 12 weeks of treatment in both group 3 and 5 being more obvious in group 3 indicating increased fibrosis (Figs. 8 and 9).
Light microscopy

Fig. 2 A photomicrograph of a section in liver of G1 rat showing the apparent normal arrangement of hepatocytes with vesicular nuclei (arrows), some cells are binucleated (B). The cells form cords around the sinusoids (S), the central vein (CV) and bile canaliculus (arrow head).
Fig. 3 Liver section of G2 rat at 4 weeks showing loss of the normal arrangement of hepatocytes with dilated sinusoids (curved arrows). Most cells have pale cytoplasm (arrows). Some cells show intracytoplasmic vacuoles (arrow heads).

Fig. 4 A photomicrograph of a section in liver of G3 rat at 12 weeks showing loss of the normal arrangement of hepatocytes with dilated sinusoids (curved arrows). Some cells shows deep eosinophilic cytoplasm with small condensed nuclei (arrows). Some cells show intracytoplasmic vacuoles (arrow heads).

Fig. 5 Liver of G4 rat at 12 weeks showing loss of the normal arrangement of hepatocytes with increased fibrosis (curved arrows). Some hepatocytes have pyknotic nuclei (arrows). Most cells show intracytoplasmic vacuoles (arrow heads).

Fig. 6 A photomicrograph of a section in liver of G5 rat at 12 weeks showing loss of the normal arrangement of hepatocytes with less amount of fibrosis (curved arrows). Some hepatocytes have eosinophilic cytoplasm and pyknotic nuclei (arrows). Most cells show intracytoplasmic vacuoles (arrow heads).

Fig. 7 A photomicrograph of a section in liver of G1 rat showing the normal arrangement of the hepatocytes in relation to the central vein (CV) and sinusoids (S). Note
the absence of blue stained fibers. (Masson trichrome stain x 100)

Fig. 8 A section in liver of G3 rat at 12 weeks showing the bluish stained fibrous tissue (curved arrows).

Fig. 9 A photomicrograph of a section in liver of G5 rat at 12 weeks showing small amount of fibrous tissue (curved arrows). Compare to G1 & G2 (Masson trichrome stain x 400)

Image analysis of the area occupied by collagen fibers:

Image analysis of Masson’s trichrome stained sections of the control rats revealed that the area occupied by collagen fibers was 3.128 ± 0.781.

The area occupied by collagen fibers was significantly increased with administration of CCL4 in group 2 (3.828 ± 0.844) compared to the control. Administration of CCl4 for 12 weeks in group 3 caused a very high significant increase in area occupied by collagen fibers (6.24 ± 0.987) as compared with the control.

ADE and CCl4 treated groups (group 4 and 5) showed high significant increase in the area occupied by collagen fibers (4.712 ± 0.797 and 4.365 ± 1.16 respectively) in comparison with the control.

Table (2):- Morphometric quantitative measurements of liver fibrosis in animal groups stained with Masson's trichrome stain.

<table>
<thead>
<tr>
<th>Group</th>
<th>Area %</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min.</td>
<td>Max.</td>
</tr>
<tr>
<td>Group 1</td>
<td>2.397</td>
<td>4.599</td>
</tr>
<tr>
<td>Group 2</td>
<td>1.181</td>
<td>3.666</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.977</td>
<td>7.214</td>
</tr>
<tr>
<td>Group 4</td>
<td>1.488</td>
<td>5.465</td>
</tr>
<tr>
<td>Group 5</td>
<td>1.045</td>
<td>4.541</td>
</tr>
</tbody>
</table>

Table 2: Area %: represent the degree of hepatic fibrosis, the results are represented as mean value± SD:-
(*) significant at (p< 0.05), (**) highly significant at (p< 0.01), (***) very highly significant at (p< 0.001).

Discussion

The presented study investigated the in-vivo protective effect of AlMadinah Alnabawiah Ajwa dates extract against hepatotoxicity induced by CCL4. Carbon tetrachloride is known to cause liver damage by inducing tissue necrosis through oxidative damage in liver cells. It is widely accepted that CCL4 causes lipid peroxidation through generating the release of reactive oxygen species which damage the mitochondrial and cytoplasmic membranes causing more severe oxidative damage in the tissues and consequently, releasing lipid hydroperoxides into circulation (Domitrovića et al., 2009).

Furthermore, protein modifications elicited by direct oxidative attack lead to the formation of protein carbonyl derivatives and protein carbonyl content (Radice, et al., 1998). This liver cell injury usual initiate activation of either resident or blood-derived phagocytes increases the steady-state concentration of fibrogenic cytokines, which recruit a large amount of fibroblasts and fibroblast-like cells for excess production of extracellular matrix.

Liver fibrosis is created not only as a consequence of the changes in the secretion of matrix, but also from changes in its degradation, which means a loss of the dynamic functional balance between fibrogensis and fibrolysis (Arthur, 2002). During the development of fibrosis, the capacity of the degradation is not eliminated, but is reduced (Batallar and Brenner, 2001). On this basis, the increase in the amount of the collagen fibers
could be the net result of two different processes formation of new fibers and degeneration of the already formed ones. With continuous and repeated injury, the formation of new fibers predominated and the degradation of the already formed fibers decreased. The terminal outcome of liver fibrosis is the formation of nodules encapsulated by fibrillar scar matrix (Tsukamoto, 1999). The progression of fibrosis observed in the CCl4 control group suggests development of an irreversible fibrosis.

Ajwa date fruits are widely consumed in the Arab world. Ajwa has been mentioned in prophetic medicine. Its strong antioxidant activity is related mainly to its rich contents of carotenoids, phenolics, melatonin and vitamins (Hoehler and Marquardt, 1996; Grosse et al., 1997; Meki and Hussein; 2001; Al-Farsi et al., 2005; Sutken et al., 2007; Chaira et al., 2009 and Abdu, 2011). Melatonin was found to be an efficient protector of DNA (Lopez-Burillo et al., 2003), protein and lipids in cellular membranes (Cuzzocrea & Reiter, 2001). It also acts as an antioxidant and suppressor of a number of endogenous and exogenous free radicals generated during cellular process (Zang et al., 1998 and Guo et al., 2003).

Looking at liver function test in the present study (Table 1), the biochemical profile showed marked increase in serum SGPT and SGOT and a marked decrease in albumin in the CCL4 intoxicated group. These results are attributed to the toxic oxidative stress of CCL4 on liver cells as shown in the histopathological results. This is in agreement with the results obtained by other investigators (Domitrovic et al., 2009). The detected significant reduction in the serum enzymes’ level by Ajwa extract could be attributed to a decrease in the lipid peroxidation of hepatocellular membrane induced by the CCL4. Furthermore, it may be attributed to the accelerated repairing and regeneration of damaged hepatocytes, this is in concurrence with other researchers (Zang et al., 1998; Cuzzocrea & Reiter, 2001; Abdu, 2011).

Histopathological evaluation showed that liver was target organ for CCL4 toxicity. Analysis of the liver sections stained with Masson’s trichrome stain revealed a progressive increase in the amount of fibrous tissue with increasing the duration of CCL4 treatment. At the 12th week, fibrosis was most obvious. However ADE combination with CCL4 decreased the area occupied by collagenous fibers (Table 2).

Comparing the biochemical and histopathological results of the animal groups show that improvement in the ADE treated groups on the biochemical level exceeds that on the histopathological level. This may be due to the fact that biochemical parameters may not reflect the degree of tissue damage (Murayama et al., 2007).

These results are of significant clinical importance, suggesting that the classical biochemical parameters such as serum minotransferase activities are not a reliable indicators of chronic liver damage (Domitrovic et al., 2009).

The overall results showed that CCL4 intoxicated group treated with ADE showed significant minimization of CCL4 induced changes in both enzyme and histopathological levels. These results could be explained by the protective role of ADE which significantly decreased the blood levels of CCL4 compared to non treated CCL4 intoxicated groups. This implies the possible chelating effect of ADE in addition to its role as a natural antioxidant, antimutagenic and immune system stimulator (Saafi et al., 2010; Rock et al., 2009 and Ragab et al., 2013).

**Conclusion**

The presented study has shown that ADE had significant protective effect against CCL4-induced changes in the liver. This effect may be due to the rich vitamins and antioxidants in the extract. This study highlights the interest to change toward the use of natural medicinal plants with antioxidant activity for protection against diseases. This may
provide a scientific base for the conventional use of ADE as a nutritional protocol of management.

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References:


