



Date Seed Oil Possesses a Protective Effect Against Paracetamol-induced Hepatotoxicity

Heba M Eltahir^{1*}

¹ Department of Pharmacology and Toxicology (Division of Biochemistry), College of pharmacy, Taibah University, Medina, Saudi Arabia

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ABSTRACT

Background: Liver is a vital organ performing major metabolic and detoxification reactions, which makes it very liable to damage and injury by a wide range of drugs and xenobiotics. Paracetamol (PCM) is an over-the-counter analgesic that is considered to be safe at therapeutic doses; however, getting beyond the therapeutic doses can induce acute hepatic and renal injury. PCM-induced liver injury is a commonly used model for investigating the hepatoprotective and therapeutic significance of drugs and natural products. Date seed oil (DSO) is extracted from the fruit seed of date palm (*Phoenix Dactylifera L*). **Aim:** the current study aims at investigating the hepatoprotective potential of DSO in PCM-induced hepatotoxicity. **Methods:** Swiss albino rats were assigned to three groups; healthy control (a daily oral dose of saline, 14 days), PCM-treated (300mg/kg, a single oral dose after 14 days of a daily oral saline dose) and DSO-PCM-treated (1ml oil/kg, daily oral dose for 14 days, followed by a single PCM dose). Animals were sacrificed and blood and liver tissue were collected for biochemical and histological evaluation. **Results:** PCM treatment induced a significant increase in liver enzymes aspartate transaminase, alanine transaminase, lactate dehydrogenase and alkaline phosphatase, in addition to total bilirubin and cholesterol levels. It significantly decreased total proteins, albumin and blood glucose levels and negatively altered liver histology. DSO treatment protected against PCM induced changes in liver enzymes, and biochemical parameters. It also protected liver tissue from histopathological changes. **Conclusion:** DSO has a potential hepatoprotective effect against PCM-induced acute liver injury.

1. Introduction:

Liver is one of the most important metabolic organs at all, as it is responsible for countless number of metabolic functions as well as detoxification processes. Being the first organ to interact with toxins, drugs, and xenobiotics causes the liver to be susceptible to damage by excessive exposure to these agents ^[1]. Several new drugs have been developed to protect the liver against these insults such as

corticosteroids and rimonabant, but they possess several side effects ^[2]. This led to the urgent need for finding alternative remedies from natural origin to protect the liver with fewer side effects as reported by several studies ^[3].

In order to evaluate the hepatoprotective effects of drugs under investigation, several drugs have been used in animal models to induce hepatic injury that mimics the pathology of hepatotoxicity in human

such as CCl_4 , thioacetamide, and acetaminophen (Paracetamol, PCM) [4].

Paracetamol is one of the most widely used analgesics, which is commonly used for all age groups and considered to be generally safe with variably high oral bioavailability. However, its use in overdose can result in hepatotoxicity and nephrotoxicity under certain conditions [5]. It is metabolized mainly by glucuronidation and sulfation into non-toxic metabolites, but a small fraction of PCM is metabolism by liver cytochrome P450 enzyme into an electrophilic intermediate that binds sulfahydril groups of glutathione and other proteins inducing PCM toxic effects. This interaction results in depletion of the antioxidant glutathione and decreasing the cell resistance against oxidative stress [6]. In addition, PCM metabolites are thought to bind mitochondrial proteins, affecting ATP production [7, 8].

One of the oldest and commonly used crops in many countries is dates, the fruits of date palm (*Phoenix Dactylifera L*), which is cultivated widely in Asia and North Africa. Despite the fact that the fruit is main nutritional product of palm trees, date fruits are not their single beneficial product, as leaves, pollen and kernel (pits/seeds) have been all used since ages for various nutritional and non-nutritional purposes [9].

Dozens of studies have reported antioxidant, anti-inflammatory, anticancer, antimicrobial, anti-hypertensive and anti-diabetic effects for palm tree products [10]. These effects have been linked to the high content of biologically active compounds such as polyphenols, flavonoids, carotenoids and phytosterols in addition to vitamins, minerals, fibers and fatty acids [11]. It is to be noted that the wide range of pharmacological effect that has been reported for dates led to increased interest in exploring the possible effects of the non-edible parts of the tree [12]. Several studies reported hepatoprotective effects for date fruits in various liver toxicity animal models e.g CCl_4 [13], dimethoate [14], dichloroacetic acid [15] and doxorubicin [16] as well as acetaminophen [17]. However, date seed extracts were less investigated for their hepatoprotective effects, as different extracts showed promising protective effects against liver toxicity induced by CCl_4 and gamma radiation [18].

Date seed represents at least one tenth of the fruit weight, containing about 80% of carbohydrates, 10% of oils and the rest includes proteins and ash [19]. Seed oil is rich in oleic acid that composes up to 45% of its

fatty acid content. In addition, the oil contains phenolic compounds such as hydroxytyrosol, protocatechuic acid, tyrosol, gallic acid, caffeic acid, p-coumaric acid and oleuropein, which add to the antioxidant potential of seed oil. Several pharmacological effects were reported for the use of seed oil as anti-inflammatory and antiatherogenic natural product [20, 21]. Aqueous seed extract (seeped seed powder) was shown to effectively reduced the level of some inflammatory cytokines such as interleukin-1 β and TGF- β and reduced levels of cyclooxygenase-1 and -2 as well as prostaglandin E2 (PGE2) in healthy females [22]. Based on published studies, the oral use of date seed oil was reported to be safe for oral doses up to 5000 gm/kg in rodents [23], which makes the use of seed oil a good and safe therapeutic option if its effects can be proven.

Up to our knowledge, no reports have investigated the effect of date seed oil in protecting liver against PCM-induced damage.

In this study, we aimed at investigating the therapeutic potential of date seed oil in combating PCM-induced hepatic injury animal model.

2. Materials and Methods

2.1. Extraction of seed oil

Date fruits were purchased from local supplier where ripe fruits, free from deformities, infections or signs of damage were selected, cleaned, and used to collect the seeds. Separated seeds were cleaned from any residual fruit flesh, washed, dried at medium heat and grinded to produce a fine dust. After sieving the date seed powder, it was stored at 4C until needed. Date fruits were identified and authenticated by a member of the pharmacognosy department, and a sample was deposited in the college of pharmacy, Taibah university, Medina, Saudi Arabia.

To extract seed oil, each 20 gm of the powder were suspended in 500 ml of methanol in a sealed container and the contents were mixed on a magnetic stirrer over night at 40C to allow for oil extraction, and the extraction was repeated three times. The extract fractions were pooled and the solvent was allowed to evaporate using a Soxhlet for 6 hrs to yield the oil according to the previously published method [24, 25]. Amount of oil obtained was about 8% v/w of dry seed powder weight.

2.2 Animal study

Eighteen, age-mate adult male Swiss albino rats (180-220 gm weight) were purchased from the animal house of the college of Pharmacy, Taibah University, Medina, KSA.

Animals were allowed to acclimatize for one week after transporting them into their new cages. They had free access to food and water and were kept at 25C at 12hrs light/dark cycles. Experimental protocol procedures were approved by the “research ethics committee of college of Pharmacy, Taibah University” under the number “COPUU-REC-90-20240226“, and all procedures fulfilled the “*National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978)*”.

2.3. Experimental design

Animals were randomly assigned into three test groups, each containing 6 animals:

Group I: healthy control where animals received a single oral daily dose of saline per oral gavage for 14 days.

Group II: PCM group where animals received a daily oral dose of saline per oral gavage for 14 days.

Group III: date seed oil (DSO)-PCM-treated group where animals received a daily oral dose of DSO (1ml/kg, equivalent to 0.907 gm/kg) per oral gavage for 14 days.

three hours after the last treatment dose, animals of group II and III received a single oral dose of PCM (300 mg/kg, Sigma-Aldrich®) to induce liver injury [26], and all animals were sacrificed 48 hrs later under light thiopental anesthesia (40 mg/kg, IP) after an overnight fasting with free access to water. Blood was collected by cardiac puncture before killing the animals, and was allowed to clot for 20 min at room temperature followed by centrifugation at 3500 rpm for 15 min to isolate serum, which was stored as aliquots at -20C until needed. Liver tissue samples were collected, rinsed quickly in normal saline and fixed overnight in 10% formalin solution followed by dehydration through a series of increasing alcohol concentrations. After being embedded in paraffin, 5 µm thick sections were cut and used for histopathological evaluation.

2.4. Biochemical investigations

2.4.1. Assessment of liver function enzymes

Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) were evaluated in serum samples of the different test groups using commercially available kits according to manufacturer’s instructions (Randox®, Randox labs, US).

2.4.2. Assessment of biochemical parameters

Serum levels of total proteins, albumin, total bilirubin, fasting blood glucose and total cholesterol were assessed spectrophotometrically using commercially available kits according to the manufacturer’s instructions (Randox®, Randox labs, US).

2.5. Histological investigation

Five µm thick, paraffin sections from liver tissue of the different test animals were de-paraffinized, rehydrated and stained with Hematoxylin & Eosin to visualize the histopathological changes. Images from the different sections were taken using a light microscope and evaluated by a histopathologist in a blind fashion.

2.6. Statistical analysis

Data presented in the current study were statistically analyzed using GraphPad Prism 6.0 software (GraphPad, San Diego, Ca, USA). All data are represented as mean ± SD. one way ANOVA test followed by Bonferroni test were used as multiple comparison tests. P values less than 0.05 indicated statistically significant difference. Normality was tested using D'Agostino & Pearson omnibus normality test. P values >0.05 indicated normally distributed data.

3. Results

3.1. Effect of the different treatments on biochemical parameters

Treating animals with paracetamol (PCM) resulted in pathological changes on the biochemical level. It induced a significant reduction in total proteins,

albumin and blood glucose levels compared to the healthy control animals ($p < 0.05$). In addition, it significantly increased the serum levels of liver enzymes ALT, AST and ALP as well as LDH levels. Also total bilirubin and total cholesterol levels were all increased upon treatment with PCM, however, in contrast to the changes in the other parameters which were significant ($p < 0.05$), the increase in total cholesterol was not statistically significant compared to healthy control ($p > 0.05$, table 1).

Pre-treating animals with date seed oil (DSO) for two weeks before PCM administration protected against the increase in the different biochemical parameters. As can be seen in table 1, DSO treatment significantly ameliorated the reduction in total proteins, albumin and blood glucose levels caused by PCM, compared to PCM-treated group ($p < 0.05$). In addition, DSO treatment protected against the increase in liver enzyme levels ALT, AST, and ALP as well as LDH, total cholesterol, and total bilirubin. The effects of DSO were significant compared to PCM effects ($p < 0.05$) except for total cholesterol and total bilirubin where the pre-treatment with DSO partially prevented the effect of PCM but the difference was not statistically different ($p > 0.05$).

Table 1: The effect of the different treatments on the biochemical parameters.

	Healthy Control group	PCM group	DSO+PCM group
Total protein mg/dl	5.583±0.71	4.343±0.38 ^a	5.608±0.35 ^b
Albumin mg/dl	4.03±0.11	3.15±0.19 ^a	3.93±0.11 ^b
Glucose mg/dl	170±24.84	80.38±15 ^a	155±32.9 ^b
Total cholesterol mg/dl	113.94±5.22	121.8±5.14 ^{n.s}	115.34±13.51
Total bilirubin mg/dl	0.46±0.18	1.45±0.21 ^a	0.63±0.24
ALT U/L	69.59±12.48	126.46±9.2 ^a	97.2±10.39 ^b
AST U/L	132.9±10.22	181.03±11.73 ^a	154.46±6.97 ^b
LDH U/L	120.56±11.28	179.84±12.79 ^a	128.73±18.72 ^b
ALP U/L	105.72±30.29	222.33±23.21 ^a	117.72±5.2 ^b

Data presented as mean±SD, n=6, significance was calculated at $p < 0.05$. a: significant compared to healthy control group, b: significant compared to PCM group. Data are normally distributed based on D'Agostino & Pearson omnibus normality test ($p > 0.2$).

3.2. Histopathological evaluation

Liver section from healthy control animals show normal architecture of liver lobules with normally presented hepatocytes arranged in regular cords radiating from the central vein, and Kupfer cells (arrowheads) within the sinusoids (S) (figure 1 A₁ and A₂).

Paracetamol treatment negatively impacted the architecture of liver tissue and distorted the

arrangement of hepatic cords showing disorganized cells around the congested central vein (C) (arrows, figure 1 B₁). Higher magnification reveals the hydropic degeneration and pyknotic nuclear changes of hepatocytes and increased number of Kupfer cells within the sinusoids (arrowheads, figure 1B). Interestingly, pretreatment of animals with DSO greatly protected the liver tissue against the PCM-induced hepatocellular damage as it reduced signs of hydropic degeneration. It also prevented the PCM-induced distortion of hepatic cords organization, allowing the liver to keep its cord arrangement around the central vein (C, figure 1 C₁). hepatocytes showed almost normal granular cytoplasm and large nuclei similar to healthy control sections This treatment kept the architecture of liver tissue of this test group very similar to the healthy control group (figure 1 A₂ and 1 C₂).

Table 2 shows the quantification of the hepatic tissues damage from the different groups. The scoring scale of tissue injury was achieved by giving the following grades: 0 = no change; 1 ≤ 25% tissue damage; 2 = 26-50% tissue damage; 3 = 51-75% tissue damage; 4 = 76-100% tissue damage. Quantification shows that DSO pre-treatment protected against congestion and dilatation of sinusoids and portal vessel. It also resulted in less necrosis and hepatocyte degeneration as well as the hyperplasia of Kupfer cells compared to animals receiving PCM without pretreatment.

4. Discussion

Liver is the main metabolic organ in the biological system as it is responsible for a wide range of metabolic activities in addition to its role in detoxification of drugs and xenobiotics. Its excessive exposure to such factors is a main cause of hepatocellular injury.

Paracetamol (PCM) is a widely used over-the-counter, safe analgesic in therapeutic dose, which is metabolized into water-soluble metabolites and excreted via the kidney. However, overdose of PCM is responsible for inducing acute hepatic and renal damage. As explained earlier, PCM is metabolized into the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQ), which depletes the reducing agent glutathione and binds SH-groups of cellular proteins forming protein adducts. As NAPQ-protein adducts accumulate, they induce a condition of oxidative stress resulting in excessive production of free radicals and reactive oxygen species (ROS), which

start a cascade of cellular damage [27]. PCM has also a non-specific inhibitory effect on cyclooxygenase-1 and 2, resulting in downregulation

of prostaglandins (PGs) production and subsequently inhibits the inflammatory response [28].

Paracetamol-induced acute liver damage in rodents is one of the most commonly used models for understanding the pathology of acute liver injury and investigating the therapeutic/protective potential of claimed hepatoprotective agents [4]. This form of acute liver injury mimics the human disease which facilitates expanding the results to human beings after the required modifications [4].

In the current research, PCM administration induced an expected significant increase in the level of multiple intracellular hepatic enzymes including AST, ALT, ALP, and LDH compared to the healthy control, indicating hepatocellular damage.

It also increased serum levels of total cholesterol, and total bilirubin compared to the healthy control. These biochemical findings were supported by the histological investigation, where PCM induced necrosis, disorganized hepatocytes in the portal area and congestion of blood vessels.

On the other hand, such treatment decreased total proteins, albumin and blood glucose levels compared to healthy animals.

Table 2: Quantification of the hepatic tissues damage in the different groups

Groups	Liver					
	Congestion	Hyperplasia of Von-kupfer cell	Necrosis	Degeneration of hepatocytes	Dilatation of portal vessels	Dilatation of hepatic sinusoids
Control group	0	0	0	0	0	0
PCM-treated group	2	3	2	2	2	3
DSO-PCM treated group	1	1	0	1	1	1

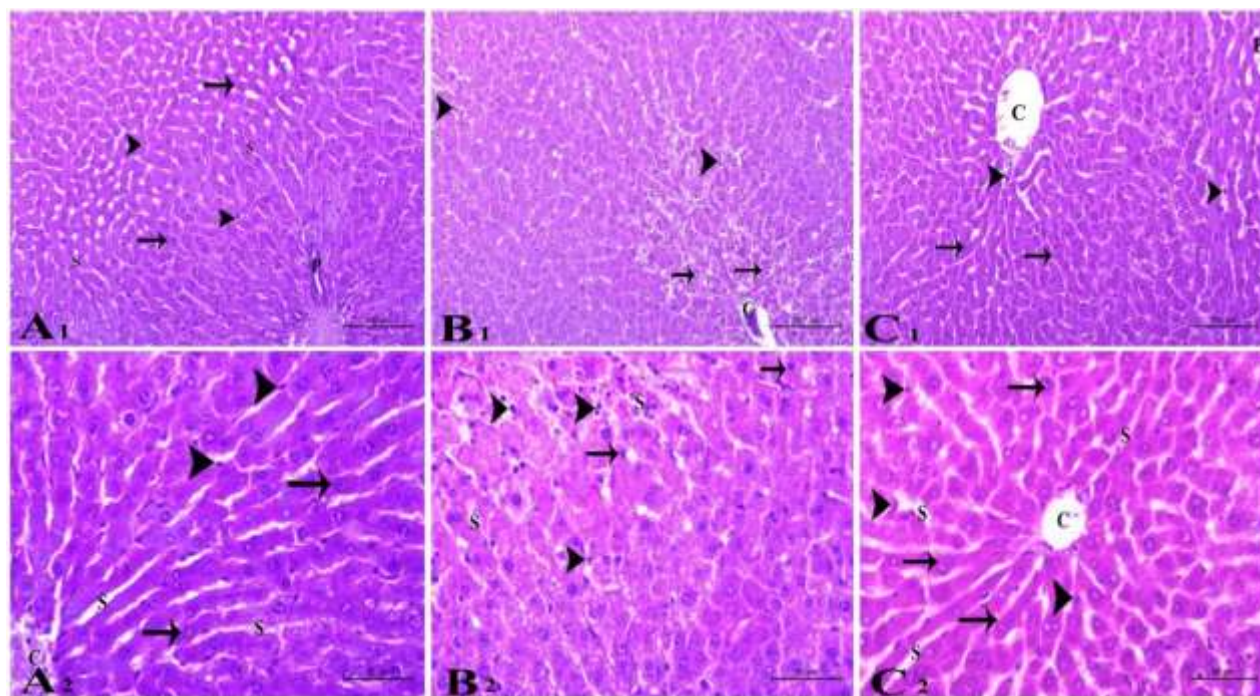


Figure 1: photomicrographs of liver sections from the different test groups.

Interestingly, pre-treating animals with DSO before administration of PCM prevented the increase in enzyme levels as well as total cholesterol, and total bilirubin to various extents and retrieved the levels of total proteins, albumin and blood glucose. The protective effect of DSO was also remarkable on the

histological level, where signs of necrosis, hemorrhage, and blood vessel congestion as well Kupfer cells were minimal.

Paracetamol-induced oxidative stress and the resulting ROS result in lipid peroxidation and damage to cellular membranes, loss of selective membrane

permeability and consequently leading to leakage of cellular contents including enzymes into the circulation [29]. This explains the up-regulation of intracellular enzyme levels AST and ALT in addition to LDH and ALP, which are all considered as markers of cellular injury in circulation after PCM intoxication. Such damage to the liver cells affects the liver capacity to perform its metabolic functions, resulting in decreased liver capacity of protein and albumin synthesis. Another possible explanation for the observed hypoalbuminemia and hypoproteinemia could be their increased excretion through the kidneys due to the PCM-induced renal damage [30]. In addition, PCM-induced liver injury decreases its ability to uptake and conjugate bilirubin for further excretion, and induces damage to hepatic bile ducts leading to leakage of bilirubin into the circulation. The increased levels of circulating bilirubin can be also regarded to its reported role as a cellular antioxidant, that can be increased in response to the oxidative insults as in case of PCM intoxication [31]. As a result one can observe a decrease in total proteins and albumin levels and an increase in levels of circulating bilirubin.

It is to be noted that the imbalance between lipid synthesis and the processes of its utilization and secretion, as well as the disturbances in hepatic lipoprotein synthesis may play a role in the observed increase in total cholesterol levels after acute PCM intoxication as reported in the current study and by other research groups [32].

It was reported earlier that PCM affects β -cells of pancreas and alters their functions resulting in the release of excessive amounts of insulin. As a result, blood glucose levels fall down substantially after PCM intoxication as reported earlier by others. In addition, several reports linked acute liver injury after PCM overdose to hypoglycemia, putting the latter as one of the "poor prognosis markers" of acute liver injury [28, 33]. These pieces of information agree with the data presented in the current work where a significant reduction in blood glucose was observed. Seed oil has been reported to possess anti-inflammatory properties in several contexts, in addition to its antibacterial and antifungal effects [34]. It was also shown to possess an antioxidant and free radical scavenging activity. Generally, date seed oil contains multiple phenolic and flavonoidal biomolecules such as chlorogenic acid and p-coumaric acid, which are responsible for the antioxidant effects of the seed oil. The oil was

reported to contain multiple amino acids and fat soluble vitamins e.g α -tocopherol, α -tocopheryl acetate and vitamin K in addition to fatty acids e.g oleic-, lauric-, myristic- and linoleic acid [35, 36].

Both anti-inflammatory and antioxidant effects of DSO allow for the proposal that it may exert its hepatoprotective effect via its ability to scavenge PCM-induced free radicals and hence hinder the inflammatory response and the hepatic cell injury [20]. It is worth to note that the observed hepatoprotective effect of DSO in the current study is in agreement with the hepatoprotective effect reported for date seed extract against CCl_4 -induced hepatotoxicity [37] and the effects observed for the aqueous seed extract in PCM-induced liver injury [38]. In addition, it was also reported earlier that DSO administration down-regulates the level of proinflammatory cytokines IL-1b, TGF-B, and COX-1 and -2, a finding that supports the hypothesis of the anti-inflammatory role of DSO in ameliorating acute liver injury induced by paracetamol [22].

Others have suggested that using antioxidant-, free radical scavenging agents provide proper protection against liver damage induced in response to the insults of PCM by interacting with the hepatic cytochrome P450 and interfering with its ability to metabolize PCM into its reactive, toxic metabolites e.g NAPQ. As a result, levels of NAPQ produced due to PCM metabolism via cytochrome P450 are reduced, ameliorating the levels of ROS that can be produced by the interaction of NAPQ with glutathione and other cellular proteins, resulting in less molecular and cellular damage [39].

In conclusion, the use of DSO as a pre-treatment for two week ameliorated the acute liver injury induced by paracetamol overdose. The protective effect was observed on the cellular and biochemical level, and can be attributed to the previously reported antioxidant, free radical scavenging and anti-inflammatory properties of DSO. However, further study(s) are needed to investigate the exact molecular mechanisms and the signaling pathways behind the observed DSO effects. Despite these promising results, the study has some limitations. The effect of PCM on tissue antioxidant capacity (reduced glutathione) and lipid peroxidation should be investigated in order to confirm the *in vivo* antioxidant effect of DSO. In addition, a future study can be performed to investigate the curative effect of DSO by administering it to animals after induction of PCM intoxication. Under this condition, the observed

effect of DSO should be compared to the standard PCM-antidote N-acetyl cysteine or similar standard drugs.

Conflict of interest

The author declares no conflicts of interest

Author contribution

The author was responsible for conception and design of the study, acquisition of data, analysis and interpretation of data, in addition to drafting and revising the manuscript and approving it for submission.

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