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Date Seeds Oil Attenuates Hepatotoxicity in High-Cholesterol-Diet-Fed Mice

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Abstract:
This study aims to investigate the hepatoprotective effect of date seeds oil (DSO) in mice subjected to a high cholesterol diet (HCD). First, the *in vivo* acute toxicity of DSO was tested. Then, the *in vivo* hepatotoxicity experiment was done on twenty-eight male mice randomly divided into four groups: a control group, an HCD group, a DSO-treated group, and a group receiving both HCD and DSO. Multiple biochemical analyses were conducted, including serum levels of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and C-reactive protein (CRP). Additionally, glutathione (GSH) and catalase (CAT) activity were analysed. Finally, a histopathological examination was done on liver tissues.
The results demonstrated that DSO significantly reduced serum TG and cholesterol levels, ameliorated oxidative stress markers such as GSH and CAT, and exhibited a hepatoprotective effect, as evidence by attenuation of liver injury through the reduction of steatosis, as well it reduced the AST, ALT, and CRP levels.
This suggests that DSO has potential as a therapeutic agent against HDC-induced hepatotoxicity.
Keywords: Hepatotoxicity, Date seeds oil, High-cholesterol diet, oxidative stress

Introduction

The date palm tree, *Phoenix dactylifera L.*, belonging to the Arecaceae family represents the most abundant arboriculture crop, since ancient times, in the oasis of North Africa and the Middle East (Bouhlali et al., 2020). According to the Food and Agriculture Organization of the United Nations (FAO), the global production of dates worldwide in 2020 is 9.75 metric tons. In Algeria, date production is of primary importance within the political program of the Algerian Ministry of Agriculture with about13 million date palm trees. It was ranked third worldwide with 1.25 metric tons per year (Shahbandeh M., 2024).

Date palm fruit is a comprehensive nutrition that provides the most essential nutrients with potential health benefits. It is considered also as a rich source of dietary fibres,secondary metabolites, essential vitamins and mineral salts (Mullassery et al., 2024).

Seeds are regarded, from the survival plant viewpoint, as the most important part of fruits, which could explain their exceptional richness in secondary metabolites compared to the edible fruit (Harkat et al., 2022), however, date seeds were considered from a long time as waste generated from date processing giving pitted dates, date paste, date syrup or date confectionery. Date seeds represent about 6.10% to 11.47% of the whole weight of date fruit (Maqsood et al., 2020).

In traditional medicine, date seed powder is commonly used for treating liver problems, diabetes, gastrointestinal disorders, cancer, pulmonary diseases, toothaches, diarrhea and various infectious diseases (Alkhalidy et al., 2023; Bouhlali et al., 2020).

Date seed oil (5 to 13 % of the seed weight) is a remarkable source of phenolic compounds, like tocopherols and phytosterols, and monounsaturated fatty acids, particularly oleic acid. With the increasing availability of date seeds as raw material, their oil extraction could be an excellent by-product with high value-added and very profitable economically. That's why it ought to be a superb opportunity for future ventures (Mrabet et al., 2020).

Nonalcoholic steatohepatitis (NASH) is a subtype of nonalcoholic fatty liver disease (NAFLD) and the leading cause of liver disease, which could lead to liver cirrhosis and even hepatocellular carcinoma (Xu et al., 2022). In the last decennium, a significant increase worldwide in the frequency of NAFLD was noted, which correlates positively with the rise in obesity. A high-cholesterol food culture promotes oxidative stress which in turn mediates cellular responses triggering NASH (Alsharari et al., 2016; Wang et al., 2014).
This study aims to assess the hepatoprotective, anti-hypercholesterolemic, anti-inflammatory, and antioxidant effects of Date Seed Oil (DSO) on the murine model of high-cholesterol-diet-induced hepatotoxicity.

Materials and Methods:

Oil preparation

Phoenix dactylifera dates were collected from the region of Adrar (Northern Algerian Sahara) in October 2020. The seeds of these dates were used to extract oil by cold pressing. After sorting, the pits were subjected to mechanical cold pressure using an oil press at room temperature. The obtained oil, the first natural juice, contains all the essential nutritional principles and does not undergo any chemical treatment or refining. Date Seed Oil (DSE) was stored in the dark at room temperature until use.

Oil extraction was carried out at the laboratory for Obtaining Therapeutic Substances (LOST), Department of Chemistry, Frères Mentouri Constantine 1 University, Algeria.

Animals

Thirty-three male *Mus musculus* mice weighing 14-18 g, aged 10 – 12 weeks, were used for *in vivo* anti-hypercholesterolemic activity and acute oral toxicity. The mice were reared and maintained in the laboratory animal facility of the "Department of Animal Biology, Faculty of Natural and Life Sciences, Frères Mentouri Constantine 1 University, Algeria". The mice were divided randomly into four groups of seven per cage (for the anti-hypercholesterolemic activity) and a group of five (for the acute oral toxicity) and kept for acclimatization seven days before starting the experiments, under standard animal laboratory conditions (12 h photoperiodic cycle (light/darkness), temperature 22 ± 2 °C and relative humidity 50 ± 5 %) and free access to water and food *ad libitum*. These protocols were applied following the Use Guidelines of Laboratory Animals Care and approved by the Ethical Committee of the DGRSDT at the Algerian Ministry of Higher Education and Scientific Research under the PRFU Project (D01N01UN250120210003).

***In vivo* acute oral toxicity test**

The acute oral toxicity test was carried out based on the guideline propounded by the OECD (2022). A dose of 2000 mg/Kg of body weight (BW) was given to a sole mouse to track mortality and clinical signs (sedation and somnolence movements, unusual aggressiveness, unusual locomotion, unusual vocalization, paralysis, convulsion, restlessness, fasciculation and/or prostration). The surveillance was done at the beginning during the first hour and then every three hours for at least two days. After the survival of this first mouse, four new mice received the same dose in a serial with an interval of 2 days. All the mice were kept under close control for 14 successive days. The number of dead mice and health disorders through the experiment was registered. If no deaths nor clinical signs are detected, the Lethal Dose 50 (LD50) is determined to be superior to 2000 mg/kg of BW.

Hepatotoxicity induced by high-cholesterol-diet

The hepatoprotective activity of the date seed oil was evaluated following the method established by Kumari et al. (2016) with some modifications. To induce hepatotoxicity, mice were fed with high-cholesterol-diet (HCD), produced by mixing 400 mg/ Kg of BW per day of cholesterol with 100 mg of flour. The HCD was administrated daily orally in the form of a ball for 21 consecutive days. Twenty-eight male mice were randomly divided into four groups:

- Group 1: daily oral administration of a ball of 100 mg of flour per mouse for 21 days

- Group 2: daily oral administration of a ball of 100mg HCD per mouse for 21 days
- Group 3: daily oral administration of a ball of 100 mg HCD per mouse and of DSO at a dose of 120 mg/ Kg of BW for 21 days
- Group 4: daily oral administration of a ball of 100 mg of flour and DSO at a dose of 120 mg/Kg of BW for 21 days.

On day 21 after a fasting period of 16 hours, the blood samples were collected on heparin tubes from the retro-orbital plexus using glass capillaries. The plasma was recovered after centrifugation and stored at – 20 °C in a freezer for biochemical investigation. All mice were dissected, and the liver was withdrawn and washed with ice-cold saline. A part of the liver and the aorta were conserved in formaldehyde solution at 10 % for histopathological investigation. The remaining parts of the liver were stored in a freezer at – 20 °C until use.

Biochemical investigation

The plasmas of mice were used to determine the lipid panel by measuring the amounts of Total Cholesterol (TC), Triglyceride (TG), High-Density Lipoprotein Cholesterol (HDL-C), and Low-Density Lipoprotein Cholesterol (LDL-C);the liver function by assessing the enzymatic activities of Aspartate Transaminase (AST) and Alanine Transaminase (ALT); and to evaluate the concentration of the C-Reactive Protein (CRP), an acute marker of inflammation. All these tests were done on the Cobas Integra 6000 plus analyzer (Roche®).

The Atherogenic Index of Plasma (AIP) was calculated according to the following equation:

$$\mathrm{AIP} = \log(\mathrm{TG}/\mathrm{HDL} - \mathrm{c})$$

Liver Homogenate Preparation

A small piece of liver (0.5 g) was added to 2 mL of Tris Buffered Saline (TBS) (Tris 50 mM, NaCl 150 mM adjusted to pH 7.4), then homogenized at 4°C with an Ultra-Turrax T18 Digital Dispenser (IKA®) at 6000 rpm for 3min.

Then, the mixture was centrifuged for 15 min at 9000g at 4°C. The supernatant was recuperated and considered as the liver tissue homogenate, which is used to estimate the total protein content, reduced glutathione content, and catalase activity.

Reduced glutathione content

The reduced glutathione content (GSH content) was assessed as defined by (Iweala et al. 2019) with some modifications. 800 µL of liver tissue homogenate were deproteinized by adding 200 µL of 5-sulfosalicylic Acid (0.25%). The supernatant was recuperated after centrifugation in refrigerated centrifuge at 4°C and 1000 rpm for 5min. Then 500 µL of this supernatant was added to 1mL of Tris-HCL Buffer (0.4 M, pH 9.61 with 20 mM EDTA) and

25 µL of DTNB (0.01M 5,5′-Dithiobis-2 Nitrobenzoic acid) and incubated for 5 min at room temperature. The absorbance was measured at 412 nm.

Catalase activity

Tissue catalase activity (CAT activity) was determined as reported in Aebi (1974). This assay is basically founded on the spectrophotometric assessment of Hydrogen peroxide (H_2O_2) rate of decomposition at 240 nm.

Histopathological analysis

The liver fixed in 10% formaldehyde solution, were washed and dehydrated before being embedded in paraffin and sliced by microtome to a thickness of 7 µm. The tissue slices were stained with hematoxylin-eosin and analysed using a Leica DFC310 FX 1.4-megapixel microscope with 400× magnification.

Statistical analysis

All data were presented as Mean ± SD from 5 mice per group. The ANOVA one-factor followed by the post-hoc Tukey HSD test was performed to compare and estimate the significance of difference ($P < 0.05$) between groups. The statistical analysis was conducted using SPSS Statistics 23.0 software (IBM® SPSS Inc.).

RESULTS:

The *in vivo* oral toxicity test revealed that the date seed oil at the dose of 2000 mg/Kg of BW was well tolerated and without recording any deaths in the 5 mice and those during the 14 days of monitoring. This result means that the LD50 exceeds 2000 mg/Kg of BW.

The serum lipid panels of different mice groups are presented in Table 1. The mice fed by HCD showed a significant increase in the level of AIP, TC and TG, and conversely decrease the level of HDL-c compared to mice belonging to the control group. The administration of the DSO in the mice of the group HCD + DSO has significantly reduced the AIP, TC and the TG levels compared to the mice that received the HCD alone. The administration of the DSO alone has significantly decreased the levels of the AIP and TG compared to the levels noted in the mice belonging to the control group.

Table .1. Effect of the date seed oil on the lipid panel in high-cholesterol-diet-fed mice

	Control	HCD	HCD + DSO	DSO
TC	1.65 ± 0.17 ^{a}	1.93 ± 0.05 ^{b}	1.18 ± 0.46 ^{a}	1.64 ± 0.19 ^{a}
TG	2.53 ± 0.86 ^{b}	3.22 ± 0.61 ^{c}	2.62 ± 0.38 ^{b}	1.63 ± 0.04 ^{a}
HDL-c	1.25 ± 0.08 ^{b}	0.85 ± 0.06 ^{a}	1.10 ± 0.19 ^{b}	1.46 ± 0.48 ^{b}
LDL-c	0.16 ± 0.09^{a}	0.18 ± 0.01 ^{a}	0.15 ± 0.03 ^{a}	0.09 ± 0.01 ^{a}

AIP	0.27 ± 0.02 [b]	0.59 ± 0.02 [d]	0.37 ± 0.05 [c]	0.07 ± 0.02 [a]

**The outcomes were presented as Means ± SD of five measurements. Analysis of variance (ANOVA one-factor and Tukey Tests) revealed statistical difference (P<0.05). Different superscripts (a, b, c…) for values in the same line are statistically different.*

The serum liver transaminase enzymes of different mice groups are presented in Table 2. The HCD group revealed a significant increase in the levels of AST and ALT relative to the control group (P<0.05). The concomitant administration of the DSO with the HCD showed that the DSO decreased significantly the AST and ALT levels compared to HCD group. The administration of the DSO alone didn't show any effect on the AST and ALT levels which were statistically comparable to the levels noted by the control group.

Table .2. Effect of date seed oil on the liver transaminase enzymes in high-cholesterol-diet-fed mice

	Control	HCD	HCD + DSO	DSO
AST	91.51 ± 5.47[a]	130.23 ± 6.22[c]	107.12 ± 3.53[b]	93.68 ± 5.13[a]
ALT	30.38 ± 1.30[a]	49.89 ± 3.52[c]	38.64 ± 3.27[b]	31.63 ± 3.39[a]

**The outcomes were presented as Means ± SD of five measurements. Analysis of variance (ANOVA one-factor and Tukey Tests) revealed statistical difference (P<0.05). Different superscripts (a, b and c) for values in the same line are statistically different.*

The levels of the hepatocyte antioxidants of different mice groups are presented in Table 3. The mice belonging to the group HCD showed a significant decrease in the level of the GSH and the activity of the catalase compared to the control group (P<0.05). The concomitant administration of the DSO with the HCD demonstrated that the DSO increased significantly the level of the GSH and the activity of the catalase compared to the levels obtained in the HCD group. The administration of the DSO alone showed a significant increase of the GSH level and the catalase activity compared to the control group.

Table .3. Effect of date seed oil on hepatocytes antioxidants in high-cholesterol-diet-fed mice

	Control	HCD	HCD + DSO	DSO
GSH (10^{-5})	11.94 ± 0.22 [c]	7.84 ± 0.16 [a]	8.90 ± 0.18 [b]	15.72 ± 0.46 [d]
Catalase activity	0.89 ± 0.07 [c]	0.61 ± 0.03 [a]	0.75 ± 0.05 [b]	1.21 ± 0.09 [d]

**The outcomes were presented as Means ± SD of five measurements. Analysis of variance (ANOVA one-factor and Tukey Tests) revealed statistical difference (P<0.05). Different superscripts (a, b and c) for values in the same line are statistically different.*

The serum CRP of different mice groups are presented in Table 4. The mice belonging to the group HCD revealed a significant increase in the level of CRP relative to the control group ($P<0.05$). The concomitant administration of the DSO with the HCD demonstrated that the DSO decreased significantly the level of the CRP compared to the levels noted with the HCD group. The administration of the DSO alone gave a similar level of CRP compared to the control group.

Table .4. Effect of date seed oil on the CRP in high-cholesterol-diet-fed mice

	Control	HCD	HCD + DSO	DSO
CRP	0.09 ± 0.03^{a}	0.33 ± 0.01^{c}	0.25 ± 0.01^{b}	0.14 ± 0.03^{a}

**The outcomes were presented as Means ± SD of five measurements. Analysis of variance (ANOVA one-factor and Tukey Tests) revealed statistical difference (P<0.05). Different superscripts (a, b and c) for values in the same line are statistically different.*

The results of histology revealed that in the control group, the liver exhibited a characteristic and undisturbed architecture indicative of a healthy state (Figure 1). The DSO group showed that the hepatic structure remains well-preserved, similar to the control group.

However, the HCD group demonstrated conspicuous lesions within the liver tissue, notably marked by the development of hepatic steatosis in response to cholesterol. This resulted in a substantial diffusion of lipids within hepatocytes, accompanied by the presence of dilated centrilobular veins, endolysis formation, and the appearance of cells with distinct nuclei.

Intriguingly, the microscopic observation of treated group with HCD + DSO demonstrated an improvement in liver tissue structure and a reduction of lipid accumulation, compared to HDC group.

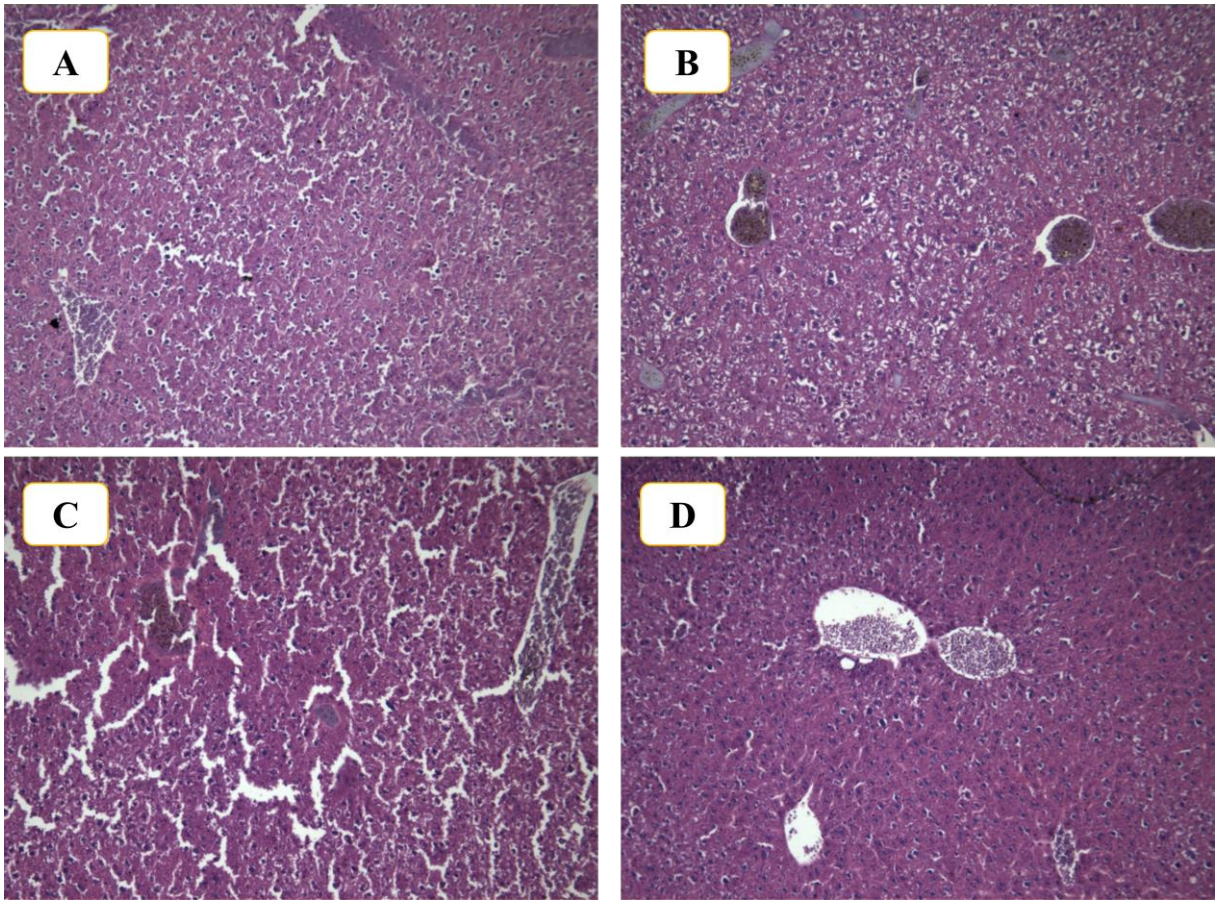

Figure 1: *Effects of Phoenix dactylifera L. seeds oil on liver histopathological changes induced by the administration of 400 mg/kg of HCD in mice. (A) Control group; (B) DSO group (120 mg/kg), (C) HCD group (400 mg/kg), (D) HCD+DSO group. All sections were stained with Hematoxylin/eosin; ×400*

DISCUSSION:

The present study was undertaken to investigate the protective effect of *Phoenix dactylifera* L. seeds oil against hepatotoxicity and hypercholesterolemia induced by a high-cholesterol diet fed to mice. HCD feeding leads to dyslipidemia precisely hypercholesterolemia that is characterized by an abnormal rise of TC, TG, and LDL-c in plasma, and an anomalous decrease in HDL-c (Hu et al., 2021). Furthermore, HCD leads to hepatotoxicity by shooting up liver enzymes, mainly ALT and AST. The strongest match to previous research, the raised levels of ALT and AST are linked to hepatic damage that could lead to oxidative stress (Du et al., 2024).

The overabundance of triglycerides in hepatocytes set up steatotic droplets in NAFLD (Xu et al., 2022). The hypertriglyceridemia, hypercholesterolemia and the lowering in HDL-c rise

the probability of NAFLD (Alsharari et al., 2016). In our study, HCD-fed mice showed significant TC, TG, AIP, ALT, AST and CRP reductions, while HDL-c, GSH and catalase activity increased after DSO administration. Meanwhile, DSO ameliorated lipid droplets and ballooning in hepatocytes. Thus, hepatotoxicity and hypercholesterolemia were prevented when treated with DSO.

The focal organ of cholesterol metabolism is the liver. In a physiological state, the excess cholesterol (e.g., on macrophages) is removed by hepatic cholesterol metabolism. In this process, HDL is responsible for transporting cholesterol to the liver to be excreted into the faeces afterwards (Wang et al., 2017). In a pathological state, due to excessive levels of cholesterol, LDL-c, causes liver injury by numerous pathways, like stimulating the liberation of the pro-inflammatory cytokine TGF-β and switching on the NLRP3 inflammasome, which could lead to liver metabolism disturbance (Ioannou et al., 2017; Tomita et al., 2014; Y. Xu et al., 2022).

Improving the HDL-c rate is considered an efficient strategy to treat hypercholesterolemia and thus liver toxicity (Marques et al., 2018). The atherogenic index of plasma, substitute small dense LDL (sdLDL), is considered as an excellent predictive biomarker for hyperlipidemia, atherosclerosis, cardiovascular disease and NAFLD (Si et al., 2021). As stated by experts, decreased sdLDL and AIP level is a rooted therapeutic approach for the treatment of dyslipidemia thus HCD induce hepatotoxicity (Santos et al., 2020). The consumption of DSO by mice has significantly improved the HDL-c rate and at the same time decreased the AIP level.

The bioactive potential of date seed oil could be partly attributed to its wealth of phenolic compounds, well known by their high biological activities, particularly antioxidant activity, like caffeic acid, catechins, epicatechins, gallic acid, oleuropein, p-coumaric acid, p-hydroxybenzoic acid, protocatechuic acid, rutin and quercetin. Many studies confirmed the presence of these phenolic compounds in all the date seed oils (0.64 -1.27 mg/g). Although, its composition differed qualitatively and quantitatively depending on the cultivar types. Moreover, DSO has a remarkable content of monounsaturated fatty acids, mostly oleic acid, with a notable antioxidant activity (Mrabet et al., 2020).

One of the main factors involved in the causation of NASH is the oxidative stress engendered by HCD intake (Al-Rejaie et al., 2013; Alsharari et al., 2016; Wang et al., 2014). Several studies noted a decreased rate of antioxidants, like catalase and GSH, in NAFLD

(Hajighasem et al., 2019; Irie et al., 2016). GSH is a central non-enzymatic antioxidant with key roles in free radical scavenging. It serves as a hydrogen donor or cofactor for antioxidant enzymes such as glutathione-s-transferases (GSTs), glutathione peroxidases (GPXs) and sulfiredoxin- 1 (Srxn1). CAT is the cytosolic antioxidant enzyme responsible for cell protection against the toxic effects of H_2O_2. Therefore, decrease in GSH levels and CAT activity exacerbate OS in NAFLD (Farzanegi et al., 2019). The same finding was indicated in our results. The significant increase in GSH level and CAT activity revealed in the HCD+DSO could be attributed to the richness of DSO on phenolic compounds with a well-known potent antioxidant activity.

The liver pentameric protein, CRP, is an excellent inflammatory biomarker which is secreted mainly in response to IL-6 and lowly to IL-1β and TNF-α by hepatocytes in the acute and chronic inflammatory phases (Feldman et al., 2023). A close association was revealed between hypercholesterolemia, fatty liver disease, hepatic steatosis and inflammation monitored by the increase in CRP level. Many studies have reported that inflammation could be the consequence and/or the cause of liver steatosis, which in turn may further stimulate the production of CRP (Luyendyk and Guo, 2011). These findings were confirmed by our results where we noted a significant increase in CRP levels in the HCD group. The significantly lower rate of CRP recorded in the HCD+DSO group could be explained by the wealth of DSO in phenolic molecules, such as gallic acid, ferulic acid, caffeic acid and p-coumaric acid, well known by their potential inhibitory effect of IL-6 and TNF-α (Nile et al., 2016). Furthermore, high-intake foods enriched by flavonoids were negatively correlated with serum CRP rates (González-Gallego et al., 2007).

Histological examination of the liver displays a potential attenuation of lesions, preserving normal hepatic architecture and indicating hepatoprotective effects. Similarly, histological observations of the heart suggest cardioprotective properties, with potential safeguarding of cardiac cell integrity and a reduction in signs of fibrosis and inflammation. Concerning the vasculature, histological results from the abdominal and iliac aorta indicate a potential improvement in vascular health, marked by a reduction in vascular lesions and conservation of elasticity. These overall findings suggest a positive impact of DSO on a histological level, highlighting its hepatoprotective, cardioprotective, and vasculoprotective properties. However, a more in-depth analysis of different cell types and histological markers is necessary for a comprehensive understanding of underlying mechanisms. Future studies could further explore these results and assess their reproducibility in clinical contexts or other experimental models.

CONCLUSION:

In summary, the potential of date fruit and date seeds in therapeutics is significant. Their hepatoprotective, anti-hypercholesterolemic, anti-inflammatory and antioxidant effects position them as promising candidates for further exploration in addressing various health concerns. Future research and clinical studies will play a crucial role in validating and expanding upon these findings.

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