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Biochemical Study on Palm Fruit as a Treatment for Liver Cancer in Rats

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Abstract: Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related death worldwide. Viral infection (particularly hepatitis C and B viruses) and alcohol are the main risk factors for HCC. In recent years, herbal medicine-based therapy has become an effective therapeutic option for treating many diseases, such as liver cancer. Our research intended to investigate the efficacy of palm date fruit extract in HCC male rats and assess its biological function as an anti-cancer agent. The anti-cancer potential of palm date fruit extract was also examined through biochemical studies of liver function tests, histopathological examination, and immunohistochemistry of α-SMA, CD34, and AFP of all groups. The CCl_4 treatment Group showed histological characteristics of HCC, while rats treated with palm date fruit extract and cisplatin were partially nearly to normal liver architecture. Overexpression of liver enzymes was observed in HCC cells compared to treated groups. These findings suggested that palm date fruit can be used as an anti-tumor therapeutic for HCC.

Keywords: hepatocellular carcinoma; palm date; cisplatin; apoptosis; CD34.

1. Introduction

Egypt is the third most populated country in Africa and the fourth most common country worldwide for hepatocellular carcinoma (HCC) [1]. HCC is considered the most challenging health condition in Egypt by health officials. Over ten years, the number of HCC patients increased from place to place [2]. Cirrhosis of the liver is a long-term condition in which normal liver tissue is replaced by fibrous tissue, resulting in liver dysfunction. Cirrhosis can be caused by a variety of reasons, including alcohol misuse, hepatitis (types B and C), fatty liver, medications, toxins, congenital disorders (fibrocystic liver disease, genetic malformations, and hemochromatosis), long-term bile duct obstruction for whatever reason, and more [3]. Early detection of HCC is crucial for lowering high HCC death rates, as early-stage HCC can be treated with potentially curative therapeutic approaches [4].

After detecting of dangerous effects of chemotherapy, radiation, and surgery, scientists focus on therapeutic natural products. Many natural products, such as fruits, ensure healthy, effective, and economic cancer prevention and treatment options [5]. The date palm tree is an essential plant in many countries. Dietary fibers, carbohydrates, proteins, vitamins, minerals, and phenolic are abundant in the palm date extract, so it has Potential anti-cancer agents [6]. As palm date is available and has a reasonable price in Egypt, research and treatment tend to

have reasonable costs with no side effects than the high cost of chemotherapeutic therapy. Palm dates contain vital anti-cancer components, which may be useful in the struggle against liver cancer.

According to a study on palm date extract, dates are high in amino acids such as glutathione, aspartic acid, proline, glycine, and lysine, which are not generated by the human body. Furthermore, dietary fiber, lipids, polyphenols, and flavonoids included in palm dates can significantly decrease cell proliferation by inducing apoptosis [7]. In a rat model, palm date extract showed strong protective benefits against HCC induced by CCl_4. This may also play a role in activating a liver function test. The result of the histological study would indicate that normal hepatocytes in the palm date and cisplatin-treated groups resemble to control group, other than HCC. In addition to the assay, the protein expression of cluster of differentiation 34 (CD34), Smooth Muscle Actin (SMA), and Alpha-fetoprotein (AFP) were detected by immunohistochemistry. The current study examined the anti-cancer effect of ethanolic palm date extract on cancer cells by assessing cell viability and proliferation, DNA damage, and changes in the treated cells' morphology compared to HCC cells. As a result, the current research findings significantly impact cancer therapies based on natural ingredients.

2. Materials and Methods

2.1. Palm date extract preparation.

Fresh palm dates were procured at a local market in the Egyptian city of Zagazig. The dates were washed twice with double distilled water before being used. The pulp was then oven dried, crushed, and extracted with ethanol at a 1:3 (w/v) ratio at 24 °C for 48 hours. Following that, the extract was run through the Whatman and Millipore filters. Finally, to make a viscous syrup, the resulting extract was condensed and stored at -80 °C [8].

2.2. Reagents and chemicals.

Cisplatin was purchased from (Sigma Aldrich), carbon tetrachloride (CCl_4) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and all of the Absolute ethanol above 99% and Sodium chloride from (Alamia, Egypt).

2.3 Phytonutrient analysis.

Basic phytonutrient screening techniques for palm date extract were used [9].

2.4. Experimental design.

Before starting the experiments, Wistar male rats (n = 32) were acclimatized for one week. The rats were divided into four experimental groups at random. (n = 8). Animals were grouped as follows:

Group I: eight rats served as a control group: normal rats, kept for three months without any treatment, received a normal chow diet and distilled water.

Twenty-four rats were injected intraperitoneally with CCl_4 (1 CCl_4:1 olive oil)1ml/kg b.wt was administered IP twice a week for three months to develop HCC and after two months of injection, were divided into three groups: (cisplatin group, Palm date group and positive control group).

Group II: The (HCC) positive control group did not receive treatment for another month. Group III: Palm date group: rats treated with date extract (400 mg/kg b.wt/day) was administered by oral gavage for another month after induction of HCC.

Group IV: cisplatin group: rats treated with cisplatin (1.5 mg/kg b.wt/i.p).was administered as a single dose per week for another month; once treatment was completed, the animals were sacrificed with an injection of urethane (1g/kg body weight) [10]. All the animals in the various experimental groups received their blood and liver tissues. The serum was separated by centrifugation (at 2500 rpm for 10 minutes) and kept at 80 °C until further analysis. For histological and biochemical investigation, a sample of the liver tissue was preserved for 24 hours in 10% neutral buffered formalin.

2.5. Measurement of serum biochemical parameters.

ELISA (Sigma kit) was used to measure liver function assays (alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP).

2.6. Histology.

The liver tissues were formalin-fixed and dehydrated using an ethanol gradient, then washed in xylene and embedded in paraffin wax. Tissue blocks were sectioned at a thickness of 5-6 micrometers, deparaffinized, and stained with hematoxylin and eosin before being examined under a microscope [11].

2.7. Immunohistochemical assay.

On sequential segments of paraffin blocks cut at a 4-m thickness, the immunohistochemical examination of alpha-smooth muscle actin (α-SMA), cluster of differentiation 34 (CD34), and Alpha-fetoprotein (AFP) immunostaining were performed. The tissue segments were deparaffinized in xylene and rehydrated in evaluated ethanol. The deparaffinized tissue areas were treated with H_2O_2 for about 11min to hinder vague peroxidase response. The microwave antigen recovery was performed for 20 min in citrate buffer 0.01M. Following a wash with phosphate buffer saline (PBS), slides were brooded for 60 min at optimal room temperature with bunny monoclonal; Alpha-Smooth Muscle Actin Monoclonal Antibody (M0851, Dako, USA), Anti-CD34 immune response [QBEND-10] (ab8536) Abcam, and Anti-alpha 1 Fetoprotein immunizer [AFP-01] (ab3980) Abcam. The coupling site of essential antibodies was visualized utilizing the Dako EnVision ™ unit (Dako, Copenhagen, Denmark). The peroxidase response was envisioned by brooding the sections with diaminobenzidine (DAB) for 12 min. The segments were counterstained with Mayer's hematoxylin [12].

2.8. Statistical analysis.

All results were done using Statistical Package for the Social Sciences (SPSS, version 23). Quantitative data were expressed as mean ± standard deviation (SD). The comparison was made using the one-way ANOVA test to calculate statistics between different groups and the Correlation coefficient test to determine whether a linear correlation was positive or negative by ranking different variables against each other. The level of significance was set at a P-value of <0.05 [13].

3. Results

3.1. The phytonutrient analysis.

Palm date extract revealed the presence of sterols, phenolic, and flavonoids and the absence of tannins and Alkaloids [14].

3.2. The effects of liver enzymes and biochemical markers on serum levels.

The data obtained demonstrated that the positive control group had significantly higher liver enzymes (AST, ALT, and ALP) than the negative control group. The administration of date extract and cisplatin to rats with HCC resulted in lower levels of liver enzymes when compared to the positive control group, as shown in Table 1.

Table 1. Measurement of serum biochemical parameters.

Parameters Groups	ALT (U/L)	AST (U/L)	ALP (U/L)
Control	50.79±4.38^{**a}	66.68±9.82^{**a}	99± 2.8^{**a}
Positive group	244.76 ±14.57	269.78± 13.60	318.2±31.9
Palm date group	96.76±4.95^{**b}	155.25±14.18^{**b}	197.2±20.3^{**b}
Cisplatin group	94.6±10.8^{**b}	99.5±12.7^{**b}	147.2±15.4^{**b}

Analysis of serum samples of rats treated with palm date extract and cisplatin following CCl_4-induced HCC. Biomarker enzymes, namely, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and (alkaline phosphatase (ALP) Values, were expressed as mean±SD. Comparisons between the four groups represented statistical significance with a P-value of ≤0.05, indicating a significant difference, and a P-value of ≤0.001, indicating a highly significant difference. (**a) indicate a highly significant difference between the negative group and the positive group. (**b) indicate a highly significant difference between the treatment groups and the positive group.

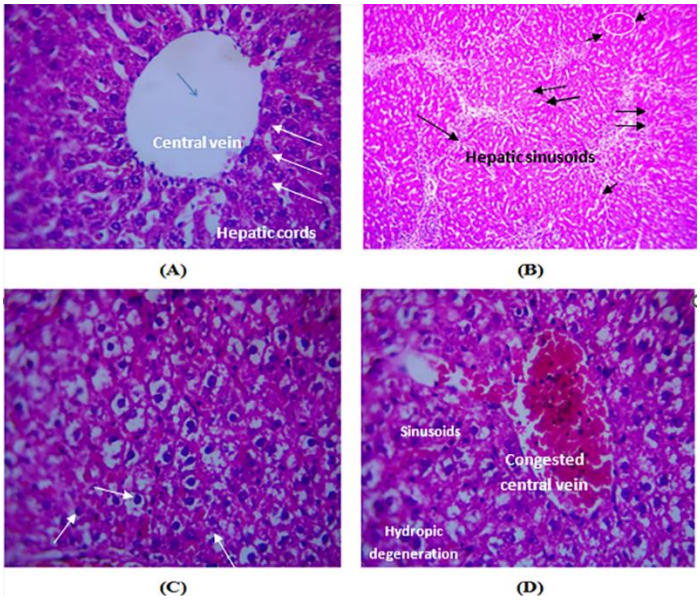

Figure 1. Haematoxylin and eosin (H&E) staining: Representative images of the liver sections (**A**, **B**, **C**, **D**) from rats treated with alcoholic date extract-induced HCC is shown above. (**A**) Control (Group A) section of normal rat liver showed normal hepatocytes, intact cell membrane, and central vein and hepatic cords formed from single cords separated by hepatic sinusoids (H&E x400); (**B**) Positive group (Group B) section of liver rat showed bridging bands of fibrosis lymphocytes. Thickened and large trabec >3cells immerging (Hcc) and hepatic sinusoids (H&E x100); (**C**) Date treated group (Group c) section of the liver of date extract showed hydropic and vascular degenerated of hepatocytes with normal liver pattern (H&E x400); (**D**) Cisplatin group (group D) section of rat liver showed hepatic congestion (congested central vein) marked hydropic degeneration and dilated sinusoids (H&E x400).

3.3. The effect on histology analysis.

Control group liver sections had a normal lobular pattern, central vein, and periphery portal veins. In contrast, animals treated with CCl_4 showed evidence of inflammatory cell infiltration. Hepatocytes with an increased nuclear-to-cytoplasmic ratio and dense connective tissue replaced the normal lobular pattern in the tissue. However, the administration of cisplatin and palm date extract promoted a partial to complete reversal of liver architecture. The histological changes in the ethanolic extract are shown in (Figure 1 and Figure 2).

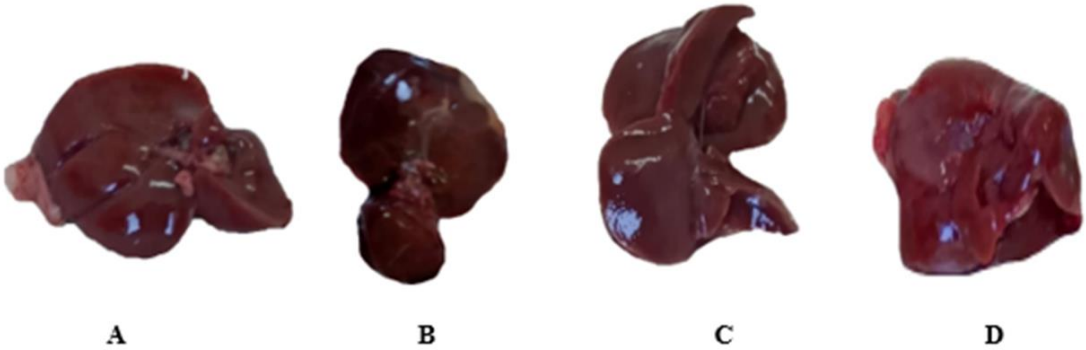

Figure 2. Photographs showing the macroscopic appearances of livers from different groups, (**A**) Control rats; Gross morphology of the healthy liver of the rat with a normal reddish-brown color. (**B**) Positive group, the structure of hepatic tissue disappeared, showing brown nuclear expression (red arrows) and cytoplasmic expression (black arrows). (**C**) Date group; (**D**) Cisplatin- showing date and cisplatin groups were similar to the normal group.

3.4. The effect on immunohistochemistry analysis.

Immunohistochemical analysis revealed a normal sinusoidal of α-SMA and a positive expression of CD34. In contrast, the negative expression of AFP in the control group, the HCC group (the positive control group), is a strong expression of α-SMA, CD34, and AFP, whereas the mild expression of α-SMA, CD34, and AFP in the date group, and a moderate expression of α-SMA, CD34, and AFP in the cisplatin as shown in Figure 3.

4. Discussion

Cancer is a complicated disease that affects worldwide health and has a significant economic impact. Although cancer treatment may include chemotherapy, radiation, and surgery, patients still suffer various side effects; even if the treatment is the same, side effects differ from person to person [5]. HCC is the world's fourth biggest cause of cancer death. It is the second-leading cause of cancer-related years lost globally, highlighting the significant disease burden of liver cancer [15], which accounts for 70–85 percent of all liver malignancies. It is the most common histological type of primary liver cancer [16]. As a result, the challenges and future research directions will be discussed, including natural product-relevant biomarkers for early detection, treatment stratification, and monitoring, as well as therapeutic approaches for prevention and treatment or reducing the risk of HCC with severe side effects.

Natural products have been used in traditional treatments and herbal therapies for a long time. They have a wide range of bioactivities components [17]. Compared to the costs of experimental approaches, chemotherapy by natural product gives effective results without side effects. In our work, we elevated the anti-cancer effect of palm date extract against HCC induced by CCl_4 in a rat model. Carbon tetrachloride causes hepatotoxicity through metabolic activation. It is regulated in the cytochrome P-450 located in the endoplasmic reticulum to

create CCl_3 (trichloromethyl free radicals), which reacts with the oxygen molecule quickly and forms a compound called (peroxy trichloromethyl free radicals) CCl_3O_2. Consequently, this induces oxidative stress causing liver damage by forming chain reactions called "propagation of lipid peroxidation.".

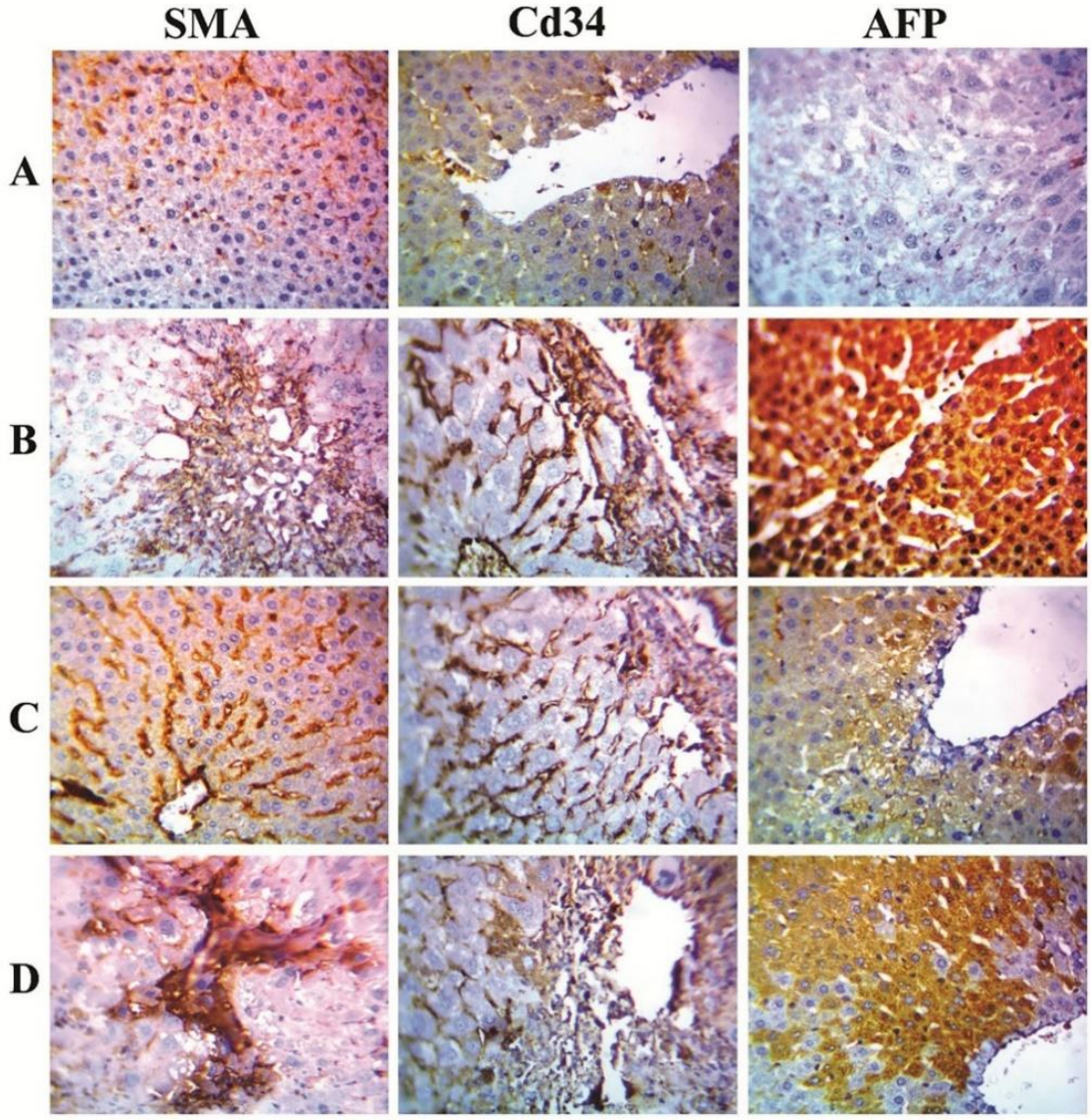

Figure 3. Immunohistochemical of alpha-smooth muscle actin, CD34, and alpha-fetoprotein expression; (**A**) Control group: Section of rat liver showed normal sinusoidal of α-SMA (IHC×400), the section of rat liver showed positive expression of CD34 in normal liver sinusoids (IHC×400), the section of rat liver showed negative expression of AFP (IHC×400); (**B**) Positive group: a section of rat liver showed strong expression of a α-SMA (IHC×400), section of rat liver showed strong expression of CD34 in fibrous bands (IHC×400), the section of rat liver showed strong expression of AFP (IHC×400); (**C**) Date group: sections of rat liver showed mild expression of α-SMA (IHC×400), the section of rat liver showed mildly increased expression of CD34 (IHC×400), the section of rat liver showed mild expression of AFP (IHC×400); (**D**) Cisplatin group: section showed moderate expression of α-SMA(IHC×400), section showed moderate expression of CD34 (IHC×400), section showed moderate expression of AFP (IHC×400).

Hepatocytes are attacked by free radicals, which activate kupffer cells, generating plentiful cytokines that activate inflammatory cells and cause inflammation [18]. The date palm is one of the most important plants cultivated in the Middle East. There are several date palm varieties, some of which produce soft fruits, like the palm date, which was recently introduced to Egypt and is now widely distributed throughout the country [19]. With a total production of nearly 1.6 million tonnes in 2019, Egypt is the top date fruit-producing country [20]. To prove

our hypothesis, we compare the component of palm date extract and cisplatin, palm dates that were high in energy and nutrition (reducing sugar and high in dietary fiber, vitamins, and minerals). According to a previous study, the glucose to fructose ratio in date palm fruit during the Khalal stage in the palm cultivar was roughly 1:1. phenolic, flavonoid, and carotenoid compounds were identified in date palm fruits at the Khalal stage of palm cultivars, according to our phytonutrient analysis results [21].

Cisplatin is a well-known chemotherapeutic drug that inhibits the proliferation of cancer cells through a specific mechanism and has remarkable clinical results [22]. Once inside the cell, it causes programmed cell death by binding to DNA, forming intra-strand DNA adducts, and blocking DNA synthesis and cell development. Despite the fact that treatment has been proven to be successful, chemotherapy has failed to eradicate cancer cells due to cancer cells' susceptibility, and individuals with cancer have died as a result [23].

Although cisplatin opened a new phase in cancer treatment, it had a double-edged sword. Furthermore, Nephrotoxicity and hepatomegaly seem to be major side effects of cisplatin. As a result, scientists have sought to develop new methods for substituting chemotherapy with natural compounds with strong anti-tumor effects. Palm date is a flavonoid with potent anti-tumor action against various malignancies and can be used instead of cisplatin to improve and help accelerate cancer therapy. Furthermore, using palm date to reduce the negative effects of cisplatin is beneficial. In this paper, we investigate the role of palm date in enhancing cisplatin's anti-tumor activity as a pharmacological property [24]. Because of its anti-cancer potential through inducing apoptosis, the safety of palm date has been confirmed in animal models, and it is documented to be nontoxic even at large dosages. The systematic use of natural compounds produced from plants in cancer therapy has resulted in low toxicity and effective therapeutic results. According to a previous study, the alcoholic extract of palm dates can suppress the development of human cancer cells [25].

Our study demonstrated that in our research, an ethanolic extract of palm date fruit improved the reversal of (CCL4) (1ml/kg b.wt) damaged liver approaching normal. Through the restoration of liver enzymes, palm date fruit extract has been demonstrated to enhance liver function and suppress HCC. According to a study, the administration of palm date 400 mg/kg b.wt/day intraperitoneally) in albino rats decreased the liver enzyme level. It is essential to evaluate the activity of the liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), which was significantly reduced after receiving palm date therapy. As a result, it can be utilized as traditional therapy for the treatment of HCC, which can, therefore, be used as conventional therapeutics for the treatment of HCC.

Following the administration of cisplatin (1.5 mg/kg b.wt/i.p). ALT and AST levels are supposed to be decreased compared to CCL4 and the control group [26]. A previous study indicated that a lower dose of date ethanolic extracts resulted in early apoptotic cells, whereas a higher dose resulted in late apoptotic stages [8]. Furthermore, a date alcoholic extract promoted apoptosis in cancer cells [27,28], which were raised following DENA delivery, indicating oxidative tissue-damaging effects, similarly found improvement of serum cytokines to approximately normal levels after treatment with date fruit. DENA-induced inflammatory cytokine changes correlated to DENA-induced hepatocarcinogenesis. Our result elevated liver enzyme levels in CCl_4-induced HCC compared to the normal values of the control group. A similar study was conducted to investigate the neuroprotective effect of aqueous *P. dactylifera* fruit [29]. As a result, date fruit contains many bioactive compounds with promising cancer-

fighting potential [30]. A substantial increase in the size of the liver and the activity of serum liver enzymes, which is high in liver cirrhosis, increased significantly. The level of liver enzymes was also significantly reduced in the palm date extract and cisplatin groups compared to the control group. As specimens stained with H&E showed severe lobular inflammatory cell infiltrations, portal bridging of fibroblasts with pseudolobulation, and many hepatocytes showed vacuolar degeneration and necrobiotic alterations. Histopathological studies confirmed liver damage. Black arrowheads show the nuclei in the CCL4 group (exhibiting abnormal morphology). The hepatoprotective efficacy of the polyphenols in palm date fruit extract against liver damage caused by CCL4 was evaluated following the administration of palm date fruit extract and cisplatin.

The results demonstrate a normal or near-normal hepatocyte architecture with regularly orientated hepatocytes close to the control. Normal histology and biochemistry are maintained after dosing extract. Our study's immunohistochemical results showed that the immunohistochemical tumor markers (α-SMA, CD34, and AFP) were strongly expressed with the hepatic disorder [31,32]. In tumor cell proliferation and migration, α-SMA expression is up-regulated. It is evident that α-SMA activated hepatic stellate cells (HSCs) can be activated or transdifferentiated into a myofibroblast-like cell in liver tumors by intercellular communication between HSCs and damaged hepatocytes. Also, its effect on hepatic immunity as a potent suppressor by affecting T-cell responses and, thus, leading cause in the progression of HCC. As over expressions of α-SMA indicate the tumor cell, normal hepatic tissues revealed normal expression of α-SMA.

In contrast, in the HCC group, treatment with palm date fruit and cisplatin produced a similar insignificant effect to the control group. However, treatment with palm date and cisplatin significantly decreased α-SMA expression. Still, the palm-treated group showed mild expression close to the control group compared to the cisplatin group, which showed moderate expression of α-SMA. The current data support the relevance of α-SMA expressing activated HSCs in carcinogenesis, which is believed to be a future target for anti-cancer therapy in HCC. CD34 is a glycosylated transmembrane protein found in hematopoietic progenitor cells and endothelial cells and plays a significant role in adhesion molecules.

CD34 is expressed by capillaries sinusoids in chronic liver disorders and malignant vascular tumors of the liver. Our findings agree with [33,34], who found overexpression of endothelial cell marker CD34 with gradual progression in the normal liver, which showed weakly expressed cirrhosis to HCC. Interpreting this result might contribute to designing efficient and safe anti-angiogenic therapy for liver disorders. Previous studies have verified that AFP regulates the growth of both normal and cancerous cells. However, increased expression suggests that AFP inhibits tumor cell development. So AFP is considered a hallmark and monitor of abnormal conditions, including (HCC) [35]. Based on previous results, we conclude that α-SMA, CD34, and AFP are highly effective biomarkers for HCC.

Finally, this study investigated the therapeutic ingredients of the palm date fruit extract in HCC progression. This study provides facts on the phytonutrient composition of date fruits and seeds and highlights palm date as a natural medicinal agent for various chronic diseases. Due to this essential fruit's functional food and nutritional features, the study also emphasizes the necessity of consuming dates as a wonderful combination for leading a healthy lifestyle. As a result, our findings may support industry and researchers in further exploring the potential date fruit for future medical and nutraceutical uses in cancer therapy [36]. The presented study

has shown that date fruit extract had a protective effect against CCL4-induced changes in the liver.

5. Conclusions

The major objective of the study is to provide an overview of the value of palm date fruit extract's putative anti-cancer actions, which are rich in minerals, vitamins, and phenolic diseases and might function as an anti-cancer agent against Hepatocellular carcinoma. The key apoptotic processes in HCC cells treated with palm date fruit extract were explored further in this work.

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Conflict of Interest

The authors declare no conflict of interest.

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