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Original Article

Amelioration of Diabetes and Nonalcoholic Fatty Liver Disease by Pistachio Kernel Protein Hydrolysate in Rats

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ABSTRACT

Diabetes Mellitus (DM) and Nonalcoholic Fatty Liver Disease (NAFLD) are prevalent conditions that affect the metabolism and can lead to liver injury. They are often associated with obesity and insulin resistance. Protein Hydrolysate of Pistachio Kernel (PHPK) is a natural product obtained from pistachio proteins by enzymatic hydrolysis. It has been reported to have antioxidant, anti-inflammatory, and antidiabetic properties. We investigated the hepatoprotective effects of PHPK in rats with type 1 DM or NAFLD induced by a high-sugar diet. We used 96 male Wistar rats and divided them into four groups: Control, NAFLD, Diabetic, and PHPK-treated (5, 50, 500 mg/kg). We fed the rats with different diets for 8 weeks and then administered PHPK orally for 4 weeks. We collected blood and liver samples for biochemical and histopathological analysis. We found that DM and NAFLD increased the levels of liver enzymes, cholesterol, and triglycerides in the blood and caused hepatic damage, as shown by distorted liver architecture, necrotic hepatocytes, sinusoidal dilatation, and kupffer cell proliferation. PHPK administration reduced the severity of these alterations and improved the liver function and morphology in rats with DM and NAFLD. Our results suggest that PHPK has beneficial effects on DM and NAFLD, indicating its potential as a natural remedy for these disorders. Future research is needed to identify the specific compound(s) responsible for its antidiabetic effects and to elucidate its mechanism of action.

Abbreviations
Protein Hydrolysate of *Pistachio Kernel* (PHPK)
Pistachio Kernel Powder (PKP)
Streptozotocin (STZ)
Fasting Blood Sugar (FBG)
Alanine Aminotransferase (ALT)
Alkaline Phosphatase (ALP)
Lactate Dehydrogenase (LDH)
Aspartate Aminotransferase (AST)
Hemoglobin A1C (HbA1C)
Plant-derived Protein Hydrolysates (PHs)
Diabetes Mellitus (DM)

INTRODUCTION

Diabetes Mellitus (DM) is a chronic metabolic condition with extensive consequences [1] . DM can have a devastating effect on various organs such as the liver [2]. It is one of the leading causes of liver failure in the United States [3]. It has been noted that the prevalence of liver disease in diabetic individuals as well as the incidence of diabetes in patients with liver disease is complicated [4] Diabetes causes a variety of liver problems, including abnormal elevations in liver enzymes, liver cirrhosis, liver cell cancer, chronic liver disease, and abrupt liver failure

[5] On the other side, it is widely recognized that a high-sucrose diet may cause nonalcoholic fatty liver disease, a collection of disorders that includes abnormal elevations in liver enzymes, liver steatosis, and liver cirrhosis [6]. As a result of their lengthy history of usage in the treatment of ailments, plants have been deemed helpful. In addition, population increase, exorbitant treatment costs, and the adverse effects of numerous synthetic medications used to treat a variety of ailments have led to a larger focus on plants used in traditional medicine [2].

Due to its high quantity of unsaturated fatty acids, antioxidants, and other physiologically active chemicals, and therefore their potential health advantages, nut intake (such as pistachios) has gained more attention in recent years [7, 8]. Due to the presence of luteolin and polyphenolic components, including flavonoids and anthocyanins, pistachio nut offers significant antioxidant effects [9, 10] Many studies have proved the health advantages of nuts, especially in connection to the improvement of dysmetabolic disorders such as type 2 diabetes. In comparison to other nuts, pistachios have the greatest quantities of unsaturated fatty acids, potassium, α-tocopherol, phytosterols, and xanthophyll carotenoids, all of which are recognized for their antioxidant and anti-inflammatory qualities [11, 12]. Pistachio nuts reduce fasting blood sugar (FBG) and homeostatic model evaluation for insulin resistance considerably, although hemoglobin A1C (HbA1c) and fasting plasma insulin may not improve much in persons with or at risk for type 2 diabetes [13]. This comprehensive array of macronutrients and micronutrients indicates that pistachio nuts should have positive effects on the inflammatory process and oxidative state [14, 15].

Since its pathophysiology closely mimics that of human type 1 diabetes, the diabetic rat generated by streptozotocin (STZ) has been extensively utilized as an animal model for the study of diabetes and its consequences. In addition, the STZ-induced animal model is more manageable and less costly than genetically engineered animals. The purpose of this research was to determine if supplementation with a protein hydrolysate of pistachio kernel (PHPK) induces beneficial changes in rats with streptozotocin (STZ)-induced diabetes or sucrose-induced NAFLD. Due to the significance of numerous pistachio byproducts and their application in many illnesses with minimal side effects, high protein, fiber, and antioxidant content, the efficiency of PHPK supplementation in rats with type 1 diabetes was also investigated.

MATERIALS AND METHODS

Preparation of Protein Hydrolysate of Pistachio Kernel

The PHPK was prepared using an enzymatic method reported by Li *et al*, with some modification [16]. Pistachios were shelled to retrieve the kernel, which was then ground in a food processor to make pistachio kernel powder (PKP). PKP was then combined with 10% w/v distilled water and digested with pepsin (5000 U/g powder, pH 1.8) for four hours at 37 °C. After 10 minutes in a bath of boiling water, the hydrolysis process was halted and the pH was corrected to 7.7. Under the same circumstances, the mixture was digested with trypsin (5000 U/g powder) for an additional six hours. After heating the hydrolyzed solution in a boiling water bath for 10 minutes, it was centrifuged at 9000 g at 4 C for 25 minutes. As pepsin–trypsin hydrolysate, the supernatant was collected and lyophilized. Pepsin hydrolysate and trypsin hydrolysate were also obtained at the same time.

Induction of Diabetes

A single intraperitoneal injection of 60 mg/kg streptozotocin was administered to rats (STZ). Following 72 h, the FBG of the treated animals was determined, and rats with FBG300 mg/dl were categorized as diabetic [17]. Using a glucometer (Media Smart, Switzerland) and a drop of blood from the tail vein (after washing the tail with 97% ethanol), the FBG levels were calculated.

Animals and PHPK Administration

We used 96 male Wistar rats of similar age and weight (250-300 g) and randomly assigned them to 12 groups (n=8). A veterinarian checked each animal for any disease signs. Then, a computer allocated and numbered the animals. We followed the ethics committee of Rafsanjan University of Medical Sciences (IR.RUMS.REC.1398.161) and kept the animals for eight weeks in a standard environment at 22±2°C, 25 - 30% humidity, and a 12-hour light and dark cycle. We divided the animals into four groups: Control (C0), Nonalcoholic Fatty Liver Disease (NAFLD) (C1), Diabetic (C2), and PHPK-treated (T1-T9). The C0 group received saline solution

intraperitoneally and a standard chow diet. The C1 and C2 groups received saline solution and a high-sugar diet (30% sucrose, 20% casein, 10% corn oil, 4% salt mixture, 3.5% vitamin mixture, and 32.5% cellulose) for 8 weeks to induce NAFLD and diabetes, respectively [6]. The PHPK-treated group had nine subgroups, each receiving a different dose of PHPK (5, 50, or 500 mg/kg body weight per day) orally, dissolved in saline [17]. The T1, T2, and T3 subgroups received PHPK and a standard chow diet. The T4, T5, and T6 subgroups received PHPK and the high-sugar diet and had NAFLD. The T7, T8, and T9 subgroups received PHPK and the high-sugar diet and had diabetes. The experiment lasted for 8 weeks [17, 18].

Body Weight

At the beginning of the study, the average body weights of the experimental groups were comparable. Following treatment, the body weights of all experimental groups were compared to those of the control group and analyzed.

Measurement of Biochemical Parameters

After eight weeks, the rats were anesthetized with ketamine (0.5 mg/kg) and xylazine (2 mg/kg). Blood samples were taken from the corners of their eyes. Serum samples were transported to the Rafsanjan University of Medical Sciences Laboratory to measure glucose, aspartate aminotransferase (AST), Alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), cholesterol, and triglyceride. These parameters were measured by autoanalyzer (BT-1500, Biotecnica instruments SPA, Roma, Italy) and corresponding assay kits (Shanghai Jianglai Biotechnology Co., Ltd., Shanghai, China).

Histopathology

Tissue samples were taken from the liver and fixated in 10% formalin solution for 48 h. After that, tissues were dehydrated through an ascending alcohol series and cleared ready with xylene for paraffin wax embedding. 4 μm-sections were taken using a microtome (Leica RM 2135); stained with hematoxylin-eosin and examined by the pathologist of Rafsanjan University of Medical Sciences.under the light microscope (Olympus CX33, Tokyo, Japan).

Statistical Analysis

The statistical analysis was performed using SPSS (Ver 19.0). The Kolmogorov-Smirnov test validated the data's normal distribution. One-way analysis of variance (One-Way ANOVA) was performed to analyze the data statistically. P-value correction was performed using the Tukey–Kramer multiple comparison test, and the significance threshold for all tests was set at $P < 0.05$.

RESULTS AND DISCUSSION

Body Weight

The ingestion of PHPK by the T1, T2, and T3 groups had no significant effect on body weight compared to the C0 group (Fig 1). Rats with NAFLD produced by sucrose had a significantly lower body weight than the control animals. Comparing the C1 group to the T4, T5, and T6 groups, consumption of PHPK substantially increased body weight. In the T7, T8, and T9 groups, the ingestion of PHPK led to a considerable rise in body weight compared to the C2 group. The STZ-induced diabetic rats had a significantly lower body weight than the control animals. The treatment of PHPK significantly raised the animals' body weight in a dose-dependent manner. Compared to the starting body weight, the rise in body weight at 500 mg/kg was greater than that at 5 mg/kg in STZ-induced diabetic rats.

Measurement of Biochemical Parameters

Compared to the control group (C0), PHPK ingestion at dosages of 50 and 500 mg/kg (T2 and T3, respectively) substantially lowered AST levels (Fig 2a, b, and c). AST, ALT, and ALP levels were considerably higher in the NAFLD (C1) and diabetes (C2) groups compared to the control group (C3) (C0). In sucrose-treated mice receiving doses of 5, 50, and 500 mg/kg PHPK (T4, T5, and T6 groups) and in diabetic animals receiving the same dosages of PHPK (T7, T8 and T9 groups), the AST, ALT, and ALP dropped considerably compared to the sucrose group (C1) and the diabetes group (D1) (C2). Our findings revealed that ingestion of 50 and 500 mg/kg of PHPK in the T2 and T3 groups substantially lowered cholesterol levels compared to the control group (C0) (Table 1). Significantly greater LDH, cholesterol, and triglyceride levels were seen in the sucrose (C1) and diabetes (C2) groups compared to the control group (C0).

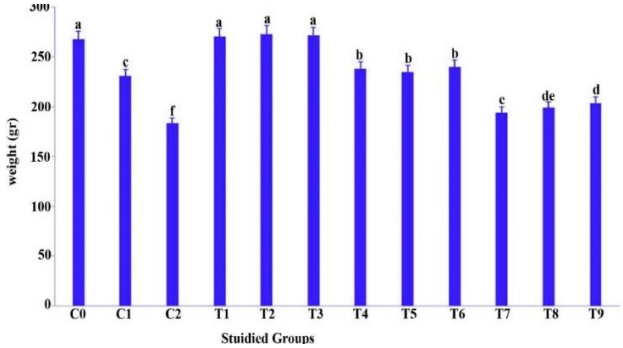

Fig. 1 Effects of PHPK on body weight in high-sugar diet and STZ-induced diabetic rats. Different letters above bars indicate significant differences among groups ($p < 0.05$). Data are expressed as Mean±SD (n=8).

The LDH, cholesterol, and triglyceride levels in sucrose-treated rats (T4, T5, and T6) ingested PHPK at dosages of 5, 50, and 500 mg/kg were considerably lower than in the NAFLD group (C1).

In addition, the levels of LDH, cholesterol, and triglycerides were considerably reduced in diabetic animals (T7, T8, and T9) after administration of PHPK at dosages of 5, 50, and 500 mg/kg compared to diabetic rats in the C2 group. Compared to the control groups (C1 and C2), T2 and T3 groups consuming PHPK at dosages of 5, 50, and 500 mg/kg shown a substantial reduction in glucose concentration (Table 1). Significantly higher glucose levels were seen in the sucrose (C1) and diabetes (C2) groups compared to the control group (C0).

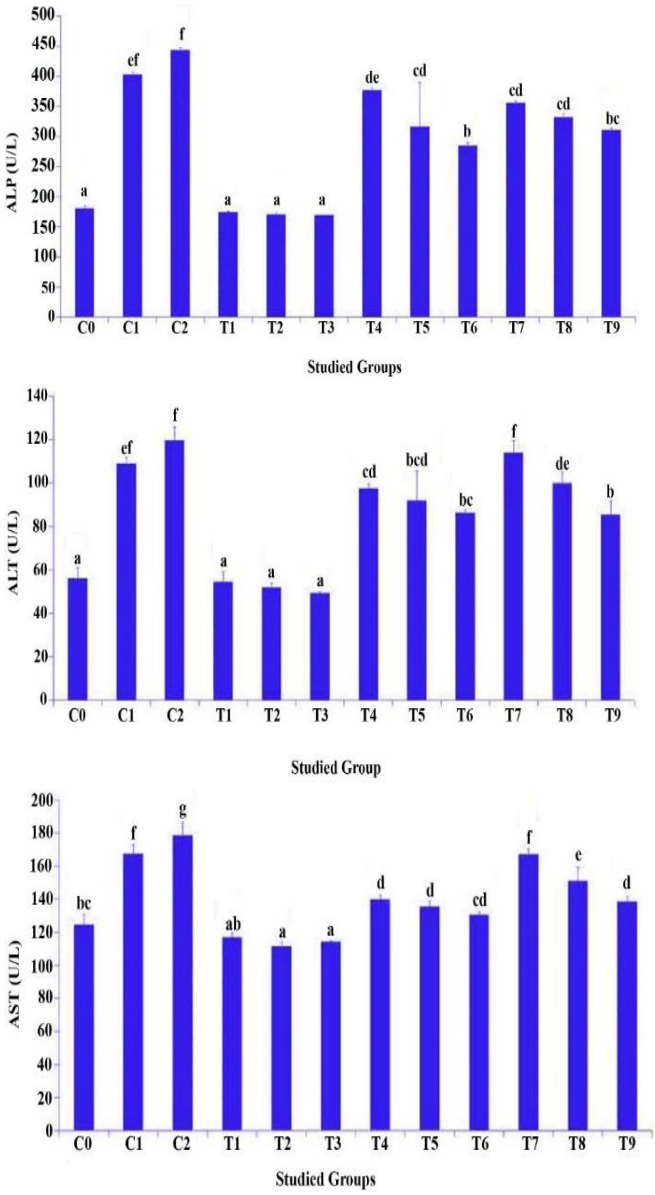

Fig. 2 Effects of PHPK on AST (a), ALT (b), and ALP (c) levels in high-sugar diet and STZ-induced diabetic rats. Different letters above bars indicate significant differences among groups ($p < 0.05$). Data are expressed as Mean±SD (n=8).

Table 1 Effects of PHPK on LDH, Triglycerides, and Cholesterol levels in rats

Test groups	Cholesterol (mg/dl)	Triglycerides (mg/dl)	LDH (U/l)	Gglucose mg/dl
C0	86.25 ± 3.77 c	73.25 ± 3.40 ab	187 ± 3.06 a	96 ± 2.16 a
C1	109.25 ± 1.26 de	107 ± 3.92 cd	460.25 ± 3.43 g	328 ± 3.06 cd
C2	133.3±5.51 f	127 ± 3.61 d	481.66 ± 4.50 h	361 ± 0.09 d
T1	83.5 ± 3.54 d	76.5 ± 6.36 abc	184 ± 1.41 a	97.01 ± 3.04 ab
T2	75.33 ± 3.06 b	72.3 ± 2.46 ab	180.66 ± 5.51 a	97.86 ± 2.06 b
T3	65.5 ± 4.95 a	57.5 ± 2.12 a	180 ± 2.82 a	98.51 ± 1.98 b
T4	103.5 ± 3.54 d	55 ± 3.71 a	427.5 ± 2.61 f	319.14 ± 1.47 cd
T5	90.25 ± 5.19 c	67 ± 2.98 ab	395.25 ± 3.63 e	316 ± 2.68 b
T6	87.00 ± 1.01 c	69 ± 5.01 ab	350.5 ± 5.51 d	310.97 ± 0.86d
T7	114 ± 5.54 e	97.3 ± 4.91 bcd	400.66 ± 3.03 e	356.05 ± 1.41 e
T8	105.00 ± 4.08 d	63.25 ± 4.45 ab	324.4 ± 5.72 c	351.16 ± 2.78 c
T9	88.5 ± 2.65 c	53.25 ± 3.14 a	279 ± 5.59 b	341.25 ± 4.18 bc

Results are expressed as Mean±SD, (n=8)

Different letters above bars indicate significant differences among groups ($p < 0.05$). Data are expressed as Mean±SD (n=8).

In sucrose-treated mice (T4, T5, and T6), the glucose level was considerably reduced following administration of PHPK at dosages of 5, 50, and 500 mg/kg compared to the NAFLD group (C1). In addition, the glucose level was considerably reduced in diabetic animals (T7, T8, and T9) following administration of PHPK at dosages of 5, 50, and 500 mg/kg compared to diabetic rats in the C2 group (Table-1).

Histopathology

The C0 control group got intraperitoneal injections of physiological saline and a regular food on the first day. Normal architecture was not seen in the liver tissue (Fig. 3a). The C1 control group was given 30% sucrose in their drinking water; liver tissue exhibited intact architecture and minimal hydropic degeneration (Fig. 3b). Significant pathological alterations, including as vacuolization, hydropic degeneration, and necrosis of hepatocytes, were generated in the animals' livers by STZ injection in the C3 control group (Fig. 3c). The T1, T2, and T3 groups were supplemented with 5, 50, and 500 mg/kg of PHPK, respectively, to their usual diets. The liver architecture was intact in T1, T2, and T3, but in T2 and T3 we observed modest hydropic degeneration and kupffer cell proliferation (Fig. 3d, e and f). The T4, T5, and T6 groups got 30% sucrose in their daily drinking water and were administered 5, 50, and 500 mg/kg of PHPK daily, respectively. The liver of those in the T4 group had hydropic degeneration (Fig. 3g). In the T5 group, we observed maintained liver architecture along with hydropic degeneration and portal vein congestion (Fig. 3h). The liver of the T6 group exhibited intact architecture, modest hydropic degeneration, and scattered infiltration of inflammatory cells into the portal system (Fig. 3i). T7, T8, and T9 target groups of STZ-induced diabetic rats fed a normal diet and administered daily doses of 5, 50, and 500 mg/kg of PHPK, respectively, were evaluated. T7 group liver tissue had an aberrant architecture, some necrotic hepatocytes (less than C2 group), sinusoidal congestion, and an increase in binucleated hepatocytes (Fig. 3j). In the T8 group, liver tissue exhibited maintained architecture, fewer necrotic hepatocytes (compared to the C2 and T7 diabetic groups), sinusoidal dilatation, and kupffer cell proliferation (Fig. 3k). We observed maintained architecture, sinusoidal dilation and congestion, kupffer cell multiplication, and central vein congestion in the T9 group (Fig. 3l).

There are lots of species of plants which are used to treat diabetes and show hepatoprotective potential [19]. Scientists have recently proved that plant-derived protein hydrolysates (PHs) have the potential to treat illnesses such as diabetes due to their nutritional value and resistance to abiotic stress. PHs are produced enzymatically from plant and plant byproducts [20].

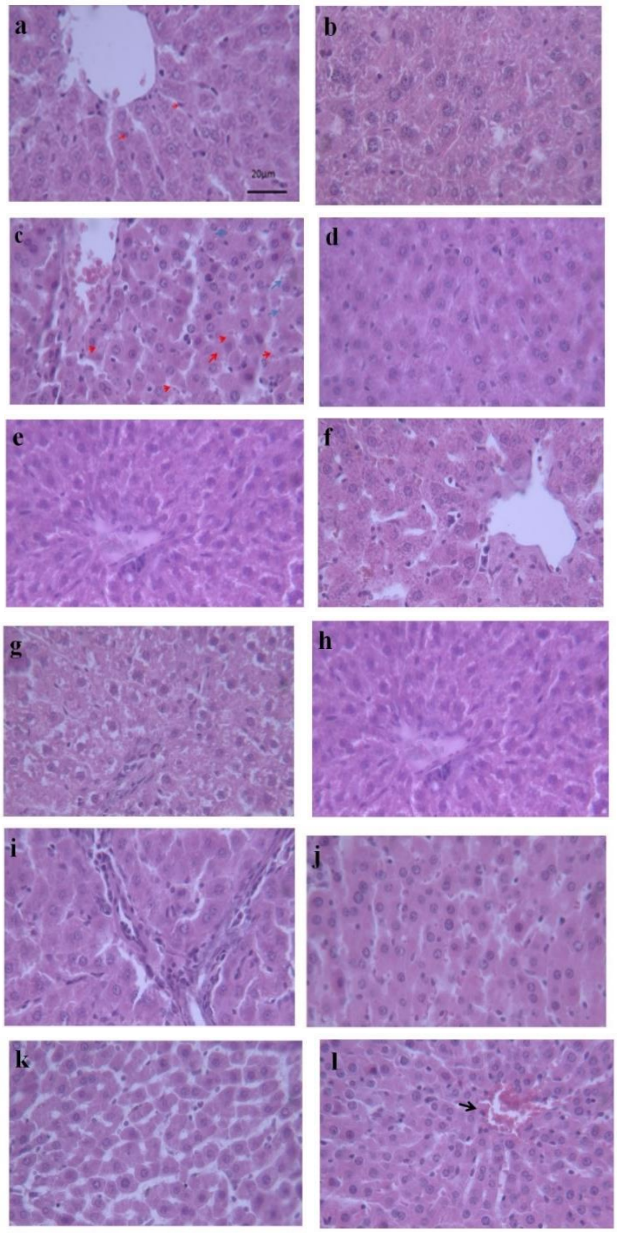

Fig. 3 hematoxylin and Eosin-stained cross-sections of the liver in high-sugar diet and STZ-induced diabetic rats and regeneration following eight weeks for seeing the effects of PHPK on liver tissue of rats (a) C0 group showed normal kupffer cells (red arrows). (b) C1 group. (c) (C2) group showed vacuolization (Blue arrows), hydropic

degeneration, and necrosis of hepatocytes (red arrows). (d) T1 group. (e) T2 group. (f) T3 group. (g) T4 group. (h) T5 group. (i) T6 group. (j) T7 group. (k) T8 group. (l) T9 group: central vein congestion (Black arrow) (×400 magnification).

Hence, PHs might be studied as a viable treatment option for some illnesses in order to examine their synergic effects in conjunction with conventional medications [21].

In accordance with previous reports, we also found that induction of diabetes reduced the weight in animals [22]. In addition, plasma levels of ALP, AST, and ALT rose due to the hepatotoxic effects of diabetes and NAFLD. In addition, diabetic and NAFLD animals have aberrant levels of LDH, cholesterol, and triglycerides [23] . We found that PHPK alleviated these harmful effects compared to diabetic or NAFLD mice in untreated groups, hence reducing liver damage and demonstrating PHPK's hepatoprotective activity.

In agreement with our findings, earlier investigations have indicated that cytoplasmic changes, inflammation, hepatic sinusoidal congestion, and necrosis occur in the hepatocytes of rats with diabetes or NAFLD [6, 24]. Additionally, the liver cell nuclei become greater and also showed nuclear inclusions [25]. Additionally, our data shown that pathological lesions in the livers of diabetic rats are reversible and return to normal after eight weeks of treatment with PHPK.

Treatment with PHPK demonstrated a potential therapeutic effect on diabetes mellitus. PHPK reduced the onset of diabetes in STZ-treated rats by decreasing HbA1c, FBG, two-hour postprandial glucose, and four-hour postprandial glucose, and by boosting insulin levels [18]. Moreover, Bagheri-Hosseinabadi *et al*., showed that treatment with PHPK has nephroprotective effects in STZ-injected Wistar rats via improving the renal function tests [26]. In contrast, PHs such as PHPK may include a variety of nutrients, including soluble peptides and proteins, carbohydrates, and phenols. Consequently, they may use many paths and processes to improve nutritional quality [27]. Total phenolic and flavonoid content and value measurements It suggests that pistachio kernel might be regarded an antioxidant-rich component [28]. In a separate investigation, 3-(8-pentadecenyl)-phenol, 3-(10-pentadecenyl)-phenol, 3-pentadecyl-phenol, and 3-(10-eptadecenyl)-phenol were shown to be the most prevalent cardanols in pistachio kernel [29]. Numerous studies on pistachio kernels reported the phytosterols, fatty acids, Anthocyanins, chlorophylls and xanthophylls [30]. According to other studies, the anacardic acids, carotenoid, chlorophyll, and chlorophyll-derived chemicals in pistachio kernels possess anti-cancer properties [31]. Moreover, it is well documented that herbal plants or agents with antioxidant properties such as linoleic acid [6], *Oenanthe javanica* [32], quercetin [33] could reduce the hepatic damage induced by NAFLD or diabetes. Likewise, PHPK's antioxidant properties may alleviate the liver damage caused by diabetes or NAFLD. Using the PHPK considerably reduced the symptoms of hepatic problems in rats with sucrose-induced NAFLD and STZ-induced hyperglycemia, according to this experimental investigation. In rats with NAFLD and diabetes, administration of PHPK increased the levels of liver enzymes and lowered the levels of triglycerides, total cholesterol, and LDH. These statistics do not include the complete spectrum of liver injuries. There are a growing number of models and medication therapies, however they have not been properly examined. Each animal model replicates just specific features of the pathophysiology of hepatic damage and may not fully represent the disease's progression in people. On the other hand, it should be underlined that accurate modeling of DM is necessary for preclinical testing of antidiabetic drugs such as PHPK, and that the use of several models permits the confirmation of experimental findings extrapolated to DM patients.

Declaration of Interest

The authors declare that there is no conflict of interest.

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