PROTECTIVE EFFECT OF POMEGRANATE PEEL EXTRACT ON DIETARY-INDUCED NON-ALCOHOLIC FATTY LIVER DISEASE

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver disorders that is generally associated with abnormal liver function test results. Pomegranate has been described as the nature's power fruit, and shown to have various health benefits. This study aims to explore the role of aqueous pomegranate peel extract (PPE) in attenuating NAFLD induced by high fat diet (HFD). 24 male guinea pigs were allocated into three groups (eight per group). Control (CON) group received normal chow diet. High fat diet (HFD) group, consumed HFD over six weeks to induce NAFLD. HFD-PPE group received HFD for six weeks followed by four weeks administration of PPE tea along with HFD. All animals survived until termination of the experiment, and were ultimately sacrificed at scheduled time (six weeks for the CON and HFD groups and ten weeks for the HFD-PPE group). NAFLD was evaluated histologically and by measuring serum levels of liver enzymes. There was a significant increase in the serum levels of cholesterol ($p=0.01$), triglyceride ($p=0.01$), alanine transaminase [ALT] ($p=0.01$) and aspartate transaminase [AST] ($p=0.001$) in the HFD group compared to the control. These levels were significantly lower in the HFD-PPE group compared to the HFD animals ($p=0.05$, 0.01, 0.01 and 0.001) for serum concentrations of cholesterol, triglyceride, ALT and AST respectively. A clear reduction in the extent of intrahepatocytic fat deposition was observed in the HFD-PPE animals as compared to the HFD group. This study demonstrates the potential role of PPE in the alleviation of HFD-induced NAFLD. While further studies are required to clarify the underlying mechanisms, these findings may provide the foundation for further nutritional and therapeutic developments.

KEYWORDS: HFD, NAFLD, Pomegranate, Liver enzymes.

INTRODUCTION

During the last few decades, the prevalence of overweight and obesity has increased substantially throughout the world. Overweight and obesity have been identified as key risk factors for a wide range of chronic diseases including Non-alcoholic fatty liver disease [NAFLD] (1). NAFLD is asymptomatic disease that is characterised by excessive fat accumulation in hepatocytes and commonly associated with abnormal liver function tests (2). NAFLD is considered to be one of the most common liver disorders in the world (3). In the United States, for instance, it is estimated that about 30% of adults are affected by NAFLD, while the rate of incidence raises to over 70% in morbidly obese individuals (4). Unfortunately, there is still a lack of accurate statistics upon which to make reliable estimates of the prevalence of NAFLD in Libya. Although there are no definite therapeutic drugs for NAFLD approved by regulatory agencies, some pharmaceutical agents have been recommended, e.g. pioglitazone and vitamin E, but these are not exempt from detrimental effects (5, 6). Recently, there has been an emphasis on the use of herbal remedies with many people around the world resorting to phytonutrients or nutraceuticals for treatment of numerous health challenges in various national healthcare settings (7).

Pomegranate (*Punica granatum*) is one of the oldest edible fruits that has been known since the ancient times in the Middle East and the Mediterranean (8). Pomegranate trees are long-lived, drought tolerant, and can adapt to adverse ecological conditions. Therefore, they are widely planted in various arid and semi-arid regions of the world. Anatomically, the pomegranate tree can be divided into different components, including: root, bark, leaf, flower, as well as peel and seed (9).

The pomegranate has been honored by being mentioned twice in the holy Quran, and designated as an example of the fruits that grow in the gardens of paradise (9). Owing to its numerous beneficial effects, the pomegranate has recently been described as the nature's power fruit (10) that has been extensively used as a medical functional food (11). Various studies have investigated the role of pomegranate fruits and extracts as possible therapeutic agents, and so far, they have shown a remarkable potential in promoting several health properties. For instance, pomegranate juice supplementation to atherosclerotic mice resulted in a significant reduction in the size of the atherosclerotic lesion as well as the number of foam cells, as compared to the control group (12). Similarly, long-term consumption of pomegranate juice has also been shown to provide a great protection against cardiotoxicity and restore cardiac properties back to normal status (13). In patients with carotid artery stenosis, daily intake of pomegranate juice for three years resulted in a remarkable improvement in the blood pressure, low-density

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lipoprotein oxidation as well as the thickness of common carotid intima-media [14].

About 50% of the total weight of pomegranate fruits corresponds to their peel [15]. Pomegranate peel is a primary byproduct which, if not reprocessed properly, may cause essential sanitary problems, and become a possible source of environmental pollution. Several studies have recently been carried out to assist in the recycling of this refuse into a wide range of valuable products [16, 17]. Compared to the pomegranate pulp, the peel seems to have more potential health promoting effects which are apparently attributable to its higher antioxidant capacity [15]. Interestingly, among various components (e.g. peel, pulp, and seed) of 28 types of commonly consumed fruits in China, the pomegranate peel has been reported to have the most powerful antioxidant and anti-inflammatory activities [18].

Although many experimental studies have demonstrated the health beneficial effects of different pomegranate extracts, only few are available on the association between pomegranate peel and liver functions, and the results seem to be contradictory [10, 19]. Indeed, the efficacy of the medicinal plant is mainly determined by its chemical composition, which has been reported to be influenced by a variety of factors including environmental and agronomic conditions, harvest time, geographical variations as well as method of extraction [20, 21]. With this in mind, we aim to explore the potential role of pomegranate peel aqueous extract (PPE) in attenuating NAFLD induced by high fat diet (HFD) in guinea pigs.

MATERIALS AND METHODS

Twenty four healthy adult male guinea pigs (*Cavia porcellus*), aged 6-8 weeks old were used in this *in vivo* study. The study protocol was approved by the Ethics Committee of the Faculty of Medical Technology, Misurata, Libya. The animals were purchased from a commercial breeder (Misurata, Libya) and housed in stainless steel cages (eight animals per cage) in the animal house (Department of Medical Laboratory, Faculty of Medical Technology, Misurata, Libya), under standard conditions of 12 hours light-dark cycle at room temperature. Cages were provided with tap water bottles and feeders, furnished with appropriate bedding material and cleaned on a daily basis.

Preparation of pomegranate aqueous peel extract:

The fresh fruits of pomegranate, free of visible marks or defects were collected from a local farm (Misurata, Libya). Pomegranate fruits were washed, the peels were removed manually and cut into small pieces. The cut pieces were left to air dry for 15 days at room temperature, and then grained to fine powder by a commercial mill. The aqueous extract of pomegranate peel was prepared daily by adding 20g of dry powder to 1000 mL of boiled distilled water. The extract was filtered to remove the residual peel particles and left to cool down slowly to room temperature. The freshly prepared extract was orally administrated to the experimental animals at a dose level equivalent to 800mg/kg.

Experimental design:

After a week of acclimatization, during which all the experimental animals had free access to drinking tap water and standard chow, the animals were randomly assigned to three groups, each comprising eight animals: first: Lean control (CON) group, which received a normal chow diet and tap water ad libitum for six weeks. Second: High fat diet (HFD) group, which had free access to tap water and HFD for six weeks to induce NAFLD. Third: HFD-PPE group, which had unrestricted access to HFD for six weeks followed by four weeks administration of PPE tea along with HFD. All animals survived until termination of the experiment, and were ultimately sacrificed at scheduled time (six weeks for the CON and HFD groups and ten weeks for the HFD-PPE group). Blood samples (about 5 mL) as well as liver tissue were harvested. All precautions were taken to reduce the potential of pain and the number of animals included in the study.

Analyses of lipid profile and liver function markers:

Serum total cholesterol, triglyceride, as well as alanine transaminase (ALT) and aspartate transaminase (AST) levels were measured by means of COBAS INTEGRA® 400 plus Analyser (Roche Diagnostics Ltd., Rotkreuz, Switzerland) using commercially available kit (32 COBAS c packs) according to the manufacturer's instructions.

Histopathological examination:

The animal's livers were carefully harvested and fixed with 10 % formalin solution at room temperature for 24 hours. Fixed liver tissue specimens were dehydrated in ascending concentrations of ethanol. Thereafter, dried tissues were embedded in paraffin wax, and then a rotary microtome (pfm 3004 M medical ag, Wankelstraße 60, 50996 Köln, Germany) was used to cut tissue slices uniformly at a thickness of 5 μm. The paraffin section was transferred onto a slide and subsequently stained with haematoxylin and eosin (H&E) staining. Microscopic examination and imaging of the stained sections was carried out using Eclipse Ci-L digital microscope camera (Nikon Instruments Inc, Melville, Tokyo, Japan).

Statistical analysis:

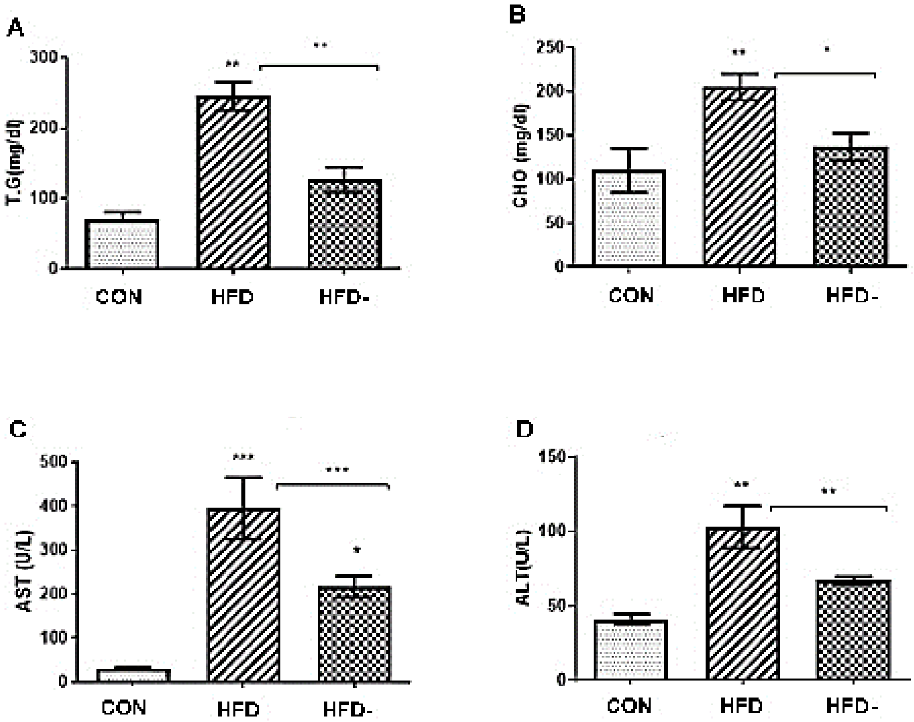
Statistical comparisons between the three experimental groups (CON, HFD, and HFD-PPE groups) were performed using one-way analysis of variance [ANOVA] (GraphPad™, version 6, Software, San Diego, CA, USA). Data were presented as Mean ± SD. Statistical significance was set at $p < 0.05$.

RESULTS

Biochemical findings:

As shown in (figure 1) (A – D), there was a significant increase in the serum levels of cholesterol (205±15 vs.

110±25 mg/dl, p=0.01), triglyceride (245±20 vs. 70±11 mg/dl, p=0.01) as well as ALT (103±14.2 vs. 41±3.4 U/L, p=0.01) and AST (395±70 vs. 31±2.5 U/L, p=0.001) in the HFD group compared to the control group. However, these levels were significantly lower in the HFD-PPE group compared to the HFD animals (137±15.5 vs. 205±15 mg/dl, p=0.05), (127±17.5 vs. 245±20 mg/dl, p=0.01), (67±2.5 vs. 103±14.2 U/L, p=0.01), (217±24 vs. 395±70 U/L, p=0.001) for serum concentrations of cholesterol, triglyceride, ALT and AST respectively.



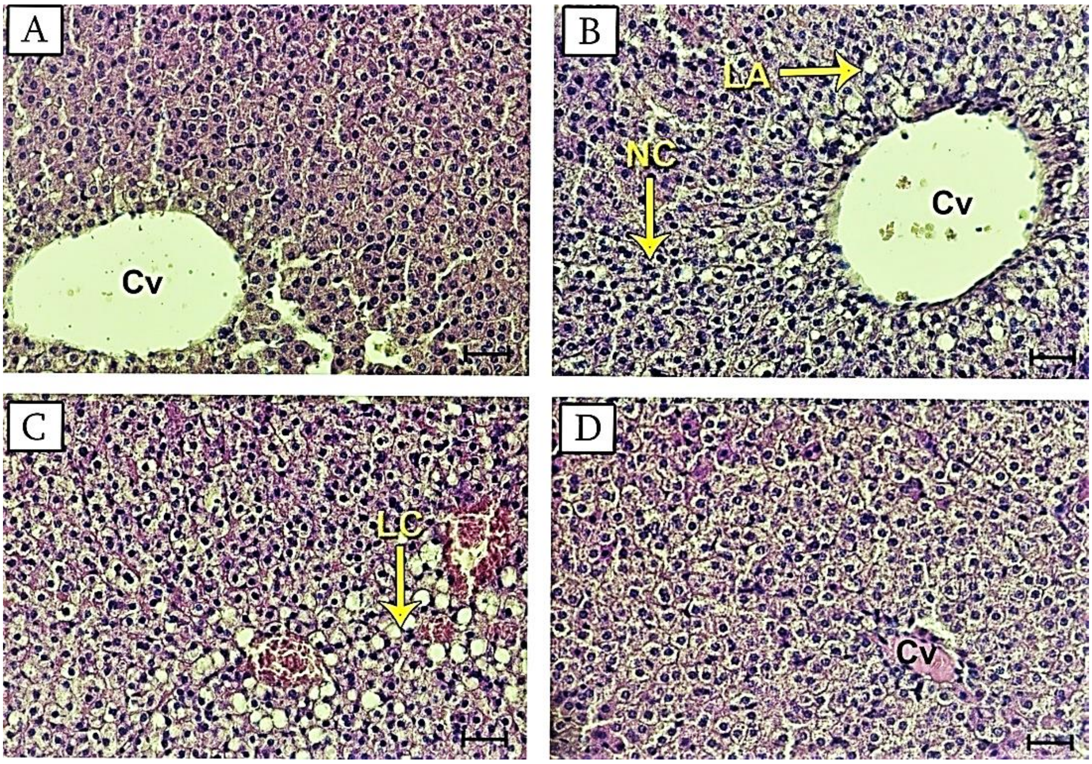
(Figure 1) Levels of biochemical findings in sera of the experimental groups.
A: Total Cholesterol; **B:** Triglyceride; **C:** Aspartate Transaminase (AST); **D:** Alanine Transaminase (ALT).
CON = control; HFD = High fat diet; PPE = pomegranate peel extract. * ($p>0.05$); ** ($p=0.01$); *** ($p=0.001$).

Histological findings:

Fatty liver was assessed histologically in the different animal groups using H&E staining. As visualized by light microscope, the control group of animals (Figure 2, A) showed typical lobular structure with normal hepatocytes and regular cords radiating from central veins. Compared to the control group, the HFD animals (Figure 2. B and C) displayed severe macrovesicular hepatic stenosis, revealed by accumulation of abnormal amounts of fat in a considerable fraction of hepatocytes. The intrahepatocytic fat droplets mostly appeared as one large vacuole that occupies the greater part of intracellular space, displacing the nucleus toward the periphery against the plasma membrane, leaving a clear halo of cytoplasm. Four weeks administration of PPE, (Figure 2, D), resulted in a clear recovery of the hepatic architecture, supported by a remarkable reduction in the severity of macrovesicular steanosis, in the HFD-PPE animals.

DISCUSSION

The health promoting effects of pomegranate fruits have attracted the attention of many researchers from all around the world. However, there is scarcity of studies available that have directly investigated the effect of the PPE on the NAFLD. The aim of the present study was to investigate the ameliorative effect of the aqueous PPE on NAFLD induced by HFD, using guinea pigs. Herein, HFD feeding resulted successfully in a considerable increase in the animal's serum concentrations of total cholesterol and triglyceride. Furthermore, the abnormal deposition of fat within the hepatocytes, along with the evident deterioration in liver function, clearly indicate the development of NAFLD in the HFD group of animals. However, after four weeks of PPE consumption, the treated animals showed a remarkable decline in the serum lipid profiles, together with an efficient alleviation of liver dysfunction, as indicated by the improvement in the histological features and serum levels of liver enzymes.



(Figure 2) H&E staining showing liver histopathology of A: control; B and C: HFD group; D: HFD-PPE group. Cv, central vein; LA, lipid accumulation; NC, necrotic cells; LC, Lymphocytic cell; Bar = 20 μm.400X.

In support of our findings, administration of HFD simultaneously with PPE for eight weeks could provide a useful means to improve liver function via reducing ALT and AST serum levels, as well as abolishing hepatic damage and hyperlipidaemia [11]. Likewise, the hepatoprotective effect of PPE has also been demonstrated in rat liver injury triggered by carbon tetrachloride, which is an efficient toxin used to induce liver pathological lesions that closely resemble hepatic features of NAFLD [22]. Moreover, the consumption of cupcakes fortified with PPE resulted in a substantial decline in body weight, as well as in serum levels of lipid profiles and hepatic enzymes. Accordingly, PPE could apparently be considered as a natural agent that protects against obesity, hyperlipidaemia and liver damage [23].

In the above cited studies, evaluation of the onset and progression of HFD-induced NAFLD was preferentially conducted using rat models, including albino wistar rats [10] and Sprague-Dawley rats [11, 23]. However, it is worth mentioning that rats are significantly different from humans, especially in terms of gene expression as well as activity levels of certain genes associated with hepatic lipid metabolism. Consequently, the disease pathogenesis in these rodents may not truly mimic that of human situation [24]. The use of guinea pigs as a realistic model for the demonstration of diet-induced fatty liver has strongly been recommended. Indeed, the striking similarities between these animals and humans, particularly in terms of hepatic lipoprotein mechanism and enzyme activities, further strengthens the prospect of being a more reliable model for exploring NAFLD aetiology and potential interventions [24, 25, 26].

The hyperlipidemia observed in the HFD group can unsurprisingly be attributable to the imbalance between high energy intake and energy expenditure [27]. Palatable food is linked to activation of the brain's dopamine reward system implicated in food craving behavior [28]. Furthermore, leptin is an adipose-derived hormone that is assumed to play a part in the development of obesity and NAFLD, probably via decreasing food intake and promoting energy expenditure. NAFLD has been shown to be associated with elevated leptin levels, suggesting the potential of leptin resistance [29]. Although the current study did not measure serum levels of leptin, it appears that it contributes to the anti-obesity and hepatoprotective properties of PPE throughout improving leptin sensitivity in the treated animals. In support of this, four weeks supplementation with pomegranate juice was associated with a remarkable reduction in serum leptin levels in rats susceptible to HFD-induced obesity [30]. This perspective may suggest an interesting avenue for further research.

When overproduction of reactive oxygen species (ROS) is accompanied by insufficient antioxidant defence, a state of oxidative stress arises leading to excessive cellular damage [31]. Oxidative stress has strongly been suggested to play a vital role in the onset and progression of NAFLD. In this regard, patients with stenosis and metabolic syndrome exhibited higher lipid peroxides as well as lower vitamin C and alpha-tocopherol serum levels compared to their normal peers [32]. Furthermore, the extent of oxidative stress, as evaluated by measuring nitrite, superoxide dismutase, and catalase activities, has been shown to differ significantly based on the disease severity and histological variabilities [33]. In NAFLD, the hepatic accumulation of triglycerides is primarily due to increased rate of free fatty acid (FFA) fluxes to the liver [34]. An overload of FFAs into the hepatocytes triggers the synthesis of highly reactive free radicals via both over-reduction of the mitochondrial electron transport chain and peroxisomal oxidation [35]. In this study, for weeks administration of PPE to animals with NAFLD resulted in a substantial improvement in liver function. Although the exact mechanism by which PPE offers hepatoprotection is unknown, there is growing evidence to suggest that pomegranate peel possess stronger antioxidant properties than most other bioactive constituents of the human diet [18]. The potent antioxidant activity of the pomegranate peel is attributable to it high contents of phenolic and flavonoid compounds, which are known to have powerful scavenging capacities for ROS [36, 37]. For greater understanding of the underlying mechanisms, future studies should include assessment of additional lipid profile and oxidative stress biomarkers.

In conclusion, the present study clearly demonstrates the potential hypolipidemic, and hepatoprotective effects of the aqueous PPE. While further studies are required to shed more light on the underlying molecular mechanisms, these findings may provide the foundation for further nutritional and therapeutic developments.

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