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Original Article

EXPERIMENTAL EVIDENCES FOR THE PROMISING THERAPEUTIC ROLE OF *VITIS VINIFERA* SEED EXTRACT AGAINST NONALCOHOLIC STEATOHEPATITIS

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ABSTRACT

Objective: The present study was planned to investigate the possible therapeutic effect of methanolic extract of *Vitis vinifera* seeds on high fat diet-induced nonalcoholic steatohepatitis (NASH) in adult female Wistar rats.

Methods: The animals were divided into four groups, (G1)was served as healthy control group and the other three groups received high fat diet for 32 weeks for induction of NASH and then assigned as follow: (G2) in which the animals bearing NASH were left untreated, (G3) in which the animals bearing NASH were treated with *Vitis vinifera* seed extract in a dose of 0.28g/kg b. wt (GSH) and (G4) in which the animals bearing NASH were treated with *Vitis vinifera* seed extract in a dose of 0.14g/kg b. wt (GSL).

Results: The results revealed significant increase in serum ALT activity, plasma glucose, insulin levels, serum resistin, NF-κB, TNF-α, HGF levels, hepatic TNF-α and HGF gene expression levels. While, serum albumin, adiponectin levels and hepatic adiponectin gene expression level were decreased significantly in NASH group. Conversely, treatment of NASH groups with GSH or GSL resulted in significant decrease in serum ALT activity, plasma glucose, insulin levels, serum resistin, NF-κB, TNF-α, HGF levels, hepatic TNF-α and HGF gene expression levels. However, serum albumin, adiponectin levels and hepatic adiponectin gene expression level were increased significantly as a consequence of treatment with GSH or GSL.

Conclusion: The efficacy of *Vitis vinifera* extract against NASH might be attributed to its strong hepatoprotective potential and powerful anti-inflammatory activity in addition to its potent role in ameliorating insulin resistance indices.

Keywords: Nonalcoholic steatohepatitis, *Vitis vinifera*, Inflammation, insulin resistance, Rats.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) covers a wide spectrum of liver pathology from steatosis alone, through the necroinflammatory disorder of nonalcoholic steatohepatitis (NASH) to cirrhosis and liver cancer. NAFLD/NASH is mostly related with visceral adiposity, obesity, diabetes mellitus 2 and metabolic syndrome. Pathogenetic concepts of NAFLD include over nutrition and under activity, insulin resistance (IR) and genetic factors. The prevalence of NAFLD has been estimated to be 17-33% in some countries; NASH may be present in about 1/3 of such cases, while 20-25% of NASH cases could progress to cirrhosis. NAFLD/NASH is not a benign disease, progressive liver biopsy has shown histological progression of fibrosis in 32%, the estimated rate of cirrhosis development is 20% and a liver--related death is 12% over 10 years. No treatment has scientifically proved to ameliorate NAFLD or to avoid its progression. The various therapeutic alternatives are aimed at interfering with the risk factors involved in the pathogenesis of the disorder in order to prevent the progression to end-stage liver disease [1].

Underlying mechanisms responsible for the progression to NASH are till unclear. A ''two-hit'' model has been proposed; peripheral insulin-resistance may represent the ''first hit'' in the pathogenesis of NASH. Combined hyperglycemia and hyperinsulinemia promote *de novo* lipid synthesis and structural defects in mitochondria within hepatocytes. Moreover, insulin-resistance of adipose tissue leads to an enhanced free fatty acid flux to the liver that contributes to steatosis. Steatotic hepatocytes may be vulnerable to a ''second hit'' induced by cytokines such as tumor necrosis factor-α (TNF-α) and oxidative stress, which lead to the development of steatohepatitis and fibrosis [2]. Oxidative stress, in particular, plays an essential role by causing peroxidation of lipids in the hepatocyte membrane to initiate liver fibrosis. Lipid peroxidation and the generation of free radicals can result in cellular death and hepatic necrosis and contribute to impaired cellular function. Antioxidant supplements could potentially protect cellular structures against oxidative stress [3].

The genetic contribution to NASH remains to be fully elucidated, but wide-ranging research is ongoing. Indeed, the broad range of NASH phenotype found in individuals with similar metabolic characteristics points to a complex genetic contribution [4].

Grape seeds are waste products of the grape *(Vitis vinifera*) juice industry. These seeds contain lipid, protein and carbohydrates. The grape seed extract is rich in bioactive phytochemicals that possess inhibitory activity on the fat-metabolizing enzymes; pancreatic lipase and lipoprotein lipase. Grape seed compounds include flavonoids, polyphenols, anthocyanins, proanthocyanidins, procyanidines and the stilbene derivative resveratrol. Grape seeds extract has been reported to have a broad spectrum of pharmacological and therapeutic effects such as antioxidative, anti-inflammatory, and antimicrobial activities, as well as cardioprotective, hepatoprotective, and neuroprotective potentials [5]. The underlying mechanisms of the anti-inflammatory property of grape seed extracts are relevant to oxygen free radical scavenging, anti lipid peroxidation, inhibiting the formation of inflammatory cytokines, alterations in cell membrane receptors, intracellular signaling pathway proteins and modulation of gene expression [6]. The current study was constructed to investigate the possible therapeutic role of *Vitis vinifera* methanolic extract in management of nonalcoholic steatohepatitis induced in the experimental model with special reference to its mode of action.

MATERIALS AND METHODS

Plant materials

Vitis vinifera seed (Grape seed) was purchased at the Harraz Herbal Drugstore (Cairo, Egypt). The plant was identified by Professor Ibrahim El-Garf, Botany Department, Faculty of Science, Cairo University.

Preparation of grape *(Vitis vinifera*) seed methanolic extract

Four kilograms (kgs) of dried seeds were ground to fine powder, then extracted with 80% methanol at room temperature for three times. The extract was then concentrated in a vacuum evaporator to obtain the crude aqueous methanolic extract (23% from the weight of the dried seeds).

Experimental animals

The present study was conducted on forty adult female Wistar rats weighing 120 - 150g obtained from the Animal House Colony of the National Research Centre, Cairo, Egypt. The animals were maintained on the standard laboratory diet and water *ad libitum* for two weeks before starting the experimental protocol. All animals received adequate human care and use according to the guidelines for Animal Experiments which were approved by the Ethical Committee of Medical Research, National Research Centre, Egypt. Nonalcoholic steatohepatitis (NASH) was induced in rats by using high fat diet which provided 30% of its energy from fat, 35% from carbohydrate and 35% from protein (casein) for 32 weeks. Supplements of vitamins and minerals were also included [7].

Experimental protocol

The animals were divided into four groups, ten animals each: (G1) Healthy control group which was fed *ad-libitum* with an isocaloric regular rat chow [8], (G2) Nonalcoholic steatohepatitis (NASH group) which was fed *ad-libitum* with high fat diet for 32 weeks and left untreated [7], (G3) NASH group treated orally with 0.28g/kg b. wt of *Vitis vinifera* seed extract daily for 8 weeks and was assigned as NASH+GSH and (G4) NASH group treated orally with 0.14g/kg b. wt of *Vitis vinifera* seed extract daily for 8 weeks and was assigned as NASH+GSL. The selected doses of *Vitis vinifera* seed extract were calculated from the chronic toxicity study for *Vitis vinifera* seed extract (data not shown).

At the completion of this round (40 weeks), the rats were fasted overnight and the blood samples were collected from the retro orbital venous plexus under diethyl ether anaesthesia [9]. Each blood sample was divided into two tubes, the first tube contains anticoagulant for separation of plasma and the second tube free from any anticoagulant for separation of serum. All blood samples were centrifuged using cooling centrifuge at 1800 xg for ten min. to obtain plasma and serum which were stored at -20°C until analysis. After blood collection, all animals were rapidly sacrificed and the liver tissues were dissected, immediately frozen in liquid nitrogen and stored at −80 °C prior to extraction for molecular genetics study.

Biochemical assays

Serum alanine transaminase (ALT) activity was estimated colorimetrically using kit purchased from Quimica Clinica Aplicada S. A. Co., Spain, according to the method of Reitman and Frankel [10]. Serum albumin level was measured colorimetrically using kit purchased from Stanbio Laboratory. Boerne, Texas, USA, according to the method of Dumas and Biggs [11]. Plasma glucose level was measured colorimetrically using kit purchased from Stanbio Laboratory, Boerne, Texas, USA, according to the method of Howantiz and Howantiz [12]. Plasma insulin level was estimated by enzyme linked immunosorbent assay (ELISA) procedure using DRG kit (Germany) according to the method of Temple et al. [13]. The homeostasis model assessment of basal insulin resistance (HOMA-IR) was used to calculate the index from the product of the fasting concentration of plasma glucose (mmol/L) and plasma insulin (mU/ml) divided by 22.5 according to the method of Duncan et al. [14]. Serum adiponectin concentration was measured by ELISA technique using kit purchased from Assay Pro, USA, according to the method of Pannacciulli et al. [15]. Serum resistin concentration was determined by ELISA technique using kit purchased from Glory Science Co., Ltd, Veterans Blvd, Suite, USA, according to the method of Schaffler et al. [16]. Serum nuclear factor kappa B-p56 (NF-κB-p56) concentration was determined by ELISA procedure using kit purchased from Glory Science Co., Ltd, Veterans Blvd, Suite, USA, according to the manufacturer's instructions. Serum TNF-α concentration was measured by ELISA procedure using kit purchased from Ray Biotech Co., Georgia, USA, according to the method of Brouckaert et al. [17]. Serum hepatocyte growth factor (HGF) level was quantified by ELISA procedure using kit purchased from Glory Science Co., Ltd, Veterans Blvd, Suite, USA, according to the method of Plum et al. [18].

Semi-Quantitative real time PCR (sqRT-PCR) detection of adiponectin, TNF-α and HGF gene expression

Isolation of total RNA

Total RNA was isolated from liver tissue of female rats by the standard TRIzol® reagent extraction method (Invitrogen, USA). Then, the complete Poly(A)+ RNA was reverse transcribed into cDNA in a total volume of 20 µl using RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An amount of total RNA (5µg) was used with a reaction mixture, termed as master mix (MM). The MM consisted of 50 mM $MgCl_2$, 5x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3; 10 mM of each dNTP, 50 µM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M- MuLV reverse transcriptase.

Reverse transcription reaction and semi-quantitative real time PCR

The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C, and the reaction was stopped by heating for 5 min at 99°C. Afterwards, the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through semi-quantitative real time-polymerase chain reaction [19]. An iQ5-BIO-RAD Cycler (Cepheid, USA) was used to determine the rat cDNA copy number. PCR reactions were set up in 25 µl reaction mixtures containing 12.5 µl 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 µl 0.2 M forward primer, 0.5 µl 0.2 M reverse primer, 6.5 µl distilled water, and 5 µl of cDNA template. Primer sequences were as follows: adiponectin: F: 5′-AGA TGG CAC CCC TGG TGA G-3′ and R: 5′-GGG TAC TCC GGT TTC ACC G-3′ [20], TNF-α: F: 5′-TCG TAG CAA ACC ACC AAG CA-3′ R: 5′-CCC TTG AAG AGA ACC TGG GAG TA-3′ [21]**;** HGF: F: 5′-AGCTCAGAACCGACCGGCTTGCAACAGGAT-3′, R: 5′-TTACCAATGATG CAATTTCTAATATAGTCT-3′ [22] and β-actin: F: 5′-CTGTCTGGCG GCACCACCAT-3′, R: 5′-GCAACTAAGTCATAGTCCGC-3′ [23]. The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle was divided into 3 steps: (a) denaturation at 95.0°C for 15 sec; (b) annealing at 60.4, 55.0, 60 and 60°C for 30, 5, 30 and 30 sec for (adiponectin, TNF-α, HGF and β-actin genes, respectively and (c) extension at 72.0°C for 30 sec. The gene expression level was calculated as follows:

First: the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formulae [24]

$$Ef = 10^{-1/slope}$$

$$Efficiency\ (\%) = (Ef - 1) \times 100$$

The relative quantification of the target to the reference was determined by using the ΔCT method if E for the target (adiponectin, TNF-α and HGF) and the reference primers (β-actin) are the same:

$$Ratio\ (reference/\ target\ gene) = Ef^{C_T(reference) - C_T(target)}$$

Statistical analysis

In the present study, all results were expressed as Mean **+** S. E of the mean. Data were analyzed by one way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 14 followed by least significant difference (LSD) to compare significance between groups [25]. Difference was considered significant when P value was < 0.05.

$$\%\ difference = \frac{Treated\ value - Control\ value}{Control\ value} \times 100$$

RESULTS

Table (1) illustrated the effect of treatment with different doses of *Vitis vinifera* seed extract on serum ALT activity and albumin level in NASH experimental model. The NASH group showed significant

elevation (P<0.05) in serum ALT activity (71.7 %) associated with the significant decline (P<0.05) in serum albumin level (-68.75%) in comparison with the healthy control group. However, treatment with *Vitis vinifera* seed extract (0.28 or 0.14 g/kg b. wt) produced significant reduction in serum ALT activity (-36.01% and -39.88%, respectively) when compared with the untreated NASH group. Serum albumin level was increased significantly (P<0.05) by 173.3% and 180% in NASH group treated with *Vitis vinifera* seed extract (0.28 or 0.14 g/kg b. wt, respectively) as compared to the untreated NASH group.

Table 1: Effect of treatment with different doses of *Vitis vinifera* seed extract on serum ALT activity and albumin level in NASH experimental model

Parameters Groups	ALT (U/L)	Albumin (g/dl)
Healthy control group (G1)	35.4± 3.2	4.8 ± 0.05
NASH group (G2)	60.8±1.7[a] (71.7%)	1.5 ± 0.04[a] (-68.75%)
NASH + GSH group (0.28g/kg b. wt) (G3)	38.9±1.5[b] (-36.01%)	4.1 ± 0.08[b] (173.3 %)
NAFLD + GSL group (0.14g/kg b. wt) (G4)	36.6±1.4[b] (-39.88%)	4.2 ± 0.06[b] (180%)

a: Significant change at P < 0.05 in comparison with the healthy control group, b: Significant change at P < 0.05 in comparison with NASH group, (%): percent difference with respect to the corresponding control value

The effect of treatment with different doses of *Vitis vinifera* seed extract on plasma glucose and insulin levels in NASH experimental model is depicted in table (2). The data showed that NASH group reveals the significant increase (P<0.05) in plasma glucose and insulin levels (80.45% and 58%, respectively) in comparison with the healthy control group. Meanwhile, treatment of NASH group with *Vitis vinifera* seed extract in the different doses resulted in the significant decrease (P<0.05) in plasma glucose and insulin levels (-41.19% and -25.67%, respectively) for *Vitis vinifera* seed extract (0.28g/kg b. wt) and (-43.05% and -25.76%, respectively) for *Vitis vinifera* seed extract (0.14 g/kg b. wt) as compared to the untreated NASH group.

The data in table (2) also showed that the induction of NASH produced significant elevation (P<0.05) in insulin resistance (185.12%) in comparison with the healthy control group. On the other hand, treatment of NASH groups with different doses of *Vitis vinifera* seed extract produced significant depletion (P<0.05) in insulin resistance (-56.31%) for *Vitis vinifera* seed extract (0.28g/kg b. wt) and (-57.76%) for *Vitis vinifera* seed extract (0.14 g/kg b. wt) when compared with the untreated NASH group.

Table 2: Effect of treatment with different doses of *Vitis vinifera* seed extract on plasma glucose and insulin levels and insulin resistance value in NASH experimental model

Parameters Groups	Glucose (mg/dl)	Insulin mU/ml	insulin resistance value
Healthy control group (G1)	83.4 ± 1.2	14.05±0.5	2.89±0.02
NASH group (G2)	150.5 ± 2.3[a] (80.45%)	22.20±0.8[b] (58%)	8.24±0.05[a] (185.12%)
NASH + GSH group (0.28g/kg b. wt) (G3)	88.5±2.0[b] (-41.19%)	16.50±1.1[b] (-25.67%)	3.6±0.03[b] (-56.31%)
NAFLD + GSL group (0.14g/kg b. wt) (G4)	85.7± 1.1[b] (-43.05%)	16.48±1.2[b] (-25.76%)	3.48±0.03[b] (-57.76%)

a: Significant change at P < 0.05 in comparison with the healthy control group, b: Significant change at P < 0.05 in comparison with NASH group, (%): percent difference with respect to the corresponding control value

The results in table (3) represented the effect of treatment with different doses of *Vitis vinifera* seed extract on serum adiponectin and resistin levels in NASH experimental model. Significant increase (P<0.05) in serum resistin level (79.2%) accompanied with the significant decrease (P<0.05) in serum adiponectin level (-33.6%) was recorded in NASH group as compared to the healthy control group. However, treatment of NASH group with *Vitis vinifera* seed extract (0.28 or 0.14 g/kg b. wt) resulted in the significant decrease (P<0.05) in serum resistin level (-40.5% and -42.39%, respectively).

The opposite was observed regarding serum adiponectin level, *Vitis vinifera* seed extracts produced significant increase (P<0.05) (36.23% for 0.28g/kg b. wt and 44.92% for 0.14g/kg b. wt) when compared with the untreated NASH group.

Table 3: Effect of treatment with different doses of *Vitis vinifera* seed extract on serum adiponectin and resistin levels in NASH experimental model

Parameters Groups	Adiponectin (ng/ml)	Resistin (pg/ml)
Healthy control group (G1)	10.4 ± 0.42	30.8 ± 0. 25
NASH group (G2)	6.9 ± 0.2[a] (-33.6 %)	55.2 ± 0.37[a] (79.2 %)
NASH + GSH group (0.28g/kg b. wt) (G3)	9.4 ± 0.3[b] (36.23%)	32.8 ± 0.29[b] (-40.5 %)
NAFLD + GSL group (0.14g/kg b. wt) (G4)	10.00 ± 0.2[b] (44.92 %)	31.5 ± 0.3[b] (-42.93 %)

a: Significant change at P < 0.05 in comparison with the healthy control group, b: Significant change at P < 0.05 in comparison with NASH group, (%): percent difference with respect to the corresponding control value.

The data in table (4) showed the effect of treatment with different doses of *Vitis vinifera* seed extract on serum NF-κBp56, TNF-α and HGF levels in NASH experimental model. Significant increase (P<0.05) in serum NF-κBp56, TNF-α and HGF levels (103.1%, 67.6% and 88.5%, respectively) was demonstrated in NASH group in comparison with the healthy control group.

On the other side, treatment of NASH group with *Vitis vinifera* seed extract (0.28 or 0.14 g/kg b. wt) induced significant decrease (P<0.05) in serum NF-κB p56 level (-44.6% and -49.2 %, respectively), TNF-α level (-34.49% and -37.16%, respectively) and HGF level (-34.77% and -37.25%, respectively) as compared to the untreated NASH group.

Table 4: Effect of treatment with different doses of *Vitis vinifera* seed extract on serum NF-κBp56, TNF-α and HGF levels in NASH experimental model

Parameters Groups	NF-κB p56 (ng/ml)	TNF-α (pg/ml)	HGF (ng/L)
Healthy control group (G1)	0.64 ± 0.04	58.1 ± 1.8	102.40 ± 1.6
NASH group (G2)	1.3 ± 0.1[a] (103.1 %)	97.4± 1.2[a] (67.6 %)	193.05 ± 1.4[a](88.5 %)
NASH + GSH group (0.28g/kg b. wt) (G3)	0.72± 0.02[b] (-44.6 %)	63.8 ± 1.5[b] (-34.49 %)	125.70 ± 1.5[b](-34.77 %)
NAFLD + GSL group (0.14g/kg b. wt) (G4)	0.66± 0.03[b] (-49.2%)	61.2 ± 1.2[b] (-37.16 %)	120.60 ± 1.1[b](-37.52 %)

a: Significant change at P < 0.05 in comparison with the healthy control group, b: Significant change at P < 0.05 in comparison with NASH group, (%): percent difference with respect to the corresponding control value

The results in table (5) illustrated the effect of treatment with different doses of *Vitis vinifera* seed extract on hepatic adiponectin, TNF-α and HGF gene expression levels in NASH experimental model. The NASH group showed significant down-regulation (P<0.05) in hepatic adiponectin gene expression level by -35.66 % in comparison with the healthy control group. While, treatment of NASH group with *Vitis vinifera* seed extracts (0.28 or 0.14 g/kg b. wt) resulted in significant up-regulation (P<0.05) in hepatic adiponectin gene expression level by 50% and 52.17%, respectively, in comparison with the untreated NASH group. NASH group showed significant up-regulation (P<0.05) in hepatic TNF-α and HGF gene expression levels by 110.52 % and 54.28 %, respectively, when compared with the healthy control group. Meanwhile, treatment of NASH group with *Vitis vinifera* seed extract resulted in significant down-regulation (P<0.05) in hepatic TNF-α and HGF gene expression levels (-40.83% and -31.48%, respectively) for 0.28g/kg b. wt, and (-50% and -33.59%, respectively) for 0.14 g/kg b. wt as compared to the untreated NASH group.

Table 5: Effect of treatment with different doses of *Vitis vinifera* seed extract on hepatic adiponectin, TNF-α and HGF gene expression levels in NASH experimental model

Parameters Groups	Relative expression of adiponectin gene (adiponectin/β-actin)	Relative expression of TNF-α gene (TNF-α/β-actin)	Relative expression of HGF gene (HGF/β-actin)
Healthy control group (G1)	1.43 ± 0.007	0.57 ± 0.003	1.05 ± 0.006
NASH group (G2)	0.92 ± 0.004[a] (-35.66 %)	1.2± 0.003[a] (110.52 %)	1.62± 0.006[a] (54.28 %)
NASH + GSH group (0.28g/kg b. wt) (G3)	1.38± 0.002[b] (50 %)	0.71 ± 0.002[b] (-40.83 %)	1.11 ± 0.005[b] (-31.48 %)
NAFLD + GSL group (0.14g/kg b. wt) (G4)	1.40± 0.003[b] (-52.17%)	0.6 ± 0.002[b] (-50 %)	1.07 ± 0.004[b] (-33.59 %)

a: Significant change at P < 0.05 in comparison with the healthy control group, b: Significant change at P < 0.05 in comparison with NASH group, (%): percent difference with respect to the corresponding control value

DISCUSSION

The results of the present study revealed the significant increase in serum ALT activity in NASH group which is in agreement with Hooper et al. [26]. Both aminotransferases (AST and ALT) are highly concentrated in the liver and the increasing serum ALT activity reflects the state of hepatocyte damage in NASH patients [27]. It has been demonstrated that oxidative stress status of the liver achieved in steato hepatitis promotes hepatocellular damage by inducing (1) severe oxidative alteration of biomolecules, with loss of their functions and impairment of cell viability; and (2) sustained activation of redox-sensitive transcription factors, such as NF-κB and AP-1, with consequent up-regulation of the expression of proinflammatory mediators at the Kupffer cell level [28]. A growing body of evidence supported the possibility that insulin resistance associated with adipose tissue inflammation and hepatic microvascular dysfunction may actually contribute to the development and/or progression of ALT activity in serum [29].

The present data showed significant reduction in serum albumin level in NASH group. This finding agrees with that of Farrelland Larter [30]. Albumin, the predominant protein in circulation, is mainly synthesized in the liver and it binds to a number of ligands. Amirtharaj et al. [31] stated that oxidative stress can cause modification of serum albumin, which influences ligand binding properties of the protein. This impact of oxidative stress might be the reason for decreasing serum albumin level in NASH group as shown in the current study. Many studies demonstrated that oxidative stress is implicated in conditions such as nonalcoholic fatty liver disease (NAFLD) and non alcoholic steatohepatitis (NASH) due to the increasing levels of free fatty acids with consequent increase in free radicals levels [32].

Treatment of NASH group with *Vitis vinifera* seed extract induced significant decrease in serum ALT activity associated with significant elevation in serum albumin level. The antioxidant and free radical scavenging property of *Vitis vinifera* seed extract may be responsible for this effect. Polyphenols present in the *Vitis vinifera* seed extract, especially (+)-catechin, possess strong antioxidant activity and they can also inhibit the oxidation of plasma lipids. Moreover, (-)-epicatechin is able to scavenge hydroxyl radicals, peroxyl radicals and superoxide radicals [33]. Furthermore, procyanidins, other

active compounds of *Vitis vinifera* seed extract, are reported to have potent antioxidant activity both *in vitro* and *in vivo* [34]. Flavonoids represent the main active components in *Vitis vinifera* seed extracts; these compounds exhibit hepatoprotective activity due to their powerful antioxidant and anti-inflammatory properties *in vitro* and *in vivo*. These lead compounds can modulate mammalian enzyme activity, enhance intracellular signaling and strengthen membranes [35]. In addition, the active principles in the *Vitis vinifera* seed extract can prevent insulin resistance, improve insulin sensitivity and increase the activity of antioxidant enzymes [36]. In view of the above mentioned properties of *Vitis vinifera* seed extract, it could be suggested that this extract is able to maintain the structure integrity of hepatocytes and decrease serum ALT activity in the treated groups. This means that *Vitis vinifera* seed extract could restore the liver functions in NASH bearing rats. The elevated serum albumin level in *Vitis vinifera* seed extract treated groups in the present study supported the hypothesis of the ability of this extract to restore the liver to nearly normal condition. This effect of *Vitis vinifera* seed extract could be attributed to its bioavailability in addition to its potent antioxidant and anti-inflammatory effects [37]. Shin and Moon [5] reported that in chronic liver diseases, the serum albumin levels are reduced due to protein synthesis disruption in the liver and the *Vitis vinifera* treatment blocked this action *via* hepatoprotective effects of grape skin and seeds where the damaged hepatocytes are potent sources of reactive oxygen intermediates and *Vitis vinifera* has potent antioxidant activity. Thus, the hepatoprotective effects of *Vitis vinifera* seed extract possibly ascribed to the increasing serum albumin level in the treated groups.

In view of our results, there was significant increase in plasma glucose and insulin levels as well as insulin resistance value in NASH group as compared to the healthy control group. These results are in agreement with Yun et al. [38]. One of the most important functions of the liver is the homeostasis of blood glucose levels by taking up and storing glucose as glycogen (glycogenesis), breaking this down to glucose when needed (glycogenolysis) and forming glucose from noncarbohydratesources such as amino acids (gluconeogenesis) [39]. The presence of high lipolytic activity due to fat accumulation results in high free fatty acids mobilization to the liver. The elevated fatty acids flux to the liver accelerates gluconeogenesis and decreases the effect of insulin on peripheral glucose disposal [40] leading to the increased plasma glucose level. NAFLD is commonly associated with an increased risk of developing Type 2 diabetes and treatable features of insulin resistance such as dyslipidaemia and dysglycaemia. A growing body of evidence demonstrated that NAFLD exacerbates hepatic insulin resistance that often precedes glucose intolerance [41]. Hepatic insulin resistance confers the failure of insulin to stimulate glycogen synthesis and to suppress gluconeogenesis, thus, insulin fails to down-regulate hepatic glucose production [30]. Hyperinsulinemia is believed to play a key role in the pathogenesis of nonalcoholic steatohepatitis (NASH) and patients with NASH had hepatic insulin sensitivity. Decreased hepatic insulin clearance develops with the increase in liver fat accumulation. It appears to be largely driven by hepatic steatosis, whereas steato hepatitis is more closely associated with reduced whole-body insulin clearance [42].

In the healthy state, insulin stimulation of insulin receptor substrate-1(IRS-1) in muscle cells leads to activation of intracellular phosphoinositide 3-kinase (PI3K) which in turn activates glucose transporter-4 (GLUT) allowing glucose entry in the muscles. In NAFLD state, the increase in intracellular metabolites (diacylglycerol), leads to decreased phosphorylation of IRS-2 *via* activation of a serine kinase cascade [43]. This in turn, results in decreasing PI3K activity with consequent decrease in glucose cellular entry. It has been proposed that a process analogous to that in muscle cells occurs in hepatic cells [44].

Treatment of NASH groups with *Vitis vinifera* seed extract produced significant decrease in plasma glucose and insulin levels as well as insulin resistance value as shown in the current results. It has been demonstrated that the existence of antioxidant compounds in *Vitis vinifera* seed extract is responsible for this effect. These compounds play an important role in the restoration of pancreatic β-cell functions and thus they can decrease blood glucose level [45]. *Vitis vinifera* seed extract has been found to increase glucose uptake in β-cells under high-glucose conditions [46]. It has been reported that the reduction of plasma glucose levels is driven as a consequence of the enhanced adiponectin expression due to treatment with procyanidins [47].

Castell-Auví et al. [48] have shown that luteolin, one of the flavonoids present in *Vitis vinifera* seed extract decreases the gene expression of sterol regulatory element-binding protein 1c (Srebf1) and fatty acid synthase (Fasn) and it enhances the phosphorylation of AMP-activated protein kinase (AMPK), a transcription factor that activates the expression of several genes involved in free fatty acids and triglycerides synthesis, as well as it affects other components of the regulatory machinery of lipid metabolism, leading to a decrease in intracellular lipid levels. Thus, the decreased plasma insulin level due to *Vitis vinifera* seed extract administration could be at least in part explained by the lipid-lowering effect of this extract. In line with these evidences, Cedó et al. [46] stated that procyanidin causes a reduction in the Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) index, suggesting the improvement of insulin resistance. Terra et al. [49] demonstrated that procyanidins significantly reduce blood glucose and insulin levels, as well as insulin resistance (HOMA-IR) values.

The present data revealed the significant decrease in serum adiponectin level as well as significant down-regulation of hepatic adiponectin gene expression level in NASH group. It has been demonstrated that normally adiponectin is found in relatively high circulating levels, but it is decreased in patients with NASH and in clinical manifestations associated with insulin resistance such as metabolic syndrome (MS) and type 2 diabetes mellitus [50]. In addition, plasma adiponectin levels have been found to be correlated inversely with the markers of systemic oxidative stress. And many studies hypothesized that oxidative stress contributes in conditions such as NAFLD and NASH due to the increased levels of free fatty acids and the consequent increased levels of free radicals [32]. It has been demonstrated that in cultured adipocytes, under oxidative stress condition, the suppressed mRNA expression and secretion of adiponectin are detected [51]. This explains the decreased gene expression level of hepatic adiponectin in NASH group as shown in the present results. Moreover, it has been found that in NASH, the local effects of adiponectin are limited due to (i) decreased adiponectin mRNA expression and (ii) decreased mRNA expression of hepatic adipoRII [20]. Furthermore, it was reported that TNF-α and adiponectin suppress each other's production and are also able to antagonise each other's action [52]. Therefore, the reduced adiponectin mRNA expression might be partially due to the suppressive effect of elevated TNF-α expression in NASH [53].

The current results demonstrated that serum resistin level shows the significant increase in NASH group. Pagano et al. [54] reported that patients with NASH are characterized by high serum resistin level. A major target organ of resistin is the liver, where resistin induces insulin resistance and increases glucose production. Therefore, resistin is related to hepatic fat content and insulin resistance [55] Thus, it has been suggested that resistin may contribute to hepatic steatosis *via* promoting insulin resistance and the increased resistin levels in NASH patients are related to histological severity of the disease [56]. Underlying liver damage and the progression of pure fatty liver to NASH and fibrosis, the hepatic stellate cells produce a variety of cytokines, including resistin. Moreover, it has been reported that a genetic polymorphism in the promoter region of the resistin gene may be an independent predictor of circulating resistin in humans. Hence, it is not possible to exclude that a gene polymorphism(s) of resistin which may be responsible for the high resistin levels in NAFLD patients [54].

Treatment of NASH group with *Vitis vinifera* seed extract led to significant increase in serum adiponectin level as well as up-regulation in hepatic adiponectin gene expression level. Décordé et al. [57] reported that *Vitis vinifera* seed markedly prevents hyperglycemia, reduces hyperinsulinemia, and increases adiponectin levels in hamsters fed a high-fat diet. Adiponectin is an adipokine that exerts a potent insulin-sensitizing effect by binding to its receptors such as AdipoR1 and AdipoR2, leading to activation of

AMPK and PPARa thus, it consequently activates glucose uptake and reduces insulin resistance [58]. Therefore, *Vitis vinifera* seed extract may induce the significant increase in serum adiponectin level [36]. Thus, the observed increase in serum adiponectin levels could be directly related to the effect of *Vitis vinifera* seed extract consumption on adipocytes to secrete adiponectin or indirectly as a result of the reduction of body weight as the secretion of adiponectin, is increased by weight loss [59]. Moreover, Georgiev et al. [60] demonstrated that regular consumption of resveratrol-rich *Vitis vinifera* extract increased serum adiponectin, due to antioxidative effect beside its antiinflammatory activity.

The local enhancement in adiponectin gene expression level produced by the treatment of NASH groups with *Vitis vinifera* seed extract might be partly attributed to the reduced proinflammatory cytokine expression levels [35]. As hepatic adiponectin expression has been found to be regulated by different factors including the proinflammatory cytokines [49]. Moreover, grape seed extract contains bioactive compounds "procyanidins" which could enhance the gene expression of the antiinflammatory molecule "adiponectin" [35]. Furthermore, *Vitis vinifera* seed extract contains another bioactive compound called "resveratrol" which reverses mRNA expression of adiponectin [61]. Additionally, Meeprom et al. [62] reported that oxidative stress inhibits the gene expression of adiponectin. *Vitis vinifera* seed extract contains procyanidins and monomeric flavanols such as (+)-catechin and (-)-epicatechin, which have potent antioxidant activities. Thus, we could suggest that these powerful antioxidants present in *Vitis vinifera* seed extract help to replenish the decreased hepatic adiponectin gene expression level.

The present findings revealed that the treatment of NASH groups with *Vitis vinifera* seed extract results in significant reduction in serum resistin level. This result could be explained by ability of *Vitis vinifera* seed extract with its active compounds "procyanidins" to reduce insulin resistance. As it has been reported that resistin is involved in the pathogenesis of insulin resistance and the reduced resistin concentration by procyanidins in *Vitis vinifera* seed might contribute to a lower risk for the development of NASH [63].

The present data indicated that serum TNF-α, NF-κB and HGF levels display significant elevation in NASH group as compared to the healthy control group. Additionally, significant up-regulation in hepatic TNF-α and HGF gene expression levels has been recorded in NASH group when compared to the healthy control group. These findings could be explained by the oxidative stress and stimulation of kupffer cell as well as stellate cell to secrete inflammatory cytokines [64]. Mitochondrial dysfunction contributes to oxidative stress and NASH is associated with mitochondrial structural defects. Oxidative stress causes various types of functional and structural damage and commonly increases TNF-α production in NASH and enhanced expression of TNF-α mRNA [65]. A preponderance of evidence supports the hypothesis that the amounts of TNF-α mRNA and TNF-α released by adipose tissue are enhanced in NASH [66]. Moreover, it has been found that NAFLD patients have elevated plasma level of lipopolysaccharide-binding protein (LBP) which is further increased in patients with NASH. This increased LBP level is related to a rise in TNF-α gene expression in the hepatic tissue of NASH patients which supports a role of endotoxemia in the development of steatohepatitis [67]. It has been stated that the appearance of liver pathology is correlated with disruption of adipokines, evidence of insulin resistance, increased hepatic oxidative stress, and increased hepatic TNF-α expression. The commonalities between different experimental models suggest that these pathways are of fundamental importance underlying the development of liver pathology in NASH [68].

High oxidative stress status in the liver of NAFLD patients with steatohepatitis leads to modulation of Kupffer cell function, through activation of transcription factors such as NF-κB [69]. NF-κB, then translocates from the cytoplasm to the nucleus to activate the expression of inflammatory cytokines perturbing the inflammatory cycle [70]. This mechanism explains the significant increase in serum NF-κB level in NASH group in the present study.

The significant increase in serum HGF level in NASH group in our study is consistent with that of Koutsogiannis et al. [71]. HGF mRNA expression in nonparenchymal cells has been found to be increased in NASH patients [72]. In NASH, the activation of Kupffer cells and macrophages within liver tissue increased the production of NF-κB which induces the expression of HGF and consequently its level [4].

Treatment of NASH group with *Vitis vinifera* seed extract resulted in significant reduction in serum TNF-α, NF-κB and HGF levels in concomitant with significant down-regulation in TNF-α and HGF gene expression levels. *Vitis vinifera* seed contains procyanidins which could reduce the TNF-α plasma level as a result of the down-regulation of TNF-α gene expression level [73]. Furthermore, procyanidin could reduce TNF-α protein level in the mesenteric adipose tissue, indicating that the local inflammation in this tissue was prevented [35]. Weisberg et al. [74] demonstrated that adipose tissue macrophages are responsible for almost all adipose tissue TNF-α expression. Procyanidins has been reported to reduce the level of macrophage present in adipose tissue. Therefore, the inhibition of the cytokine expression including TNF-α might be due to a decrease in the number of macrophages [35].

Procyanidin has been found to decrease NF-κB level in liver, which is directly associated with the decreased hepatic expression of the other inflammatory molecules as TNF-α and C-reactive protein. Also, procyanidins have been hypothesized to reduce MCP-1 secretion in adipocytes, partially, due to the diminished TNF-α levels which might be a consequence of the inhibitory effects of procyanidins on NF-κB activation [35].

Furthermore, the existence of procyanidins in *Vitis vinifera* seed plays an important role in preventing low-grade inflammation *in vivo*, by adjusting adipose tissue cytokine imbalance, enhancing antiinflammatory molecules and diminishing proinflammatory mediators beside the down-regulation of TNF-α and IL-6 expression i. e., these compounds could inhibit NF-kB cascade [49]. Additionally, *Vitis vinifera* seed contains resveratrol which could reduce the expression of the inflammatory mediators (TNF-α, IL-6, and COX-2) in mature adipocytes, inhibit TNF- α -activated NF-kB signaling, and reverse the TNF-α secretion [61].

Serum HGF levels have been reported to be strongly associated with insulin resistance and all components of metabolic syndrome. Insulin resistance and metabolic syndrome are the most specific findings of NASH, and HGF might be the possible messenger of the disease between adipocytes and hepatocytes [75]. The antioxidant and free radical scavenging ability of *Vitis vinifera* seed extract active constituents (polyphenols) especially (+)-catechin may be responsible for the decreasing serum level of HGF in NASH-treated groups. (+) – catechin possesses antioxidant activity by inhibiting the oxidation of plasma lipids.

Moreover, (-)-epicatechin is able to scavenge hydroxyl radicals, peroxyl radicals, superoxide radicals [33]. Thus *Vitis vinifera* seed extract can ameliorate insulin resistance and improve insulin sensitivity [36]. Besides that, *Vitis vinifera* seed has been found to block NF-κB expression [35] and in turn it can indirectly inhibit the stimulant of HGF expression and consequently its level [4].

CONCLUSION

The current results provide a clear experimental evidence for the promising role of *Vitis vinifera* seed extract in management of nonalcoholic steatohepatitis (NASH). The active contituents of *Vitis vinifera* namely flavonoids, polyphenols, anthocyanins, proanthocyanidins, procyanidines and the stilbene derivative resveratrol may be responsible for this effect through their hepatoprotective activity, antioxidant capacity and anti-inflammatory property. Beside that, *Vitis vinifera* seed extract has proved its potential in modulating insulin resistance status associated with nonalcoholic steatohepatitis. Therefore, *Vitis Vinifera* seed extract could have potent therapeutic implication in chronic liver diseases accompanied with insulin resistance and severe inflammation.

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CONFLICT OF INTERESTS

Declared None

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