


ORIGINAL CONTRIBUTION

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Anti-diabetic effects of pomegranate extracts in long-term high fructose-fat fed rats

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Abstract

Background: A high-sugar or/and high-fat diets is a major risk factors for obesity. However, increased fruit and vegetable intake has been recently linked to obesity prevention. The aim of the present study was to investigate the preventive effects of pomegranate leaves (PL), juice (PJ) and peel (PP) extracts, on insulin resistance and oxidative stress in high fat and high fructose diet-induced obese rats.

Methods: Obesity in rats was induced by consumption of diet high in saturated fat and fructose (HFD) for a long period (12 weeks).

Results: Compared to the control group (CG) fed chow diet, the high fat and high fructose diet (HFD) group showed a significant increase in the fasted plasma levels of glucose (29.8%), insulin (45%), amylase (70%) and lipase (54%). Moreover, HFD feeding has increased lipid peroxidation and protein carbonylation and decreased antioxidant enzymes levels. However, PL, PJ and PP treatment markedly prevents glucose intolerance, insulin resistance and oxidative stress and decrease amylase and lipase levels.

Conclusion: These findings highlight that a long-term intake of pomegranate extracts might be a potential alternative strategy for the prevention of a HFD induced insulin resistance and oxidative stress.

Keywords: Pomegranate parts, A high fructose-fat diet, Insulin resistance, Oxidative stress, Amylase, Lipase

Background

Obesity is a risk factor for several metabolic diseases, particularly type 2 diabetes [1, 2]. Most patients with type 2 diabetes are overweight or obese, and the dramatic increase in the prevalence of type 2 diabetes was mostly related to an excessive body weight gain [3]. In fact, obesity is a condition marked by an expansion in subcutaneous and /or visceral adipose tissue. This fat accumulation results in an increase in a chronic systemic inflammation. This long term low grade inflammation induces a dysregulation in glucose and fatty acid metabolism, leading to insulin resistance [4, 5]. Several previous studies, have

reported that HFD consumption enhances the accumulation of reactive oxygen species in liver resulting in chronic inflammation [1, 6, 7] leading to insulin resistance and metabolic syndrome. Therefore, decreasing of HFD consumption can reduce liver inflammation and that may be a convenient strategy for preventing obesity-associated metabolic syndromes [6]. Recent epidemiological studies provided evidence linking dietary daily rations to incidence of obesity. Indeed, it has been reported that the increased consumption of high-sugar diets especially fructose-enriched diet [8, 9] or of high fat [10, 11] generated metabolic syndrome, rapidly promotes lipogenesis and worsens insulin resistance [12].

Currently there is a growing popularity of herbal therapies over conventional drugs, 40% of the compounds used in the pharmaceutical industry are derived from

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medicinal plants [13]. Moreover, the World Health Organization (WHO) announced that 80% of people in developing countries frequently use medicinal plants for their primary health care for various diseases. A wide variety of medicinal plants have been found to have a healthful effects in the treatment and the prevention of metabolic syndromes like diabetes and obesity [14–16]. In this context, much more attention has been focused particularly in fruit and vegetables consumption which was generally associated with lower prevalence of metabolic syndrome, like grape [9], green tea [17] and avocado oil [18].

The pomegranate, *Punica granatum* L. belongs to the punicaceae family and is cultivated in several countries, especially in the Mediterranean region. The juice, the seed oil, and the flower extracts have been described to have several beneficial effects in vitro as well as in vivo, such as antidiabetic, anti-obesity, anti-inflammatory, antioxidant, and antitumor effects [19]. They were highly rich in natural antioxidants such as flavonoids [20] and polyphenols [21] compared with any other fruit. In a previous study on the beneficial effects of pomegranate, Harzallah et al., [7] investigated the effect of three extracts (flowers, seed oil and peel) in C57bl/6 male mice, fed high fat and high fructose diet for 28 days. The authors observed that only pomegranate seed oil improved insulin sensitivity following the 4 weeks of treatment. Pomegranate flower and peel extracts have no effect on glucose homeostasis and insulin sensitivity, however they have exhibited a significant anti-inflammatory effect by decreasing the plasma levels of the pro-inflammatory cytokines IL-6 and TNF- α , and increasing the anti-inflammatory cytokine IL-10. Taking these findings together, pomegranate extract particularly peel extract might have a beneficial effect on insulin sensitivity and glucose tolerance if it has been used for a longer period (over 6 weeks). Thus, the aim of our study is to investigate the effect of long term treatment (12 weeks) with pomegranate leaves (PL), juice (PJ) and peel (PP) extracts in male *Wistar* rats fed high fructose-high fat diet, on the glucose tolerance, the insulin sensitivity, the hepatic markers of oxidative stress, and on the activity of α -amylase and lipase.

Materials and methods

Plant material

Pomegranate leaves and fruits were reaped from *Tounsi* trees in Mahdia region, Tunisia. Authenticity of *Tounsi* cultivar was identified by Dr. Faten Zaouay, a taxonomist from the Higher Agronomic Institute (University of Sousse, Tunisia) and a voucher specimen was deposited in herbarium at the Faculty of Pharmacy (University of Monastir, Tunisia). Pomegranate juice and methanolic

extracts of pomegranate leaves and peel were prepared as described in our previous study [22].

Lipid extraction and analysis of fatty acid methyl esters (FAMES)

Protocols of lipid extraction and FAMES analysis were carried out according to the protocol as described previously by our team [23].

Experimental design

Male *Wistar* rats (200–250 g) were obtained from the central pharmacy of Tunisia. For acclimatization conditions and maintenance requirements of rats, standard diet and HFD compositions, show data in our previous study [22]. Five groups (6 animals in each group) were divided. One group was fed with a standard diet only (control group CG). One group was fed with HFD only (obese control group HFD). The three remaining groups were fed with HFD and received daily per gavage one of three pomegranate extracts at a dose of 250 mg/kg of BW, pomegranate juice (Group HFD+ PJ) pomegranate peel (group HFD+ PP) and pomegranate leaves (group HFD+ PL). Throughout the study, body weight and food intake were monitored respectively twice a week and daily. At the end of the experiment, and after an overnight fast the rats were sacrificed by decapitation and the blood was taken for biochemical analysis.

Insulin tolerance test (ITT) and Oral glucose tolerance test (OGTT)

ITT and OGTT were carried out respectively at 10th and 11th week of treatment with pomegranate extracts. For the ITT, the overnight fasted rats received 0.75 IU/kg of insulin solution by intraperitoneal injection, and blood glucose was measured at 0, 30, 60 and 90 min following the injection.

For the OGTT, 2.5 g/10 ml/kg of glucose dissolved in water were loaded to overnight fasted rats by oral gavage and blood samples were collected by incision from the tail for glucose measurement at 0 min before, and, 30, 60, 90 and 120 min after the glucose load. Glucose was determined immediately by a portable glucometer.

Biochemical analysis

The levels of plasma glucose were measured using the glucose oxidase-PAP kit (Biomaghreb, Tunis City, Tunisia). The levels of plasma insulin were measured using RayBio Mouse Insulin ELISA kit (RayBiotech. Catalog #: ELM-Insulin). The α -amylase activity was evaluated according to kinetic method using a commercial kit (BIOLABO ref. 80,023, Maizy, France). The lipase activity was evaluated also according to kinetic method using a commercial kit (BIOLABO ref. 99,891, Maizy, France).

Hepatic markers of oxidative stress

Liver malondialdehyde (MDA) as a marker of lipid peroxidation was measured according to reference [24]. Protein carbonylation (PC) as a marker of protein oxidative damage was quantified according to reference [25]. Superoxide dismutase (SOD) activity was carried out following the method cited in reference [26]. Glutathione peroxidase (GPx) activity was evaluated as described by reference [27].

Statistical analysis

Statistical analysis was performed using SPSS version 21. Duncan's test was used to analyze the data and the criterion for statistical significance was $p < 0.05$.

Results

Fatty acid composition

The fatty acid composition of the studied pomegranate extracts are shown in Table 1. Results showed 33 identified fatty acids ranging from C12:0 to very long chain fatty acids such as C24:0. Unsaturated fatty acids (UFA) were the main fraction in the all extracts and it contained MUFA, Di-UFA and Tri-UFA. Furthermore, four different families of unsaturated fatty acids (UFAs) were detected in our samples including omega 3 (w-3), omega 6 (w-6), omega 7 (w-7) and omega 9 (w-9) fatty acids. Polyunsaturated fatty acids (PUFAs) are reported very important for human health. They prevent and reverse high-fat-diet induced adipose tissue inflammation and insulin resistance [28, 29], decrease blood cholesterol levels, prevent CVD mortality [30] and inhibit apoptosis and neuroinflammation [31]. PJ was more concentrated in UFA and PL presented the highest percentage of SFA. In generally no significative difference was shown in SFA/UFA ratio. The dominant UFA are C18:1 w9 (cis) for PP, C18:1 w7 (cis) for PJ and C18:2 (t9, c12) for PL. The major SFA are C16:0 and C17:0. Unlike to pomegranate seeds oils, these pomegranate extracts contained fewer amounts of Conjugated Linolenic acids (Clna) especially punicic acid C18:3 (c9, t11, c13) which was reported by several researchers as the predominant FA in pomegranate seed oil and its content can exceed 70%. The *trans/cis* ratio seems very low in generally. Researchers reported that the low *trans/cis* ratio has health benefits. In fact, high *trans* fatty acids intake affect serum lipid levels and increased coronary heart disease mortality, and cardiovascular disease (CVD) incidence [32].

Effect on glucose homeostasis and insulin sensitivity

When compared to the group fed chow diet, the fasted levels of plasma glucose and insulin were increased respectively by 30% and 44% in HFD group after the 12 weeks of High fat and high fructose diet feeding (Table.2). The treatment with pomegranate juice leaves

Table 1 Fatty acid composition of individual lipid classes of pomegranate seeds

FATTY ACIDS	PL	PJ	PP
C12:0	0,49 ± 0,13	2,28 ± 0,27	0,74 ± 0,08
C14:0	0,71 ± 0,06	5,24 ± 1,13	1,61 ± 0,41
C16:0	20,71 ± 1,64	14,63 ± 0,64	16,01 ± 1,1
C17:0	16,75 ± 0,12	12,90 ± 2,5	18,77 ± 2,06
C18:0	4,76 ± 0,27	2,96 ± 0,07	3,96 ± 0,12
C20:0	0,39 ± 0,06	0,17 ± 0,06	0,82 ± 0,13
C22:0	0,20 ± 0,02	0,21 ± 0,05	0,63 ± 0,08
C24:0	0,16 ± 0,01	0,84 ± 0,11	0,21 ± 0,01
C14:1 w9	3,48 ± 0,75	0,96 ± 0,12	1,77 ± 0,06
C16:1 w7 (trans)	1,55 ± 0,008	0,33 ± 0,04	1,06 ± 0,004
C16:1 w7 (cis)	0,67 ± 0,08	0,19 ± 0,04	0,57 ± 0,04
C17:1w8	0,58 ± 0,15	0,25 ± 0,01	0,24 ± 0,12
C18:1 w9 (cis)	7,57 ± 0,11	10,93 ± 0,5	18,04 ± 2,51
C18:1 w9 (trans)	9,8 ± 0,5	0,14 ± 0,03	8,54 ± 0,19
C18:1 w7(cis)	0,74 ± 0	31,60 ± 0,1	0,26 ± 0,13
C20:1 w9	0,36 ± 0,15	0,36 ± 0,11	0,3 ± 0,15
C24:1 w9	0,2 ± 0,07	0,21 ± 0,07	0,04 ± 0,01
C18:2 (t9,c12)	19 ± 1,39	8,42 ± 0,06	11,67 ± 0,12
C18:2 (c9,t12)	0,62 ± 0,2	1,42 ± 0,4	1,70 ± 0,05
C18:2 w6 (c9,c12)	0,55 ± 0,13	0,24 ± 0,13	0,33 ± 0,07
C18:3 w6	0,31 ± 0,09	1,56 ± 0,11	0,72 ± 0,32
C18:3 w3 (cis)	1,63 ± 0,12	1,42 ± 0,05	1,69 ± 0,09
C18:2 (t9,t11)	1,96 ± 0,11	0,16 ± 0,03	0,62 ± 0,04
C18:2 (c11,t13)	0,88 ± 0,73	0,09 ± 0,01	2,4 ± 0,15
C18:2 (t10,c12)	0,04 ± 00	0,3 ± 0,06	0,96 ± 0,01
C20:2	0,28 ± 0,09	0,14 ± 0,002	0,25 ± 0,005
C20:3 w6	0,64 ± 0,03	0,04 ± 0,01	0,45 ± 0,08
C20:3 w3	0,93 ± 0,05	0,36 ± 0,05	0,68 ± 0,1
C18:3 (c9,t11,c13)	0,16 ± 0,02	0,08 ± 0,008	1,23 ± 1,5
C18:3 (c8,t10,c12)	2 ± 0,16	0,05 ± 0,02	0,9 ± 0,18
C18:3 (c9,t11,t13)	1,43 ± 0,36	1,52 ± 0,01	1,67 ± 0,12
C18:3 (t9,t11,c13)	0,36 ± 0,02	0,41 ± 0,03	0,47 ± 0,10
C18:3 (t9,t11,t13)	0,53 ± 0,11	0,035 ± 0,01	0,39 ± 0,03
ΣSFA	44,19 ± 1.73 ^a	39,26 ± 2 ^b	42,8 ± 1.6 ^a
ΣPUFA	56,04 ± 1.9	60,32 ± 1,63	57,06 ± 3.5
UFA/SFA ratio	1,26 ± 0,05	1,3 ± 0,07	1.1 ± 0.12
<i>trans/cis</i> UFA ratio	0.77 ± 0.04 ^a	0.015 ± 0.002 ^c	0.4 ± 0.04 ^b

Each value represents the mean of three determinations ($n = 3$) ± SD. Means with different letters within the same row column were significantly different at the level of $p < 0.05$

and peel resulted in a significant reduction in fasted plasma glucose and insulin levels. Consequently, the homeostatic index of insulin resistance (HOMA-IR) which is used to quantify insulin resistance [33, 34], was

Table 2 Effect of HFD and pomegranate extracts administration plasma glucose, insulin levels and index (HOMA-IR)

	CG	HFD	HFD + PJ	HFD + PL	HFD + PP
Glucose (mg/dl)	90.5 ± 9.02**	129 ± 6.32	104.83 ± 9.17**	114 ± 9.40*	113.66 ± 14.26*
Insulin (μIU/ml)	30 ± 11.06**	54.60 ± 8.32	33 ± 6.84**	31 ± 6.93**	37 ± 7.14*
Insulin sensitivity index (HOMA-IR)	2600.5 ± 883.9**	7013.03 ± 1306.8	3459.20 ± 518.59**	3494.9 ± 697.9**	4270 ± 670.7**

Results are shown as the mean ± SEM (n = 6). **p* < 0.05; ***p* < 0.01 versus HFD group, HFD High Fructose-fat Diet, pomegranate leaves (PL), pomegranate juice (PJ) and pomegranate peel (PP)

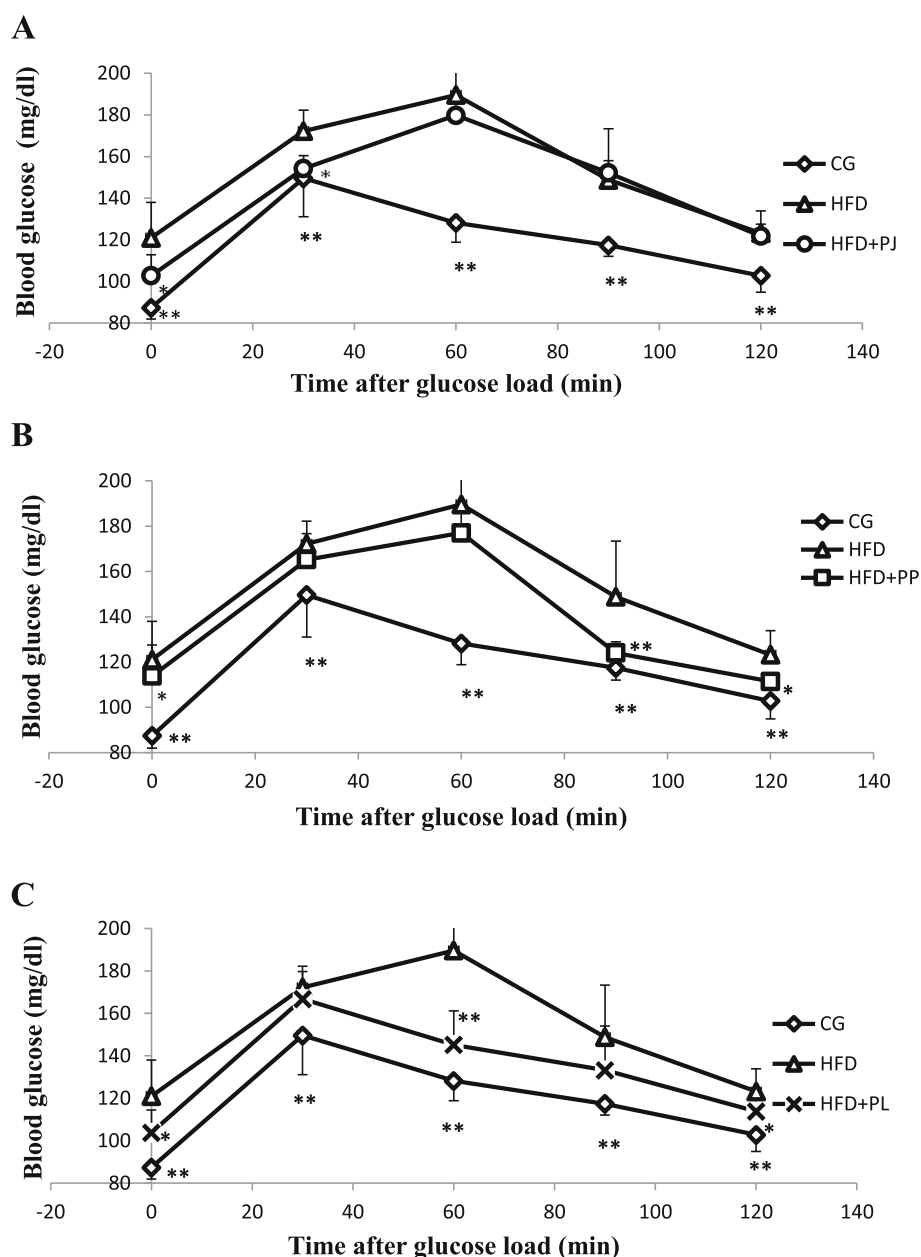


Fig. 1 Effect of pomegranate extracts supplementation on glucose tolerance curve in rats during 11 weeks experimental period. Results are shown as the mean ± SE (n = 6). **p* < 0.05; ***p* < 0.01 versus HFD group, pomegranate juice (a), pomegranate peel (b) and pomegranate leaves (c)

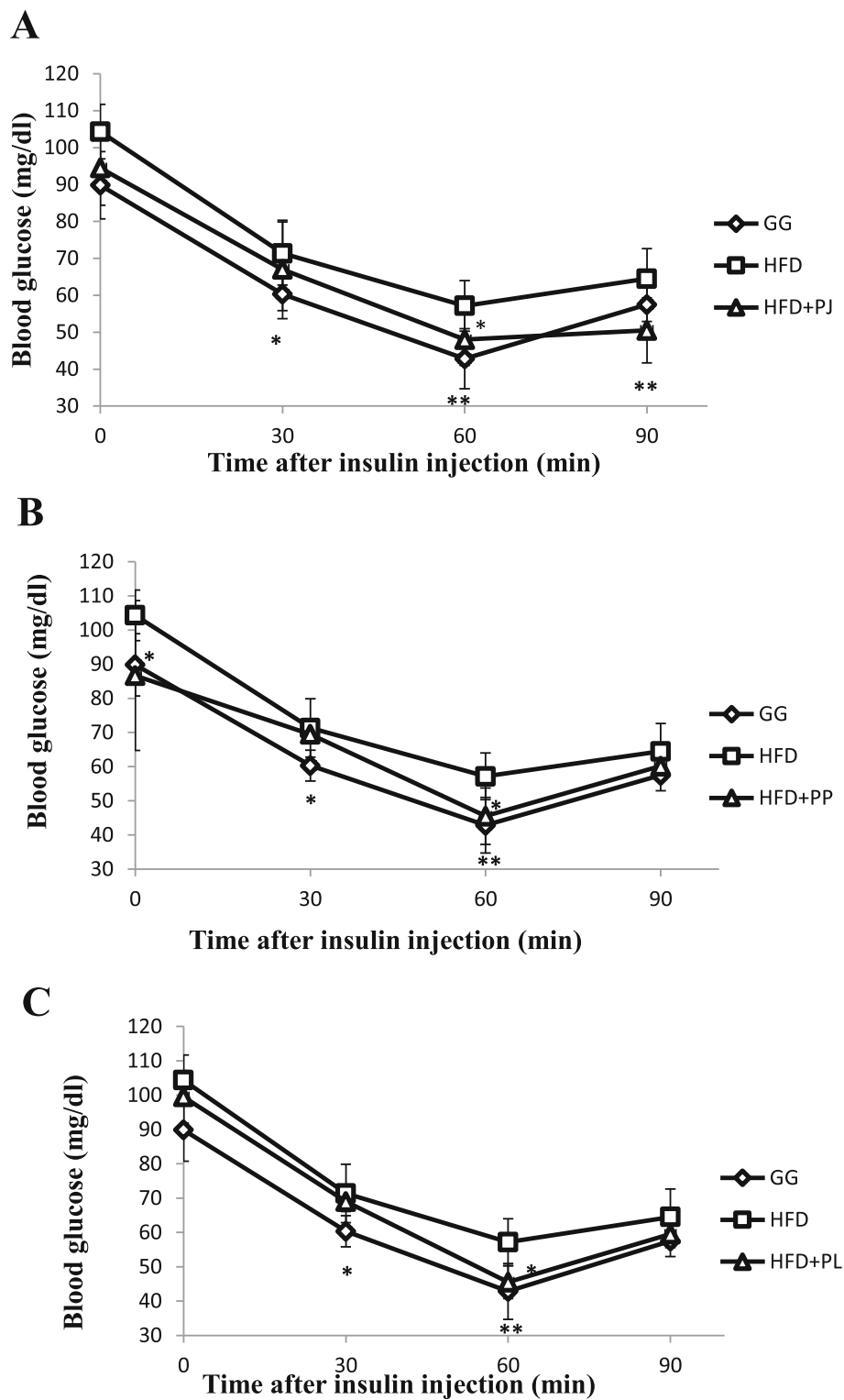


Fig. 2 Effect of pomegranate extracts supplementation on the insulin tolerance curve in rats during 11 weeks experimental period. Results are shown as the mean \pm SE ($n = 6$). * $p < 0.05$; ** $p < 0.01$ versus HFD group, pomegranate juice (a), pomegranate peel (b) and pomegranate leaves (c)

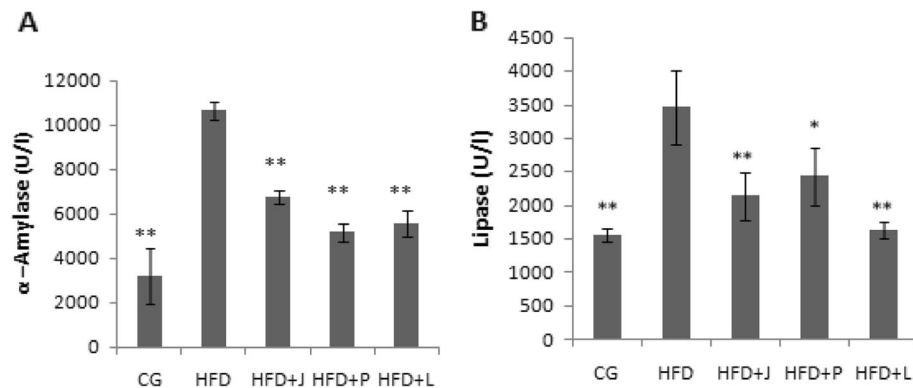


Fig. 3 Effect of pomegranate extracts administration on serum amylase (a) and lipase (b) levels. Results are shown as the mean \pm SE ($n = 6$). * $p < 0.05$; ** $p < 0.01$ versus HFD group, pomegranate juice (PJ), pomegranate peel (PP) and pomegranate leaves (PL)

respectively reduced by half for juice and leaves extracts and by 39.1% for peel extract indicating a significant improvement in insulin sensitivity by the three pomegranate extracts.

To further evaluate the effect of pomegranate extracts on glucose metabolism and insulin sensitivity, we have performed an oral glucose and insulin tolerance tests. Both tests resulted in incremental changes in animal plasma glucose.

After animals received glucose solution, the incremental plasma glucose peaked at 60 min. Results illustrated in Fig. 1; show that HFD feeding impaired glucose tolerance in rat. In fact, HFD group had higher level of blood glucose than control group after 30, 60, 90 and 120 min after oral glucose gavage (Fig. 1). Pomegranate leaves-treated HFD rats had significantly lower levels of plasma glucose than HFD group at 60 min after oral glucose load ($p < 0.01$) and the levels tended to remain low (Fig. 1c). Pomegranate peel-treated HFD rats showed also a slight decrease in plasma glucose at 60 min and a significant decrease after 90 and 120 min after oral glucose gavage compared with HFD group (Fig. 1b). Although, results in Fig. 1a demonstrated that HFD rats treated with pomegranate juice were glucose intolerant. In fact, there were no remarkable differences in the glucose level at any time-point with HFD group after glucose administration.

During insulin tolerance test, results show that HFD feeding impaired insulin sensitivity in rat (Fig. 2). In fact,

the plasma glucose concentrations were decreased significantly at 30 and 60 min for all HFD feeding groups then restored slightly at 90 min ($p < 0.05$). The HFD group had higher level of blood glucose than control group after 30 and 60 min of insulin injection. All pomegranate extracts have preventive effect and decreased significantly the levels of plasma glucose at 60 min after insulin injection ($p < 0.05$) compared to HFD group. This indicates that pomegranate extracts consumption can significantly improve glucose homeostasis and insulin resistance.

Effect on the plasma levels of amylase and lipase

The effect of pomegranate extracts treatment on α -amylase and lipase activities are reported respectively in Fig. 3a and b. The high fat and high fructose feeding induced a significant increase in the serum α -amylase activity by 70% when compared to control rats ($p < 0.001$). Interestingly, the oral treatment with PJ, PP and PL resulted in a significant decrease in α -amylase activity by 30%, 50% and 49% respectively when compared to the HFD group.

Compared to the control group fed chow diet, the HFD feeding induced a potent increase ($p < 0.01$) in the plasma lipase activity. However, the long term treatment with PJ, PP and PL extracts reverted back the lipase activity in plasma by 37%, 51% and 28% respectively.

Table 3 Effect HFD and pomegranate extracts administration on hepatic oxidative markers

	CG	HFD	HFD + PJ	HFD + PP	HFD + PL
GPX (U/g P)	5.20 \pm 0.83**	2.8 \pm 0.23	3.4 \pm 0.7	2.13 \pm 0.54	4.04 \pm 0.91*
SOD (U/mg P)	8.24 \pm 0.45**	3.62 \pm 0.56	5.92 \pm 0.36**	7.32 \pm 0.41**	7.73 \pm 0.78**
MDA (nmol/mgP)	0.29 \pm 0.02**	0.5 \pm 0.07	0.3 \pm 0.06*	0.25 \pm 0.07**	0.30 \pm 0.1*
PC (μ M)	13.67 \pm 1.27**	18.51 \pm 2.63	15.12 \pm 2.15*	12.43 \pm 1.72**	14.42 \pm 0.48*

Results are shown as the mean \pm SEM ($n = 6$). * $p < 0.05$; ** $p < 0.01$ versus HFD group, HFD High Fructose-fat Diet, pomegranate leaves (PL), pomegranate juice (PJ) and pomegranate peel (PP)

Effect on hepatic oxidative markers

Table 3 shows the beneficial effect of pomegranate extracts on hepatic oxidative stress status in HFD fed rats. Results revealed that HFD increased oxidative injury to lipids (by + 42% for MDA level) and proteins (by + 26.14% for PC level) and reduced significantly levels of SOD (by 56%) and GPx (46%) compared to control group. Whereas, the levels of MDA and PC were significantly decreased in all pomegranate extracts treated HFD groups. This indicates that pomegranate juice, peel and leaves alleviated lipid peroxidation and protein carbonylation. Furthermore, pomegranate extracts consumption improved significantly hepatic antioxidant enzymes levels such as SOD and GPx in treated groups compared to HFD group. PL exhibited a significant increase in GPx level, however the increased effects generated by PP and PL were statistically non-significant.

Discussion

Fructose, a dietary monosaccharide is known as a contributor to nearly all of the classic manifestations of the metabolic syndrome, including insulin resistance, hyperinsulinemia, hypertension, and dyslipidemia [35]. For that, several previous researches have used high fructose diet supplementation as an excellent animal model to study metabolic syndrome, type 2 and obesity-induced inflammation and insulin resistance, as well as to investigate the modulating effects of dietary components on progression of these diseases [36–38]. Within this framework, we utilized this model to investigate the potential beneficial effects of the long term treatment with pomegranate leaves, juice and peel extracts. Our results show that the consumption of HFD for 12 weeks markedly induces an increase in body weight (Show our previously published results), glycemia associated with insulinemia and, consequently, an insulin resistance. These findings are consistent with previous studies [36].

Body weight reduction shown in HFD rats may be explained by the consumption of energetic food rich in saturated fats (lard) and carbohydrate (fructose) which stimulates appetite by increasing ghrelin levels [39] and blocked satiety by decreasing leptin levels [40]. During OGTT and ITT, the incremental plasma glucose and insulin concentrations were significantly increased in HFD groups, indicating that HFD feeding markedly impaired insulin-stimulated glucose uptake in peripheral tissues. The action mechanism of fructose-fat feeding on insulin resistance remain incomprehensible but some explanations have been proceeded including insulin signaling alteration [17], the enzymatic activities of carbohydrate metabolism [12] and excessive reactive oxygen species (ROS) production. In this study, we may explain fructose-induced hyperglycemia by increase of ROS and the decrease of the antioxidant protection system in

various tissues like liver. In fact, our findings show that excessive fructose intake causes lipid and protein peroxidation and thus the inhibition of hepatic antioxidant enzyme like SOD and GPx. Others previous investigations reported similar results the same rats model [41, 42].

Results show that pomegranate leaves, peel or juice supplementation prevent body-weight gain (Show our previously published results [22]), hyperglycemia and hyperinsulinemia. Furthermore, they remarkably attenuate the impairment of insulin-stimulated glucose disposal in insulin resistant rats [9]. Moreover, pomegranate extracts protect hepatic antioxidant enzymes and decrease lipid and protein peroxidation. The preventing effect of pomegranate extracts can be related to the antioxidant properties of their active components such as polyphenols, flavonoids and anthocyanins [21, 43].

The inhibition of amylase activity by pomegranate extracts (which has been also reported by other authors [44, 45]) resulted in a decrease in intestinal absorption of carbohydrates by suppressing their breakdown [46]. This reduction of the intestinal absorption of carbohydrates may explain the lowering effect of pomegranate extracts in fasted plasma glucose. Moreover, the decrease in intestinal carbohydrates absorption contributes to the reduction in energy intake leading to the weight loss observed in pomegranate extracts-treated HFD rats (Show our previously published results [22]).

The improvement of insulin sensitivity observed in pomegranate treated animals might be related to the anti-inflammatory properties of the fruit extracts described by Harzallah et al. [7]. Indeed the decrease in pro-inflammatory cytokines such as TNF- α or IL-6 may have a beneficial effect by improving glucose uptake in target tissues such as skeletal muscle and/or adipose tissue.

Moreover, the restoration of plasma lipids profile (Show our previously published results [22]) by pomegranate extracts supplementation may be attributed to the diminution of the fat and cholesterol absorption resulting from the inhibition of lipase, the key enzyme in lipid metabolism. Pomegranate extracts may contain active compounds considered as pancreatic lipase inhibitors [47]. Our findings are in line with a previous report. In fact, a screening of in vitro pancreatic lipase inhibition by extracts of fruits, vegetables, legumes and cereals, containing high levels of anthocyanin reported that pomegranate juice had the highest level of anthocyanin and was the best inhibitor of pancreatic lipase [48].

Conclusion

Our study indicates that the long-term treatment of high fat and high fructose fed rats with pomegranate leaves, peel or juice extracts, improves glucose tolerance, prevents insulin resistance and reduces carbohydrates and lipid absorption by decreasing α -amylase and lipase activities.

Abbreviations

HFD: High Fat High Fructose Diet; BW: Body Weight; CG: Control Group; PL: Pomegranate leaves; PJ: Pomegranate Juice; PP: Pomegranate Peel; MDA: Malondialdehyde; SOD: Superoxide Dismutase; GPx: Glutathione peroxidase; PC: Protein Carbonylation; ROS: Reactive Oxygen Species (ROS); ITT: Insulin Tolerance Test; OGTT: Oral Glucose Tolerance Test; HOMA-IR: Homeostatic Index of Insulin Resistance

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Authors' contributions

Z. Amri designed the study, participated in data collection, analysis, interpretation, and writeup. MR. Ben Kheder participated in study design, data collection and write-up. MS. Zaibi participated in study design and critically revised the manuscript. W. Kharroubi and M. Turki drafted the manuscript and critically revised the manuscript. A. Elfeki and F. Ayadi provided materials and reagents. M. Hammami critically revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

The study was approved by Animal Ethics Committee of the University of Sfax (Sfax, Tunisia) for the care and use of laboratory animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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