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Protective Effect of Gallic Acid against Nonalcoholic Fatty Liver Disease Induced by High Fat Diet

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Authors' contributions

This work was carried out in collaboration among all authors. Author BSI designed and conducted the research. Authors HAS and BM revised and edited the manuscript. Author ESA wrote the initial draft of the manuscript. The authors declare that all data were generated in-house and that no paper mill was used. All authors have read and approved the final version of the manuscript.

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ABSTRACT

Liver is considered as significant organ within body. Aims: Our survey aimed in illustrating protective effectiveness of gallic acid (GA) against high fat regimen nonalcoholic fatty liver disease (NAFLD). Study design: In our study**,** Rats were classified into 3 groups; control, orally given fatty-sucrosed diet, gallic acid treated groups. Methodology: They were evaluated through measuring hepatic cholesterol and triglyceride, alanine and aspartate aminotransferases and gammaglutamyl-transferase; total, direct and indirect bilirubin; total protein, albumin and globulin; hepatic and adipose malondialdehyde, glutathione-S-transferase, superoxide dismutase, catalase, reduced glutathione and glutathione peroxidase activities; glucose, insulin, homeostasis model assessment of insulin resistance, leptin and adiponectin; tumor necrosis factor alpha, interleukin-17 and interleukin-1beta; fatty acid synthase, acetyl-Coenzyme A carboxylase-α and HMGCoA reductase. Results: Our results demonstrated that GA ameliorated the elevated lipid, serum liver function enzymes, bilirubin and the decreased L.glycogen levels and serum protein profile. GA improved the hepatic and adipose antioxidants activities by decreasing MDA and increasing GST, SOD, Cat, GSH and GPx activities. GA ameliorated the elevated Glu, INS, HOMA-IR, LEP and the decreased

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adiponectin levels. Moreover, GA ameliorated the elevated TNF-α, IL-17, IL-1β, FAS, ACC-α and HMGCR levels. Liver and adipose histopathologies confirmed our results.
Conclusion: Gallic acid intake exhibited a beneficial therapeutic effect on nonalcoholic fatty liver disease rats as anti-inflammatory and antioxidant agent.

Keywords: Gallic acid; non-alcoholic fatty liver disease; high fat diet; antioxidants; histopathologies.

1. INTRODUCTION

Metabolic diseases in human can be imitated in rodents by the usage of dietary interventions like high lipid regimen [1]. Chronic exposure to HFD resulted in hepatic steatosis in conjunction with obesity and impaired glucose tolerance condition characterized by dyslipidemia, hyperinsulinemia, liver damage elevated markers, and hypoadiponectinemia [2]. Also high lipid regimen encourage a liver disease called NAFLD that is characterized by increased fat in liver, cumulation of hepatocytes fats, and inflammatory immune cells infiltration in parenchyma of liver and pro-inflammatory cytokines secretion resulting in damage of liver [3]. Nonalcoholic fatty liver disease (NAFLD) is a clinical condition characterized by significant precipitation of lipid in liver and persistent disorders in enzymes of liver [4]. NAFLD is considered to be a series of complex, multifaceted pathological processes including oxidative stress, inflammation, apoptosis, and finally metabolism [5]. Gallic acid (GA) is trihydroxybenzoic acid having the $C_7H_6O_5$ molecular formula and is abundantly found in tea leaves, oak bark, sumac, gallnuts, witch hazel and other plants [6]. It has been shown to have powerful anti-obesity and anti-oxidative action [7]. Also, it is considered to be associated with curing the malady of lipid metabolism including NAFLD [8]. Therefore, the purpose of our survey was to evaluate protective influence of gallic acid against high lipid diet nonalcoholic fatty liver disease.

2. METHODOLOGY

2.1 Experimental Animals

White male wistar rats (*Rattus norvegicus*) weighs about 90-110 g, sixty day old were used in our survey. They were gained from Helwan town's animal house, Cairo, Egypt and kept under observation for 1 week before the onset of the experiment for acclimatization and excluding any intercurrent infection. The animals which were chosen individually and housed in standard polypropylene cages and maintained under normal atmospheric R.T (20-30°C), illumination (12 h light/12 h dark cycles), humidity (50-60%). Rats had free water access.

2.2 Dietary Formula and Tested Bioactive Plant Constituents

Ingredients of diet like casein, cholesterol, corn starch and sucrose were purchased from Oxford laboratories, Mumbai, India; bile salts, DL-Methionine, calcium carbonate and cellulose were purchased from S.D. Fine-Chem Ltd., Mumbai, India; sodium chloride, calcium phosphate and potassium citrate are from Pharmaceutical Chemicals Co. Egypt; other components were obtained from commercial sources. Diets were prepared in the nutrition department, faculty of veterinary medicine, Beni-Suef University, Beni-Suef, Egypt, at intervals according to requirements and stored at four degree celsius till use. Gallic acid (GA) was obtained from Sigma-Aldrich Co. (USA).

2.3 Doses and Treatment

During the entire study, rats fed on fatty-sucrosed diet (FSD) daily for ten weeks. Gallic acid was administered by gastric intubation in a dosage of one hundred mg / kg.bw. dissolved in dist.H_2O Chao et al. [7] daily for ten weeks during the experimental study.

2.4 Experimental Design

In the present study, the rats number is 30 rats. They were categorized into 3 groups; intended as following:

Group 1: Ten rats fed on normal diet (ND) daily for ten weeks.
Group 2: Ten rats fed on fatty-sucrosed regimen daily for ten weeks.
Group 3: Ten rats fed on FSD daily for ten weeks, parallel with gallic acid.

At the end of ten weeks, animals were sacrificed under reasonable diethyl ether numbness. In a

centrifuge tube, Blood was collected from jugular vein of each rat and left to clot at R.T for 45 minutes. After centrifugation at 3000 r.p.m. at 30ºC for fifteen minutes, sera were separated and kept frozen at -30ºC till biochemical analysis. Fresh liver and adipose tissue from each animal were extirpate rapidly. Livers were weighed and parts of them were kept in RNA latter for gene expression analysis. Parts of liver and adipose tissue were fixed in 10% neutral buffered formalin for paraffin section preparation and histopathological examination. 0.5g from liver and adipose tissue was homogenized in five ml 0.9% sterilized sodium chloride by using teflon homogenizer (Glas-Col, Terre Haute, USA) for determination of some oxidative stress and antioxidant parameters. Parts of fresh liver samples were stored at -20◦C to be used for glycogen content determination and for determination of some biochemical parameters.

2.5 Biochemical Analysis

Hepatic cholesterol (H.Chol) and hepatic triglyceride (H.TG) were measured according to Young, [9] method. Glycogen content was determined in the liver according to Seifter et al. [10] method. Liver index was calculated as; Liver index equal Liver weight divided by body weight) x 100%. Serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) were determined according to the kinetic procedure of Tietz, [11]. Gamma-glutamyltransferase concentricity was determined in serum according to the procedure of Young et al. [12]. Serum total bilirubin, direct bilirubin, total proteins and albumin concentrations were determined according to Young, [9] procedure. Globulin concentration in serum was calculated according to Doumas et al. [13] from the equation: Globulin (g/dL) equal total proteins (g/dL) minus albumin (g/dL). Liver and adipose lipid peroxidation were determined by thiobarbituric acid reactive substances (TBARS) measurement according to Ohkawa et al. [14] procedure. Liver and adipose tissues oxidation parameters were assayed according to methods of Habig et al. [15] for glutathione-S-transferase (GST) with little modifications; Nishikimi et al. [16] for superoxide-dismutase activity (SOD); Aebi, [17] for Catalase (Cat) activity with some modifications; Beutler et al. [18] for reduced glutathione (GSH) concentrations and Paglia and Valentine, [19] for glutathione peroxidases (GPx).

Glucose concentration in serum was determined according to the procedure of Young, [9]. Serum insulin, leptin and adiponectin were examined by sandwich ELISA using reagent kit that purchased from BioSource Europe S.A. (Belgium) according to the methods of Templer et al. [20]; Maffei et al. [21] and Hotta et al. [22] respectively. HOMA-IR describes the glucose-insulin homeostasis by the following equation Matthews et al. [23]. HOMA-IR = [(Serum Insulin) × (Fasting Glucose)]/22.5, where 22.5 is the normalizing factor. Real time-Polymerase Chain Reaction (RT-PCR) was used for determination of gene expression of tumor necrosis factor-α (TNF-α), interleukin-17 (IL-17) and interleukin-1β (IL-1β) according to Pfaffl, [24] method. Fatty acid synthase (FAS), acetyl-Coenzyme A carboxylase-α (ACC-α) and HMG-CoA reductase (HMGCR) levels were determined by using western blotting analysis according to Harlow and Lane, [25] method. Finally liver and adipose histopathological study were examined according to Banchroft et al. [26].

2.6 Statistical Analysis

The Social Sciences Statistical Package (IBM SPSS (One Way Anova))for WINDOWS7, version twenty; SPSS Inc, Chicago) was used for the statistical analysis of the results. By using the general linear models procedure (IBM SPSS), comparative analysis was conducted. *P* more than 0.05 were considered statistically non-significant, while *P* less than 0.05 were considered statistically significant.

3. RESULTS

Data regarding effectiveness of high fat diet (HFD) and gallic acid (GA) administration on hepatic lipid profile are presented in Table 1. Our results revealed that the intake of high fat diet produced marked impairment demonstrated by significant increase in hepatic cholesterol, hepatic triglyceride, liver index and significant decrease in liver glycogen content as compared to normal rats, while oral gallic acid administration significantly decreased these elevated levels when compared with the high lipid regimen feeding rats and recorded a noticeable results compared to normal ones.

Gallic acid effectiveness on serum liver function enzymes (ALT, AST, GGT) and serum liver biochemical tests (total, direct and indirect bilirubin) of HFD feeding rats was illustrated in Table 2. GA treatment of HFD feeding rats significantly decreased these elevated values of

liver enzymes and liver biochemical tests in serum and becomes near to those of normal values. Data regarding the high fat diet (HFD) effectiveness and administration of gallic acid on serum protein profile parameters (total protein, albumin and globulin) are presented in Table 3. Our results revealed that the intake of high fat diet produced a marked impairment demonstrated by significant serum total protein, albumin and globulin decrease as compared with normal rats, while oral gallic acid administration significantly increased these decreased levels when compared to the high fat diet feeding rats recording a noticeable amelioration.

Tables 4 and 5 show the effect of gallic acid on the hepatic and adipose tissues oxidative stress and antioxidant markers in HFD feeding rats. Lipid peroxidation (LPO) product was increased significantly in both of them as a result of HFD feeding while the curing of HFD feeding rats with GA significantly decreased these elevated values and becomes near to those of normal values. On the other hands, rats fed with HFD exhibited a noticeable decrease in values of glutathione-S-transferase (GST) activity, activity of superoxide dismutase (SOD), activity of catalase (CAT), reduced glutathione content (GSH) and glutathione peroxidase (GPX) level as compared to normal rats. While, their treatment with GA with HFD feeding produced a significant increase of these antioxidants values as compared to the corresponding HFD administered group pointing to a marked normalization as compared to normal group.

Systemic and hepatic insulin resistance expressed in glucose, insulin, homeostasis model assessment of insulin resistance (HOMA-IR), leptin and adiponectin levels were presented in Table 6. Our results revealed that the feeding with HFD produced marked impairment demonstrated by significant increase in all the mentioned parameters except adiponectin which significantly decreased while oral administration of gallic acid significantly ameliorated these changes.

Data summarized in Table 7 demonstrate the effectiveness of HFD feeding and treatment with GA on hepatic cytokines; TNF-α, IL-17 and IL-1β markers. The results revealed that gallic acid significantly decreased these elevated levels of hepatic TNF-α, IL-17 and IL-1β when compared with the HFD feeding rats recording noticeable amelioration as compared to normal ones.

Effectiveness of gallic acid intake on lipid metabolic regulators; fatty acid synthase (FAS), acetyl-Coenzyme A carboxylase-α (ACC-α) and HMG-CoA reductase (HMGCR) after high fat diet (HFD) are presented in Table 8 and illustrated in Fig. 1. The results exposed that the intake of high fat diet produced a marked impairment demonstrated by a significant increase in FAS, ACC-α, HMGCR levels as compared to normal rats. While oral gallic acid administration significantly decreased their levels.

Table 1. Effect of gallic acid treatment on hepatic lipid profile on high fat diet feed rats

Parameter / Groups	H.Chol (mg/dL)	H.TG (mg/dL)	Liver Index	Liver glycogen (mg/ g liver)
Control	143.57 ± 8.64[a]	428.29 ± 20.004[a]	2.23 ± 0.18[a]	7.88 ± 0.68[c]
HFD	464.05 ± 16.98[c]	785.44 ± 27.73[c]	4.93 ± 0.08[c]	3.88 ± 0.168[a]
HFD+ GA	309.08 ± 24.28[b]	571.35 ± 33.67[b]	4.178 ± 0.06[b]	5.57 ± 0.165[b]
LSD	25.207	39.183	0.168	0.594
P value	.00	.00	.00	.00

- Data are expressed as Mean ± SE, - Numbers of samples in each group is six.
- Values, which share the same superscript symbol, are not significantly different.
- F-probability: P <0.001, - C (control), HFD (high fat diet), HFD+GA (high fat diet + gallic acid).

Table 2. The effect of gallic acid treatment on serum liver function enzymes and serum liver biochemical tests of high fat diet feed rats

Parameter / Groups	ALT (U/L)	AST (U/L)	GGT (U/L)	T.bilirubin (mg/dl)	D.bilirubin (mg/dl)	In.bilirubin (mg/dl)
Control	26.97 ± 0.74[a]	32.97 ± 3.32[a]	14.495 ± 1.362[a]	0.225 ± 0.009[a]	0.097 ± 0.005[a]	0.080 ± 0.01[a]
HFD	52.36 ± 3.45[b]	54.07 ± 4.57[b]	42.751 ± 3.565[c]	0.503 ± 0.086[b]	0.233 ± 0.050[b]	0.264 ± 0.061[b]

Parameter / Groups	ALT (U/L)	AST (U/L)	GGT (U/L)	T.bilirubin (mg/dl)	D.bilirubin (mg/dl)	In.bilirubin (mg/dl)
HFD+ GA	33.05 ± 3.92[a]	32.89 ± 2.16[a]	27.10 ± 3.00[b]	0.275 ± 0.014[a]	0.101 ± 0.010[a]	0.17 ± 0.019[ab]
LSD	4.311	4.942	3.967	0.072	0.0426	0.053
P value	.00	.01	.00	.01	.05	.01

- Data are expressed as Mean ± SE. - Numbers of samples in each group is six.
- Values, which share the same superscript symbol, are not significantly different.
- F-probability: P <0.001, - C (control), HFD (high fat diet), HFD+GA (high fat diet + gallic acid).

Table 3. The effect of gallic acid treatment on serum protein profile parameters on high fat diet feed rats

Parameter / Groups	T.P (g/dL)	Alb (g/dL)	Glb (g/dL)
Control	9.98 ± 0.23[c]	7.89 ± 0.41[c]	2.80 ± 0.37[c]
HFD	6.36 ± 0.20[a]	5.25 ± 0.23[a]	0.78 ± 0.06[a]
HFD+ GA	7.74 ± 0.08[b]	6.71 ± 0.17[b]	1.59 ± 0.05[b]
LSD	0.266	0.419	0.315
P value	.00	.00	.00

- Data are expressed as Mean ± SE. - Numbers of samples in each group is six.
- Values, which share the same superscript symbol, are not significantly different.
- F-probability: P <0.001, - C (control), HFD (high fat diet), HFD+GA (high fat diet + gallic acid).

Table 4. The effect of gallic acid treatment on liver oxidative stress and antioxidant defense system on high fat diet feed rats

Parameter / Groups	Liver					
	MDA (nmol/g.T)	GST (U/g.T)	SOD (U/g.T)	Cat. (U/g.T)	GSH (mg/g.T)	GPx (U/g.T)
Control	22.25 ± 1.62[a]	14.335 ± 0.914[b]	670.87 ± 7.16[c]	19.00 ± 0.15[c]	1.820 ± 0.007[c]	142.65 ± 10.85[b]
HFD	42.56 ± 0.56[c]	10.116 ± 0.435[a]	451.36 ± 15.48[a]	9.60 ± 0.08[a]	0.270 ± 0.020[a]	53.16 ± 10.77[a]
HFD + GA	35.74 ± 2.35[b]	14.033 ± 1.098[b]	607.94 ± 29.57[b]	14.96 ± 0.23[b]	0.577 ± 0.006[b]	126.44 ± 3.55[b]
LSD	2.384	1.220	27.877	0.242	0.0189	12.816
P value	.00	.01	.00	.00	.00	.00

- Data are expressed as Mean ± SE.- Numbers of samples in each group is six.
- Values, which share the same superscript symbol, are not significantly different.
- F-probability: P <0.001, - C (control), HFD (high fat diet), HFD+GA (high fat diet + gallic acid).

Table 5. Effect of gallic acid treatment on adipose oxidative stress and antioxidant defense system on high fat diet feed rats

Parameter / Groups	Adipose					
	MDA (nmol/g.T)	GST (U/g.T)	SOD (U/g.T)	Cat (U/g.T)	GSH (mg/g.T)	GPx (U/g.T)
Control	9.76 ± 0.029[a]	12.540 ± 0.541[b]	704.07 ± 11.17[c]	19.36 ± 0.14[c]	0.216 ± 0.031[b]	126.44 ± 3.55[c]
HFD	38.07 ± 3.05[b]	9.113 ± 0.562[a]	112.24 ± 13.20[a]	9.43 ± 0.08[a]	0.086 ± 0.010[a]	58.35 ± 7.10[a]
HFD + GA	11.62 ± 0.51[a]	11.246 ± 0.413[b]	659.68 ± 12.71[b]	15.17 ± 0.13[b]	0.214 ± 0.029[b]	106.99 ± 3.55[b]
LSD	2.526	0.721	17.531	0.176	0.036	7.103
P value	.00	.01	.00	.00	.00	.00

- Data are expressed as Mean ± SE.- Numbers of samples in each group is six.
- Values, which share the same superscript symbol, are not significantly different.
- F-probability: P <0.001, - C (control), HFD (high fat diet), HFD+GA (high fat diet + gallic acid).

Table 6. Effect of gallic acid treatment on systemic and hepatic insulin resistance on high fat diet feed rats

Parameter / Groups	Glu (mg/dL)	INS (µU/ml)	HOMA-IR index	LEP (pg/ml)	Adiponectin (pg/ml)
Control	84.29 ± 1.93^{a}	2.736 ± 0.093^{a}	10.26 ± 0.48^{a}	2.913 ± 0.290^{a}	17.20 ± 1.23^{c}
HFD	135.98 ± 8.04^{c}	4.953 ± 0.021^{b}	29.96 ± 1.88^{c}	11.066 ± 0.327^{c}	7.18 ± 0.67^{a}
HFD + GA	120.71 ± 2.21^{b}	2.820 ± 0.164^{a}	15.10 ± 0.86^{b}	4.170 ± 0.171^{b}	12.86 ± 0.63^{b}
LSD	6.990	0.155	1.738	0.384	1.261
P value	.00	.00	.00	.00	.00

- Data are expressed as Mean ± SE. - Numbers of samples in each group is six.
- Values, which share the same superscript symbol, are not significantly different.
- F-probability: P <0.001, - C (control), HFD (high fat diet), HFD+GA (high fat diet + gallic acid).

Table 7. Effect of gallic acid treatment on cytokines on high fat diet feed rats

Parameters / Groups	TNF-α	IL-17	IL-1β
Control	1.006 ± 0.004^{a}	1.005 ± 0.002^{a}	1.006 ± 0.004^{a}
HFD	5.850 ± 0.514^{c}	6.033 ± 0.152^{c}	5.433 ± 0.600^{b}
HFD + GA	2.580 ± 0.171^{b}	2.300 ± 0.289^{b}	1.966 ± 0.111^{a}
LSD	0.442	0.267	0.498
P value	.00	.00	.00

- Data are expressed as Mean ± SE. - Numbers of samples in each group is six.
- Values, which share the same superscript symbol, are not significantly different.
- F-probability: P <0.001, - C (control), HFD (high fat diet), HFD+GA (high fat diet + gallic acid).

Table 8. Effect of gallic acid treatment on the expression of lipid metabolic regulators in the liver on high fat diet feed rats

Parameter / Groups	fatty acid synthase (FAS)	acetyl coenzyme A carboxylase-alpha (ACC-α)	HMG-CO A reductase (HMGCR)
Control	1.010 ± 0.006^{a}	1.003 ± 0.002^{a}	1.013 ± 0.008^{a}
HFD	6.243 ± 1.156^{b}	6.566 ± 0.657^{b}	7.133 ± 0.354^{b}
HFD + GA	3.066 ± 0.327^{a}	2.100 ± 0.073^{a}	1.533 ± 0.091^{a}
LSD	0.981	0.540	0.299
P value	.00	.00	.00

- Data are expressed as Mean ± SE.- Numbers of samples in each group is six. - Values, which share the same superscript symbol, are not significantly different. - F-probability: P <0.001, - C (control), HFD (high fat diet), HFD+GA (high fat diet + gallic acid).

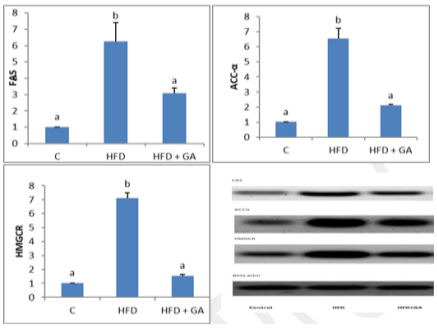

Fig. 1. Illustrated and Western blotting analysis showing the effect of gallic acid treatment on liver fatty acid synthase, acetyl coenzyme A carboxylase-alpha and HMG-CO A reductase levels on HFD feed rats

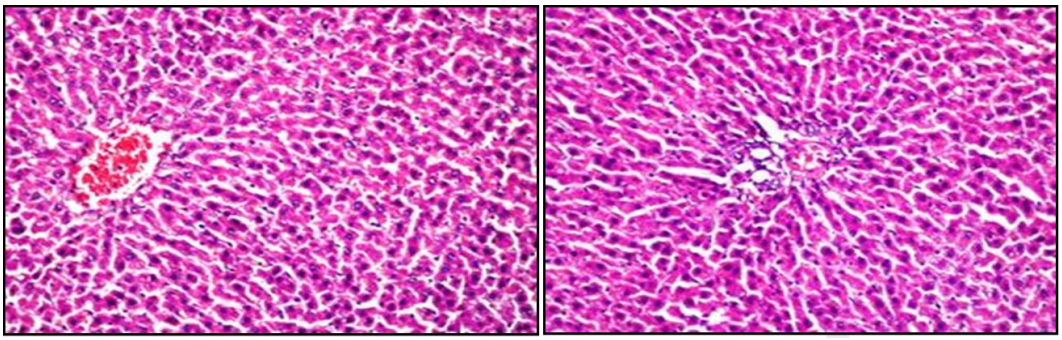

Fig. 2. Photomicrographs of liver sections of control rats showing normal histological structure of the central vein as well as the portal area with portal vein, hepatic artery and bile ducts with the surrounding hepatocytes in the parenchyma

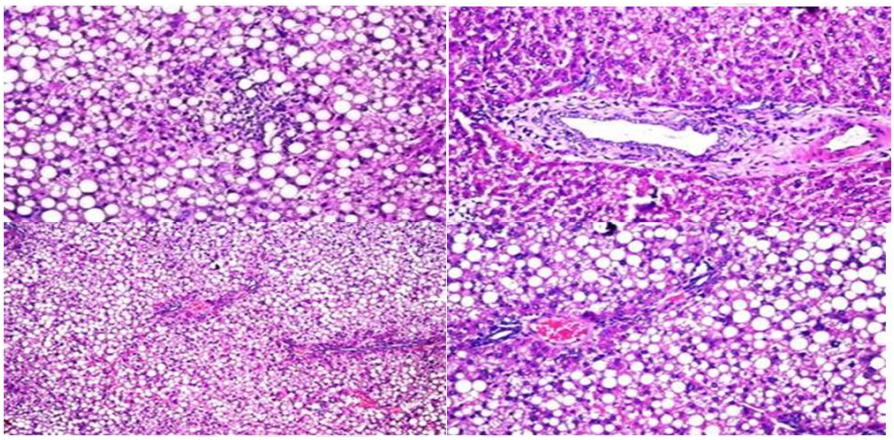

Fig. 3. Photomicrographs of liver sections of rats fed with high fat diet showing fatty change in diffuse manner all over the hepatocytes in the parenchyma associated with congestion in the portal vein and fibrosis in the portal area. Focal inflammatory cells infiltration in the parenchyma. The portal area showed periductal fibrosis surrounding the hyperplastic bile ducts

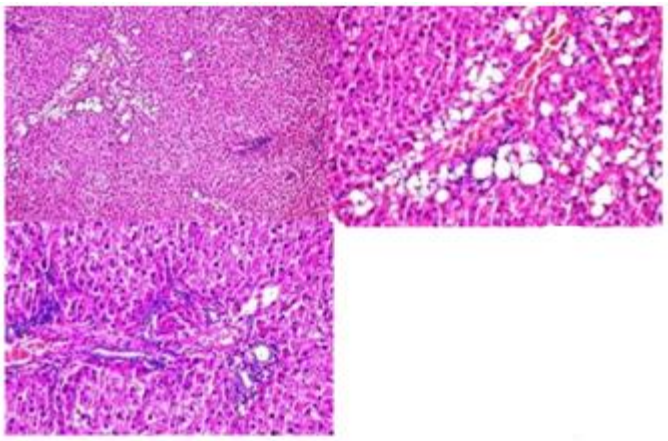

Fig. 4. Photomicrograph of liver section of rat fed with high fat diet and treated with gallic acid showing fatty change in some few hepatocytes in focal manner I the parenchyma associated with few fibrosis in the portal area and congestion in the portal vein

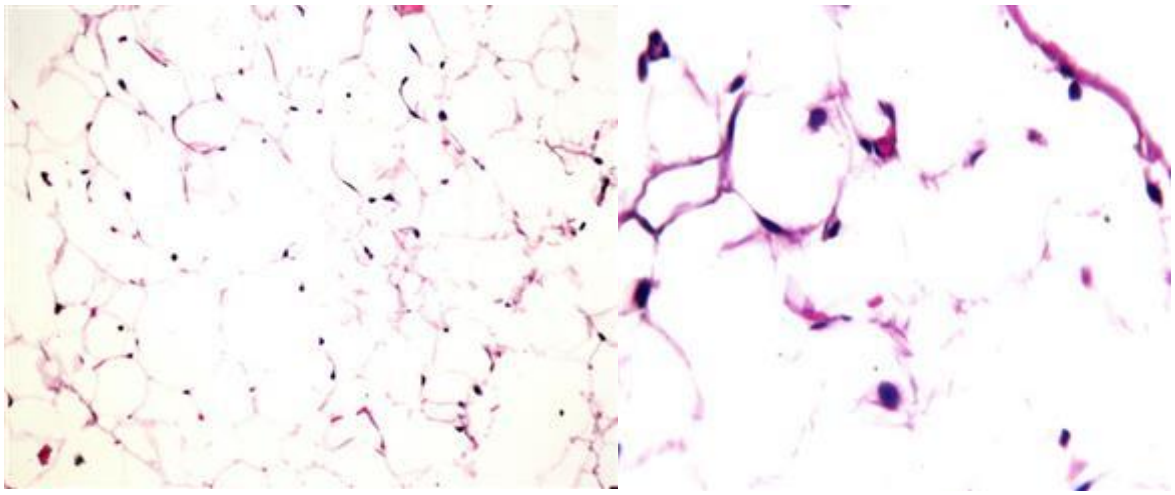

Fig. 5. Photomicrographs of adipose sections of control rats showing no histopathological alteration and normal histological structure of the hexagonal adipoblasts cells in the lobules with flattened nuclei

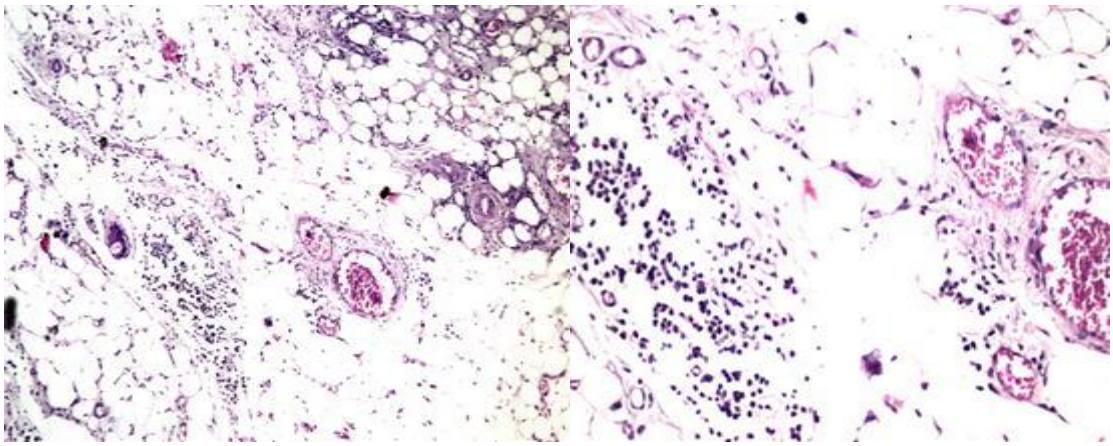

Fig. 6. Photomicrographs of adipose sections of rats fed with high fat diet showing inflammatory cells infiltration between the lobules and cells with dilatation and congestion in the blood vessels

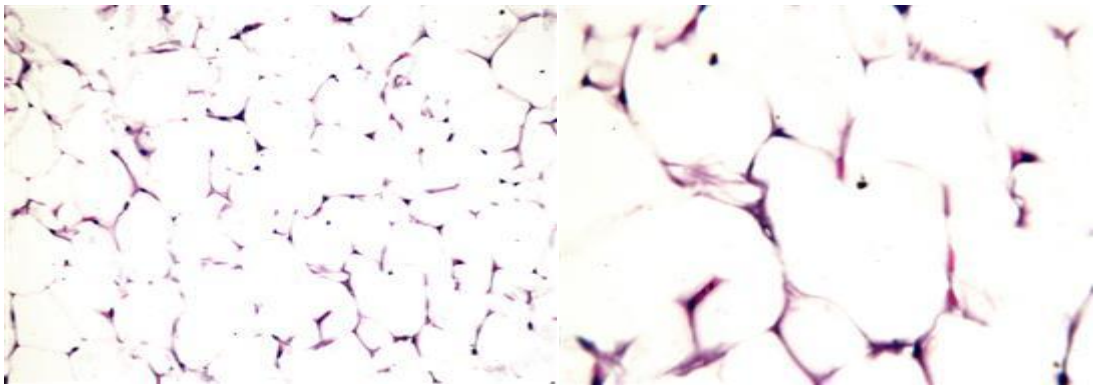

Fig. 7. Photomicrograph of adipose section of rat fed with high fat diet and treated with gallic acid showing no histopathological alteration

Normal liver histology and liver histopathological study showing the effect of HFD feeding and treatment with GA are presented in Figs. 2, 3, 4 and revealed that the liver of animals treated with high fat diet showed change in fats in a diffuse manner all over the hepatic cells in the parenchyma associated with congestion in the portal vein and portal area fibrosis and showed focal inflammatory cells infiltration in the parenchyma and the portal area showed periductal fibrosis surrounding the hyperplastic bile ducts. Oral administration of GA showed fatty change in some few hepatocytes in focal manner I the parenchyma associated with few fibrosis in portal area and congestion in portal vein. Normal adipose histology and adipose histopathological study showing the effect of HFD feeding and treatment with GA are presented in Figs. 5, 6, 7 and revealed that the adipose of animals treated with high fat diet showed inflammatory cells infiltration between the lobules and cells with blood vessels dilatation and congestion. Oral GA administration showed no histopathological alteration in adipose tissue.

4. DISCUSSION

The fatty liver disorders spectrum that not resulting from abuse of alcohol, autoimmune, drug, viral and genetic etiologies is formerly named nonalcoholic fatty liver disease (NAFLD) but newly has been renamed metabolic dysfunction associated fatty liver disease (MAFLD) [27]. NAFLD is manifested through the accumulation of lipids in the liver in the excess consumption of alcohol absence, resulted from the imbalance between input of lipid (uptake of fatty acid as well as *denovo* lipogenesis) and output (very lowdensity lipoprotein (VLDL) export and fatty acid β-oxidation) [28]. The progressive transmission from steatosis to nonalcoholic steatohepatitis was explained initially by a 2-hit hypothesis, although new studies have suggested a modified 'multiple-hit' paradigm. In this condition, the 1st hit is insulin resistance (IR) and disturbance of metabolic, which results in steatosis of liver [29]. This is followed by a spectrum of hits inclusive oxidative stress, proinflammatory cytokine-mediated hepatocyte damage, changed lipid partitioning and toxicity of liver mediated by free fatty acids (FFAs), unnatural loading of cholesterol inside liver, high level of insulin in blood, elevated level of leptin in blood and hypoadiponectinaemia [30]. Diet with high-fat and diet with methionine choline-deficiency are mostly used to produce steatosis of liver and NASH in experimental animals [31]. Unlike other various animal samples, animals that fed an high fat diet mimic both the pathogenesis and histopathology of NAFLD human because they have the note features observed in human patients with NAFLD including fatness and insulin resistance (IR) [29]. The results obtained in the present work can be discussed in two main aspects: first, the development of nonalcoholic fatty liver disease model induced through feeding with high lipid diet; second, gallic acid effects against changes induced by feeding with high fat diet. Our results are in accordance with those acquired by Jung et al. [32]; Zhihong et al. [33]; Lihua et al. [34]; Shing-Hwa et al. [35] in the changes of serum and hepatic lipids, insulin resistance (IR) and hepatic glycogen in the rats fed HFD. Insulin resistance (IR) obtained after feeding HFD explains some of these obtained changes as it results in elevation in the level of triglyceride in liver and in the end steatosis of liver via different mechanisms. 1st, insulin fails to inhibit lipolysis of adipose tissue by hormone-sensitive lipase, leading to increased efflux of FFAs into the circulation and subsequently uptake by the liver [36]. Second, hyperinsulinaemia and hyperglycaemia associated with IR promote synthesis *of de novo* lipid in liver through the membrane-bound transcription factor sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate response element-binding protein (ChREBP) upregulation [37]. Third, high levels of insulin in blood directly inhibits FFAs β-oxidation. Simultaneously, these phenomena encourage hepatic accumulation of FFA and result in accumulation of triglyceride in liver and steatosis through esterification [29].

Glycogen content decrease in liver is a marker of liver's IR [33]. This may be due to an insulin deficiency state because it depends on insulin for influx of glucose [38]. A previous study reported that HFD fed mice developed IR, displayed elevated blood levels of glucose and insulin, as well as decreased glycogen synthesis, phosphorylated glycogen synthase kinase 3β (p-GSK3β) and phosphorylated Akt (p-Akt) expression and glycogen synthase (GS) mRNA levels [39]. Also we know that, insulin inhibits glycogenolysis and if there is a lack of insulin liver glycogen content will decrease [40]. In Jing et al. [41] study, alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities and liver index increased in HFD fed rats indicating the lesions in their liver. The highly palatable, energy rich HFD elicited significant increased levels of body weight, serum glucose, leptin and

insulin along with a correspondent increase in liver, heart, pancreas, kidney, spleen and mesenteric, perirenal, gonadal and subcutaneous fat pad weights. This extra fat accumulates around the subcutaneous, mesenteric, perirenal and gonadal fat pads, which are easily prone for the fat accumulation and the increase in weight [42]. In our study, administration of gallic acid to high fat diet feed rats resulted in decreased levels of hepatic cholesterol, triglycerides and liver index and on the other hands it increased the level of liver glycogen. These results are compatible with those obtained by Jung et al. [32]; Chin-Lin and Gow-Chin, [43] and Yuanyuan et al. [8]. Also, Punithavathi et al. [38] stated that the overall gallic acid protective effects in rats with type II diabetes might be due to lowering in blood glucose and hepatic glycoprotein components, lipid peroxides, lipids and increase in plasma insulin and hepatic glycogen, antioxidant systems. Jung et al. [32] proved that protective effect of GA in liver is partly due to elevation in the liver fatty acids β-oxidation. Yuanyuan et al. [8] explained that gallic acid effectively reduced the intracellular level of TG in the NAFLD model, enhanced the level of AMP-activated protein kinase (AMPK) and ACC2 phosphorylation, up-regulated the expression of CPT1A, which suggested that GA enhanced the effect of lipid metabolism through AMPK/ACC2/CPT-1A signaling pathway regulation. Moreover, activation of AMPK induces autophagy directly through the unc-51 like autophagy activating kinase 1 complex phosphorylation or indirectly via the mammalian target of rapamycin (mTOR) signaling inhibition [44]. Autophagy prevents the lipid droplets accumulation and therefore regulates metabolism of lipid [45]. GA being an antioxidant decreases the stress to a great extent thereby decreasing the excess sugar desire and consequently recoups hepatic and renal glycogen content [46].

Liver function tests (LFTS) are typical biochemical markers to identify patients with nonalcoholic steatohepatitis (NASH) and cirrhosis, without showing steatosis utility [47]. The data of the present study showed that feeding with high fat diet resulted in a significant rise in ALT, AST and GGT activities. These results agreed with those acquired by Banderas et al. [48]; Zhihong et al. [33]; Lihua et al. [34] and Shing-Hwa et al. [35]. Del et al. [49] stated that lipid peroxidation products such as malondialdehyde, GGT and oxidized LDL which are increased in patients with NAFLD may be related to antioxidants decreasing. Fatma et al. [50] explained that high fat diet may increase the liver fatty acid synthesis and the free fatty acids (FFAs) delivery to the liver. Also, it may decrease free fatty acids β-oxidation that in turn may cause hepatic accumulation of fat. Liver is bombarded by FFAs that pour out into the portal blood from the adipose tissue [51]. This can directly cause liver cells inflammation that then librate further pro-inflammatory cytokines resulting in more hepatocyte damage and therefore affecting the liver cells solidity [52]. Injury of hepatocyte caused impairment in the permeability of liver cell membrane [53]. Damage of the cell membrane caused leakage of these enzymes which are normally situated in the cytosol into the stream of blood, thus manifesting damage effected to liver and other tissues [54]. Gallic acid amelioration in these previous liver function biomarkers corresponds to Mahaboob et al. [55]; Vijaya et al. [56] and Jung et al. [32] who suggest that antioxidant and free radical scavenging properties, reported previously may account for the gallic acid hepatoprotective effect. The reversal of increased enzymes by GA may be due to the prevention of the intracellular enzymes leakage by its antioxidant and membrane-stabilizing activity which was supported by histological amelioration as indicated in our results. In the present study, our results of increased levels of serum total, direct and indirect bilirubin in HFD go parallel with Kumar et al. [57]; Ryoko et al. [58]. Treatment with GA indicate the effectiveness of the drug in maintaining the liver functional homeostasis [59].

The decreased levels of total protein, albumin and globulin in serum may result from dysfunction of insulin that is caused by feeding with HFD and inevitably results in increased catabolism of protein [32]. This produces precursors for gluconeogenesis and generation of energy through tricarboxylic acid (TCA) cycle, and lowered production of protein [60]. Atef et al. [61] demonstrated that depletion is due to albumin and amino acids leakage from the damaged renal tubules. Also, Murray et al. [62] explained this depletion by losing the anabolic effect of insulin. Also, Amel, [53] stated that synthesis of protein is lowered in all tissues because of lowered production of ATP responding to absolute or relative deficient insulin and alkaline phosphatase activity. Cecily and Daisy [63]; Jung et al. [32] and Adel et al. [64] also demonstrated an ameliorative effect of GA on protein levels HFD feeding rats.

An important risk factor in NAFLD pathogenesis is oxidative stress [65]. Cytokine challenge and oxidative stress are substantial in mediating NAFLD progress from steatosis to nonalcoholic steatohepatitis (NASH), fibrosis and finally cirrhosis [34]. Increased FFAs in plasma are taken up by the liver resulted in accelerated hepatic β-oxidation rate, that is the main resource of production of ROS in liver [33]. In the body, white adipose tissue (WAT) is found around numerous internal organs [66]. The main metabolic pathways of adipose tissue are lipogenic (triglycerides synthesis) and lipolytic (triglycerides degeneration into glycerol and free fatty acid) [67]. It is possible that this change in metabolic activity will affect the oxidant-antioxidant balance in the adipose tissue [66]. The present study data showed that feeding with HFD caused a significant elevation in level of liver and adipose lipid peroxidation products (LPO) and caused a considerable decrease in their antioxidant defense system. These data paralleled with numerous investigators; Lai et al. [68]; Zhihong et al. [33] and Evelyn et al. [69]. ROS hit polyunsaturated fatty acids and start cellular peroxidation of lipid leading to the forming of aldehyde by-products, like MDA. These molecules have the power to diffuse from their origin sites to reach remote intracellular and extracellular targets, that way amplifying the effects of oxidative stress [34]. A main oxidative stress source in NASH is the surplus load of FFA output from IR and obesity [29]. In the state of FFA load, β-oxidation in mitochondria can become overwhelmed, resulting in rise in production of ROS [70]. In the presence of IR, an increase in FFAs induces the elevated expression of the cytochrome enzyme, P4502E1, in liver microsomes, enhances mitochondrial β oxidation, and generates a substantial quantity of ROS, which exceeds the elimination ability of the anti-oxidative system [8]. In addition, oxidative stress may be rised in metabolic syndrome because of dyslipidemia resulting from elevated TGs and FFA levels which led to increased foam cells forming, rendering LDL less dense and extra vulnerable to oxidation and uptake by macrophages [71]. Also, Cemil et al. [66] study showed that the high-fat diet (HFD) increased the oxidative stress in the adipose tissues of the rats whose retroperitoneal adipose tissues were denervated. Excessive free fatty acids (FFAs) results in an overflow of electrons in the electron transport chain (ETT) pending oxidative phosphorylation leading to their leakage and $O_2^{\bullet -}$ generation followed by the production of another reactive oxygen species molecules [72]. When the reactions of SOD and CAT are taken into consideration, the decrease in activity of SOD in the HFD group causes the inability to remove the superoxide radical, which is formed due to metabolism. As a result of that, it is highly possible that the superoxide radical will attack the lipids and cause the MDA level to increase [66]. Antioxidant enzyme and GSH decrease may result from fast consumption and exhaustion of storage of this enzyme in combating free radicals produced during development of obesity [54,73]. Elevated activity of the polylol pathway inhibition of the pentose phosphate pathway because of hyperglycemia resulted in decreased intracellular NADPH grades that is required for restoration of GSH from its oxidized form GSSG [74]. Moreover, Videla et al. [75] stated that the raise in activity of pro-oxidant is associated with a decrease in the antioxidant potential (activity of SOD and glutathione content). In adipose tissue, obesity can stimulate oxidative stress fundamentally via dysfunctional mitochondrial oxidative phosphorylation or through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) enzyme catalytic activity [76]. NOX stays the major way for production of ROS in adipose cells and also increased pro-inflamatory cytokines in adipose tissue [6]. It is recognized that cumulation of WAT in obese subjects results in motivation of MCP-1 [77]. This causes rise in TNF-α secretion which triggers NF-κB activation. This protein controls many pro-inflammatory cytokines and cyclooxygenase-2 transcription which play important role in oxidative stress [78]. GA has a strong antioxidant with the ability to eliminate free radicals and also has an effect of anti-oxidative [79] and anti-inflammatory activities [80]. This is due to the inhibitory activity of GA on production of free radical could be due to its action on cytochrome P450-dependent mono-oxygenase activities and the epoxide hydrolase activity enhancement [55]. Also, it is via upregulation of expression of peroxisome proliferator-activated receptor (PPAR)γ and activation of NAD-dependent deacetylase sirtuin-1 (SIRT1)/ peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC1α) pathway, this phenolic acid can stimulate the adipose tissue browning [81]. GA has been shown to specifically target the adipose tissue to inhibit lipogenesis, ameliorate insulin signaling, and concomitantly combat raised pro-inflammatory response and oxidative stress [6].

Insulin resistance (IR) is the 1st step and physiopathological key toward NAFLD growth

[82]. Elevations in serum free fatty acids (FFA), in several inflammatory cytokines (ex; tumor necrosis factor-alpha (TNF-α)), as well as reactive oxygen species (ROS) are remarkable mediators of IR in obesity and NAFLD as they stimulate IR at the insulin resistance substance (IRS) proteins level [83]. The index of homeostasis model assessment of insulin resistance (HOMA-IR) is used to appreciate systemic IR, and increased HOMA-IR values mark increased IR grade [33]. A major target tissue of insulin action is the liver [84]. Leptin (LEP) is accountable for regulation of nutrition attitude via satiety promotion [29]. Adiponectin is a secretory protein produced by WAT adipocytes [85]. It is a potent anti-inflammatory and TNF-α-neutralizing adipocytokine. Experimental animal and In vitro studies have elucidated the significance of this mediator in revocation of IR and inflammation [34]. The present research data showed that feeding with HFD resulted in significant increased serum insulin, leptin, homeostasis model assessment of insulin resistance (HOMA-IR), glucose levels and caused a significant decreased adiponectin level. These results agreed with Evelyn et al. [69]; Tahereh et al. [77] and Ayat et al. [86]. HFD decreases the receptors of insulin expression, prohibits the oxidation of fatty acids (FA) in skeletal muscles, reduce the expression of GLUT4 mRNA and its protein content inside cells, and decreases the translocation of GLUT4 to the membrane of the cell, factors that may be responsible for the observed high blood glucose ad insulin levels in animals [87]. The explanation for these results is that obesity is associated with low degree of chronic systemic inflammation which probably results in IR [42]. Tumour necrosis factor (TNF)-α overexpression in puffy patients stimulate IκB kinase β, which play remarkable fuction in development of IR via preventing insulin receptor substrate (IRS)-1 and IRS-2 phosphorylation [36]. Under physiological conditions, excessive FFA is transformed into TGs or cholesterol esters and thereafter stocked in lipid droplets inside adipocytes [88]. The initial TGs deposition takes place in subcutaneous adipose tissue, and as the deposition rises in size, IR increases and borders further accumulation of lipid under the skin [89]. Thereafter, TGs are diverted to the visceral fat store and to non-adipose tissue [90]. The latest is likewise recognized as ectopic lipid cumulation and may be linked to IR and cellular dysfunction [91]. In the liver and skeletal muscle, deposition of fat has been associated with the IR development [92]. Maria et al. [93] stated that in humans, increased leptin levels are observed in patients with NAFLD/NASH and in obese individuals. The positive correlation between serum leptin and body fat is probably explained by the increased leptin release from large fat cells [94]. Also, there is decreased production of adiponectin, which has insulin-sensitizing, anti-inflammatory, vascular-protective properties [95]. Adiponectin (an adipocyte-derived hormone) is produced and secreted via nuclear receptor PPARγ activation, and its production is decreased by caloric excess, by means of a mechanism which is supposed to be associated with reduction or impedance of leptin [96]. The lowering of the levels of glucose via GA can be demonstrated by variation of various signalling pathways inclusive AMPK/Sirt1/PGC1α stimulation [81] and Akt-marking and raised glucose transporter-4 translocation may furthermore play a function [97]. Together, activation of AMPK reduces IR and serves as a potential curative involvement for obesity and T2DM therapy by regulating metabolism of fatty acid, via improving mitochondrial function, and via clearing the damaged mitochondria by autophagy [98]. The observed activation of Akt in liver by GA might partly participate in the reduction of gluconeogenic genes like PEPCK and G6Pase and finally supply the useful effects observed in metabolism of insulin and glucose [99].

The equilibrium between cytokines/adipocytokines and pro-inflammatory and anti-inflammatory activities plays a key role NAFLD growth [34]. There is much evidence supporting a focal role for pro-inflammatory cytokines, specially tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), in nonalcoholic steatohepatitis (NASH) growth [100]. Our results demonstrated that feeding with HFD caused a considerable rise in the levels of tumor necrosis factor-α (TNF-α), interleukin-17 (IL-17) and interleukin-1β (IL-1β) in the liver. These data are in accordance with those obtained by Tilg, [101]; Do-Geun et al. [102] and Jennie et al. [29]. Also, Cai et al. [103] revealed that steatosis induced with HFD, increased activity of nuclear factor kappa beta (NF-κB) is associated with high expression of hepatic inflammatory cytokines, inclusive TNF-α and Kupffer cells stimulation. In the setting of intake of excessive dietary fat, high free fatty acids (FFAs) levels are delivered to the liver. Stimulation of hepatocyte by FFAs leads to the intracellular translocation of Bax to the lysosome. Permeability of lysosomes is increased, leading to Cathespin B release.

Cathespin B presence in the cytosol causes translocation of NF-κB into the nucleus with increased production and release of TNF-α which inhibits action of insulin [104]. Administration of GA to high fat diet feeding rats caused a considerable decrease in TNF-α, IL-17 and IL-1β. In accordance with our results, Yuanyuan et al. [8] stated that GA decreased levels of cytokine and regulated the expression of enzymes related to metabolism of lipid at the protein and mRNA levels. In the model with NAFLD, GA can significantly down-regulate the intracellular TNF-α and IL-8 levels, suggesting that GA is capable of mitigating NAFLD incidence and progression by decreasing the relevant cytokines levels [8]. Ashok et al. [105] found that GA probably repressed pro-inflammatory cytokines (TNF-α, IL-17, IL-1β and interferon- gamma (IFN-γ)) and inflammatory mediators, like COX-2 and iNOS. Therefore, GA attenuates rats liver injury induced with NDEA probable by inducing Nrf2-mediated antioxidant enzymes and attenuating the inflammatory mediator COX-2 through inhibition pathway of NF-kB [59].

The enzymes of ACC and FAS are involved in *de novo* lipogenesis in liver, which contributes to NAFLD pathogenesis [106]. Elevated FAS, Acly, SREBP1c and other genes participate in the de novo synthesis and fatty acids uptake have been reported in NAFLD animal paradigms beside human studies [107]. Also, NAFLD has been found to be associated with the increased HMGCR activity which is a cholesterol synthesis rate limiting step enzyme [108]. The present research data showed that high fat diet supply rats exhibited a considerable raise in fatty acid synthase (FAS), acetyl-Coenzyme A carboxylase (ACC-α), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) reductase levels. These data go parallel with Zhihong et al. [33] who revealed that in the route of *de novo* synthesis, protein expressions of FAS, ACC-α and sterol regulatory element binding protein-1c (SREBP)-1c were more elevated in high fat diet (HFD) rats than in control rats. Insulin (INS) is responsible for regulation of the transcription and activation of liver SREBP-1c, that participates in hepatic synthesis of fatty acid by activating the expression of FAS and ACC1 [109]. Zhihong et al. [33] also, showed that in the route of metabolism of cholesterol, the increased expression of HMGCR and decreased CYP7A1expression were seen in rats feed with HFD compared with the control rats. Mao et al. [109] also, showed that FAS, mature SREBP-1c, immature SREBP-1c protein and ACC1 mRNA elevated in obese rats livers when compared with the control group. In the same way, Yinrun et al. [110] revealed that mRNA expression of FAS, ACC1, HMGCR, acyl-coenzyme A: cholesterol acyltransferase (ACAT2) and apolipoprotein B (ApoB) were down-regulated significantly, while expression of CYP7A1 was up-regulated significantly in the livers of treated rats as compared to untreated rats with hyperlipidaemia. Angulo, [111] stated that in NAFLD period, the liver increases the fat uptake and synthesis. Consequently, obese rats overexpress genes involved in *de novo* lipogenesis, inclusive FAS, ACC and SREBPF1 [112]. As total cholesterol increases in the body, the synthesis rate of cholesterol tends to decrease, due to a negative feedback regulation of the HMGCR enzyme [113]. Sterol regulatory element-binding proteins (SREBPs) are the main transcriptional factors in expression of lipogenic gene inclusive FAS [114]. SREBP1 is stimulated by elevated levels of insulin, and increased carbohydrate diet feeding rapidly stimulated expression of FAS in livers of rats [115]. Furthermore, levels of SREBP1 mRNA expression have been found to be elevated in NASH animal samples [116]. In line with our data, Caballero et al. [117] revealed that a paradoxical increase in hepatic HMGCR mRNA expression has been found in NAFLD obese patients liver. The results have obviously proved that during feeding with HFD, elevated Sp1 mediated SREBP-2 expression up-regulates HMGCR resulting in increased biosynthesis of cholesterol in the liver. In the present study it has been found that increased levels of fatty acid synthase, acetyl-Coenzyme A carboxylase, HMG-CoA reductase due to high fat diet feeding were decreased as a result of treatment with gallic acid. These results may be explained as GA can interfere with synthesis of cholesterol by blocking β-Hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase activity [38]. Ou et al. [118] revealed that further mechanistic studies showed that GA stimulated the AMP-activated protein kinase (AMPK) and eNOS activation and the FAS suppression after stimulation with oleic acid. GA effectively reduced the level of intracellular triglyceride (TG) in the NAFLD model, enhanced the level of phosphorylation of AMPK and ACC2, up-regulated the CPT1A expression, which suggested that GA enhanced the lipid metabolism effect through regulation of the AMPK/ACC2/CPT-1A signaling pathway [8].

In the sitting study, the histolopathological changes due to HFD adminstration showed liver

fatty change in diffuse manner in the parenchyma associated with congestion in the portal vein and fibrosis in the portal area. There was focal inflammatory cells infiltration in the parenchyma. The portal area showed up periductal fibrosis surrounding the hyperplastic bile ducts. These results are compatible with Lihua et al. [34]; Zhihong et al. [33] who observed that the histopathology of HFD-fed rats liver revealed severe steatosis of liver with damaged lobular structures and showed considerable accumulation of lipid droplet, acinar and portal inflammation, macrophages and lymphocytes infiltration, hepatic sinusoid absence, liver cells enlargement, blurred boundaries between cells, cell membranes damage. Steatosis of liver results from too much importation or diminished export or oxidation of free fatty acids (FFAs). Steatosis presence is stimulated by the overaccumulation of FFA/TGs and cholesterol is closely associated with chronic inflammation of liver that is partially mediated by the stimulation of the inhibitor of the NF-κB kinase subunit β/NF-κB signalling pathway [119]. In addition, feeding with HFD results in a considerably elevated level of liver triglyceride, fatty acids and cholesterol and a considerably reduced PUFA to MUFA proportion. These findings indicated that supply with HFD caused a considerable hepatic steatosis and its damage in mice [32]. Moreover, reactive oxygen species (ROS) have been found to be one of the main factors causing steatosis of liver [120]. Gallic acid potentially amended the histological deterioration produced by high fat diet since fatty change was noticed in some few hepatocytes in focal manner and the parenchyma associated with few fibrosis in the portal area and congestion in the portal vein. These amelioration in liver histological architecture is compatible with Chin-Lin and Gow-Chin, [43]; Jung et al. [32] whose results revealed that GA administration reduced the lipid droplets number, reversed the surplus cumulation of fat in liver intracellular vacuole, reduced the levels of liver cholesterol, TG and fatty acids increased the PUFA-to-MUFA ratio. Also treatment with GA preserved function of liver and decreased the level of ALT. Taken together, above results indicate that GA improves steatosis of liver in NAFLD mice stimulated with HFD [32]. In our survey, the changes of histolopathology due to HFD adminstration showed inflammatory cells infiltration between the lobules and cells with dilatation and congestion in the blood vessels in adipose tissue. These results agreed with Julio et al. [121]; Haripriya and Vijayalakshmi, [122] who revealed that in HFD mice, the morphometry of adipocytes showed hypertrophy, the fat pads were significantly large, the adipose tissue shows increased adipocytes deposition and increased peri-adrenal fat. Gallic acid potentially amended the histological deterioration in adipose tissue produced by HFD since no histopathological alteration was observed. These amelioration are confirmed by Chin-Lin and Gow-Chin, [43] who illustrated that GA decreases the adipose tissue weights of epididymal and peritoneal fat, therefore it decreases the body fat content of HFD-fed rats. GA administration may reduce accumulation of perirenal fat via repressing proliferation of adipocytes resulting in reduced perirenal adipose tissues weight and level of triglyceride in plasma in high fructose diet rats and also hypothesized that GA may improve impaired insulin signal transduction and promote utilization of glucose by ameliorating sensitivity of insulin and modulate metabolism of lipid via the increase of ATGL expression leading to decreasing hyperglycemia and cumulation of fat in perirenal adipose tissues of high fructose diet rats as the result [123].

5. CONCLUSION

Gallic acid administration exhibited a beneficial therapeutic effect on rats with nonalcoholic fatty liver disease due to its anti-inflammatory and antioxidant action. It is our view that further work into gallic acid treatment to prevent or slow progression of NAFLD is needed and important to pursue. in the future, large-scale samples and appropriate duration of gallic acid treatment for NAFLD should be performed. With this approach, it is conceivable to distinguish various NAFLD phenotypes within the definition of the general metabolic syndrome.

CONSENT

It is not applicable.

ETHICS APPROVAL

The present study was approved by the Animal Ethics Committee of Beni-Suef University.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and

producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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