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GALLIC ACID-LOADED PRONIOSOMES: FORMULATION AND EVALUATION IN HIGH-FAT DIET-INDUCED NON-ALCOHOLIC FATTY LIVER DISEASE

VB Pokharkar[1*], S Padher[2], S Bhalerao[3], and S Giramkar[4]

[1*]Poona College of Pharmacy, Bharati Vidyapeeth Deemed to be University, Pune, Maharashtra, India. varsha.pokharkar@bharatividyapeeth.edu
[2]Poona College of Pharmacy, Bharati Vidyapeeth Deemed to be University, Pune, Maharashtra, India
[3]Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth Deemed to be University, Pune, Maharashtra, India
[4]Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth Deemed to be University, Pune, Maharashtra, India

ABSTRACT:
Non-alcoholic fatty liver disease (NAFLD) is a common metabolic liver disorder associated with obesity, insulin resistance and dyslipidemia. Gallic acid (GA), a polyphenol with reported hepatoprotective, antioxidant and lipid-modulating activity, has poor aqueous solubility and low oral bioavailability. We prepared GA-loaded proniosomes using Span 60 and evaluated them in a high-fat diet (HFD)-induced NAFLD model in male Wistar albino rats (Rattus norvegicus). Rats received an HFD (36% fat) for 120 days and were allocated to four groups (n = 6): normal control, disease control (HFD), positive control (saroglitazar, 4 mg/kg/day, orally) and test formulation (GA proniosomes, 150 mg/kg/day, orally), with treatment during the final 60 days. Body weight, fasting blood glucose, serum insulin, lipid profile, liver and adipose tissue weights and liver histology were assessed. Compared with the disease control group, GA proniosomes lowered fasting blood glucose (105.6 ± 16.12 mg/dL [5.86 ± 0.89 mmol/L] vs. 149.1 ± 28.55 mg/dL [8.28 ± 1.58 mmol/L]; $P < 0.05$) and serum triglycerides (101.0 ± 20.8 mg/dL [1.14 ± 0.23 mmol/L] vs. 184.4 ± 36.1 mg/dL [2.08 ± 0.41 mmol/L]; $P < 0.05$), and increased HDL cholesterol (33.30 ± 6.52 mg/dL [0.86 ± 0.17 mmol/L] vs. 17.70 ± 4.99 mg/dL [0.46 ± 0.13 mmol/L]; $P < 0.05$). Histology showed a mild decrease in macrovesicular steatosis in treated animals. Body weight, serum insulin, total cholesterol and liver weight did not differ significantly from the disease control group. Because no free GA group was included, the contribution of the proniosomal carrier to these effects could not be determined. These findings indicate that GA proniosomes improve glycemic and lipid parameters and mildly reduce macrovesicular steatosis in HFD-fed rats, and support further mechanistic evaluation, including gene expression analysis.

KEYWORDS: Non-alcoholic fatty liver disease; Gallic acid; Proniosomes; Span 60; High-fat diet; Hepatic steatosis

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) affects approximately 25% of the adult population worldwide (Tilg and Moschen, 2010; Younossi et al., 2016). It is defined by excessive hepatic lipid accumulation in the absence of significant alcohol intake and spans a spectrum from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and hepatocellular carcinoma (Friedman et al., 2018). Its pathogenesis is closely linked to metabolic syndrome, with obesity, insulin resistance, dyslipidemia and oxidative stress as central drivers (Ipsen et al., 2018).

At the molecular level, steatosis develops when hepatic lipid acquisition, through fatty acid uptake and de novo lipogenesis, exceeds lipid disposal through fatty acid oxidation and export (Ipsen et al., 2018). These processes are controlled by transcriptional regulators such as sterol regulatory element-binding protein-1c (SREBP-1c) and the peroxisome proliferator-activated receptors (PPARs), and they are modulated by insulin signaling and AMP-activated protein kinase (AMPK) activity (Li et al., 2011; Ipsen et al., 2018).

Approved pharmacological options for NAFLD remain limited which has directed attention to phytochemicals as multi-targeted agents. Gallic acid (GA; 3,4,5-trihydroxybenzoic acid), a polyphenol widely distributed in fruits, nuts and medicinal plants, has shown antioxidant, anti-inflammatory, antidiabetic and hepatoprotective activities in preclinical studies (Locatelli et al., 2013; Nabavi et al., 2013). It has also been reported to suppress hepatic lipogenesis through SREBP-1c (Locatelli et al., 2013; Huang et al., 2016). However, its use is limited by poor aqueous solubility, susceptibility to oxidative degradation and rapid systemic elimination, which result in low oral bioavailability (Yu et al., 2018). Our earlier in silico screen of 27 phytoconstituents against PPAR-α and PPAR-γ predicted GA to be not orally bioavailable, which supports a delivery-system approach, but also gave GA weak binding scores (−5.7 and −5.9 kcal/mol), well below those of glycyrrhizic acid and silymarin (−8.3 to −8.9 kcal/mol) (Padher et al., 2025). Any effect of GA on lipid metabolism may therefore not depend mainly on direct PPAR binding.

Proniosomes are dry, free-flowing, proliposome-like systems in which a non-ionic surfactant is coated on a carrier. On hydration they convert into niosomes, which are closed bilayer vesicles able to entrap hydrophilic and lipophilic drugs, and they are more stable on storage than niosomal dispersions (Uchegbu and Florence, 1995; Rajera et al., 2011). Span 60 (sorbitan monostearate) is widely used for this purpose because of its high phase transition temperature, vesicle-forming ability and chemical stability (Moghassemi and Hadjizadeh, 2014). The solvent evaporation method, in which

surfactant and drug are dissolved in an organic solvent that is then evaporated, is a well-established route to these systems and offers ease of scale-up, reproducibility and high entrapment efficiency (Rajera et al., 2011).
We therefore prepared GA-loaded proniosomes by solvent evaporation with Span 60 and evaluated their effects on glycemic and lipid parameters, organ weights and liver histology in a high-fat diet (HFD)-induced NAFLD model in rats, using saroglitazar as a reference drug. GA proniosomes improved fasting glucose, triglycerides and HDL cholesterol and mildly reduced macrovesicular steatosis, whereas body weight, insulin, total cholesterol and liver weight were not significantly altered.

MATERIAL AND METHODS

Materials

Gallic acid (purity ≥98%) was obtained from Sigma-Aldrich. Span 60 (sorbitan monostearate), cholesterol (analytical grade) and chloroform were obtained from Loba Chemie,Mumbai. Saroglitazar magnesium (reference standard) was used as the positive control drug. High-fat diet pellets (36% fat) were obtained from VRK nutrition solutions. All other reagents and solvents were of analytical grade.

Preparation of gallic acid proniosomes

GA-loaded proniosomes were prepared by the solvent evaporation method. Span 60 and cholesterol were dissolved in chloroform in a round-bottom flask at a 1:3 ratio, and GA was dissolved in the same organic phase using maltodextrin as a carrier. The solvent was removed under reduced pressure in a rotary evaporator at 60°C until a thin, dry film formed on the wall of the flask, and the film was dried under vacuum overnight to remove residual solvent. For characterization, the dried film was hydrated with phosphate-buffered saline (pH 7.4) at 60°C and probe-sonicated to obtain uniform vesicles.

Physicochemical characterization

Mean vesicle size, polydispersity index (PDI) and zeta potential of the reconstituted niosomal dispersion were determined by dynamic light scattering and laser Doppler electrophoresis, respectively, using a Malvern Zetasizer (Malvern Panalytical, UK. Samples were diluted with double-distilled water and analyzed in triplicate at 25°C. Encapsulation efficiency (EE%) was determined indirectly by separating unentrapped GA from the dispersion by and quantifying free drug in the supernatant or dialysate by UV-visible spectrophotometry at the λmax of GA (approximately 271 nm) against a validated calibration curve. EE% was calculated as shown in Equation 1. Vesicle morphology was examined by scanning electron microscopy.All characterization studies were performed in triplicate (n = 3), and results are expressed as mean ± SD.

EE (%) = [(Total drug added − Free drug in supernatant) / Total drug added] × 100 (1)

Ethical approval and animals

All animal experiments were approved by the Institutional Animal Ethics Committee (IAEC), Bharati Vidyapeeth Deemed to be University Medical College, Pune (Ref. No. BVDUMC/3016/2022/001/005), and were conducted in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Male Wistar albino rats (Rattus norvegicus; 5 weeks old; body weight 120-150 g) were housed at 22 ± 2°C under a 12 h light/dark cycle with free access to water and food throughout the study.

Induction of NAFLD and experimental design

NAFLD was induced by feeding a commercial HFD containing 36% fat ad libitum for the entire 120-day experimental period. Animals were randomly allocated to four groups (n = 6 per group; Table 1). Treatments were given by oral gavage during the final 60 days (days 61-120), while the respective diets were continued.

Table 1. Experimental group design

Group	Designation	Animals (n)	Treatment
I	Normal control (NC)	6	Standard chow diet throughout
II	Disease control (DC)	6	HFD (36% fat) throughout; no treatment
III	Positive control (PC)	6	HFD + saroglitazar 4 mg/kg/day, orally (days 61-120)
IV	Test formulation (TF)	6	HFD + GA proniosomes 150 mg/kg/day, orally (days 61-120)

HFD, high-fat diet; GA, gallic acid.

Body weight

Body weights were recorded twice weekly from day 0 to day 120 using a calibrated electronic balance.

Blood collection and biochemical analysis

Blood was collected by retro-orbital puncture under light ether anesthesia before the start of treatment and at the end of the study (day 120). Serum was separated by centrifugation at 3000 rpm for 10 min. Fasting blood glucose, serum insulin, total cholesterol, triglycerides and HDL cholesterol were estimated. (Tietz, 1995). LDL cholesterol was calculated using the Friedewald equation.

Organ weight

At the end of the study (day 120), animals were sacrificed the liver and retroperitoneal adipose tissue were excised, blotted dry and weighed on a calibrated analytical balance. Tissues were then stored at −80°C for further analyses, including oxidative stress estimation.

Histopathology

Liver samples were fixed in 10% neutral buffered formalin, processed by routine dehydration and paraffin embedding, cut at 5 µm and stained with hematoxylin and eosin (Bancroft and Gamble, 2008). Sections were examined by light microscopy at 10× and 40× magnification by a blinded pathologist. Hepatic steatosis, hepatocellular hypertrophy and inflammatory infiltration were assessed and graded.

Statistical analysis

Data are expressed as mean ± SD. Comparisons were made by two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test .P < 0.05 was considered statistically significant. Analyses were performed with GraphPad Prism version 9.0 (GraphPad Software, USA).

RESULTS

Physicochemical characterization of GA proniosomes

On hydration, the optimized GA proniosomal formulation yielded niosomes with the vesicle size, PDI, zeta potential and EE% shown in Table 2.

Table 2. Physicochemical characteristics of the optimized GA proniosomal formulation

Parameter	Value (mean ± SD, n = 6)
Vesicle size (nm)	500 nm
Polydispersity index (PDI)	0.2
Zeta potential (mV)	-36 mV
Encapsulation efficiency (%)	76%

GA, gallic acid; PDI, polydispersity index.

Body weight

On day 120, body weight in the DC group was significantly higher than in the NC group (P < 0.05), consistent with adiposity induced by chronic HFD feeding (Figure 1). Body weights in the PC and TF groups were numerically higher than in the NC group and lower than in the DC group, but neither difference from the DC group was significant. A larger sample or a higher dose may be needed to detect an effect on body weight.

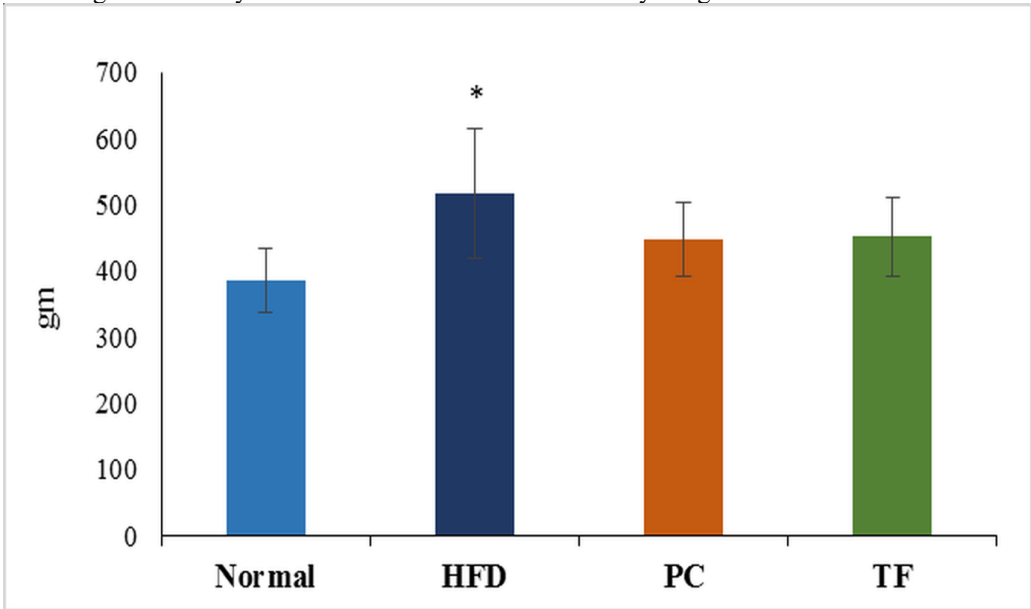

Figure 1. Body weight of the experimental animals on day 120. Data are mean ± SD (n = 6). *P < 0.05 vs. NC (two-way ANOVA with Dunnett's multiple comparison test). NC, normal control; DC, disease control; PC, positive control (saroglitazar); TF, test formulation (GA proniosomes).

Biochemical parameters

The biochemical parameters measured on day 120 are summarized in Table 3.

Table 3. Effect of GA proniosomes on biochemical parameters on day 120 (mean ± SD, n = 6)

Parameter	NC	DC (HFD)	PC (saroglitazar)	TF (GA proniosomes)

Parameter	NC	DC (HFD)	PC (saroglitazar)	TF (GA proniosomes)
Fasting blood glucose, mg/dL (mmol/L)	98.2 ± 13.25 (5.45 ± 0.74)	149.1 ± 28.55** (8.28 ± 1.58)	110 ± 25.64# (6.11 ± 1.42)	105.6 ± 16.12# (5.86 ± 0.89)
Insulin, mIU/L	43.18 ± 15.29	56.87 ± 9.84	42.63 ± 17.97	41.44 ± 8.38
Total cholesterol, mg/dL (mmol/L)	54.56 ± 4.79 (1.41 ± 0.12)	62.91 ± 6.83 (1.63 ± 0.18)	50.63 ± 4.20 (1.31 ± 0.11)	51.90 ± 15.29 (1.34 ± 0.40)
Triglycerides, mg/dL (mmol/L)	100.3 ± 55.5 (1.13 ± 0.63)	184.4 ± 36.1* (2.08 ± 0.41)	129.2 ± 21.8 (1.46 ± 0.25)	101.0 ± 20.8# (1.14 ± 0.23)
HDL cholesterol, mg/dL (mmol/L)	23.34 ± 4.60 (0.60 ± 0.12)	17.70 ± 4.99 (0.46 ± 0.13)	25.15 ± 7.00 (0.65 ± 0.18)	33.30 ± 6.52# (0.86 ± 0.17)

**P < 0.01 and *P < 0.05 vs. NC; #P < 0.05 vs. DC (two-way ANOVA with Dunnett's multiple comparison test). 1 mIU/L = 1 μIU/mL. NC, normal control; DC, disease control; HFD, high-fat diet; PC, positive control; TF, test formulation.

Fasting blood glucose

HFD feeding significantly increased fasting blood glucose in the DC group (149.1 ± 28.55 mg/dL (8.28 ± 1.58 mmol/L)) relative to the NC group (98.2 ± 13.25 mg/dL (5.45 ± 0.74 mmol/L; P < 0.01)). GA proniosomes reduced fasting blood glucose to 105.6 ± 16.12 mg/dL (5.86 ± 0.89 mmol/L; P < 0.05 vs. DC), and saroglitazar reduced it to 110 ± 25.64 mg/dL (6.11 ± 1.42 mmol/L; P < 0.05 vs. DC), a similar effect.

Serum insulin

Serum insulin was numerically higher in the DC group (56.87 ± 9.84 mIU/L) than in the NC group (43.18 ± 15.29 mIU/L), but the difference was not significant. Insulin in the TF group (41.44 ± 8.38 mIU/L) and the PC group (42.63 ± 17.97 mIU/L) was like that in the NC group, and no significant differences among groups were detected.

Total cholesterol

Total cholesterol was slightly higher in the DC group (62.91 ± 6.83 mg/dL (1.63 ± 0.18 mmol/L)) than in the NC group (54.56 ± 4.79 mg/dL (1.41 ± 0.12 mmol/L)), without statistical significance. Values in the PC group (50.63 ± 4.20 mg/dL (1.31 ± 0.11 mmol/L)) and the TF group (51.90 ± 15.29 mg/dL (1.34 ± 0.40 mmol/L)) were slightly below the NC value, and no significant differences among groups were found.

Serum triglycerides

HFD-fed animals had significantly higher serum triglycerides than NC animals (184.4 ± 36.1 mg/dL (2.08 ± 0.41 mmol/L) vs. 100.3 ± 55.5 mg/dL (1.13 ± 0.63 mmol/L; P < 0.05)). GA proniosomes lowered triglycerides to 101.0 ± 20.8 mg/dL (1.14 ± 0.23 mmol/L; P < 0.05 vs. DC), within the range of the NC group. Saroglitazar lowered triglycerides to 129.2 ± 21.8 mg/dL (1.46 ± 0.25 mmol/L), which was not significant versus the DC group.

HDL cholesterol

HDL cholesterol was numerically lower in the DC group (17.70 ± 4.99 mg/dL (0.46 ± 0.13 mmol/L)) than in the NC group (23.34 ± 4.60 mg/dL (0.60 ± 0.12 mmol/L)). GA proniosomes raised HDL cholesterol to 33.30 ± 6.52 mg/dL (0.86 ± 0.17 mmol/L; P < 0.05 vs. DC), above the NC level. Saroglitazar gave 25.15 ± 7.00 mg/dL (0.65 ± 0.18 mmol/L), which was not significant versus the DC group.

Organ weights

Liver and retroperitoneal adipose tissue weights are shown in Table 4. HFD feeding significantly increased liver weight (18.78 ± 6.58 g) and adipose tissue weight (16.67 ± 8.51 g) relative to the NC group (11.9 ± 1.8 g and 7.2 ± 3.8 g, respectively; P < 0.05 for both). Neither saroglitazar nor GA proniosomes significantly changed these weights relative to the DC group, and adipose tissue weight in the TF group (15.56 ± 4.50 g) remained significantly higher than in the NC group (P < 0.05).

Table 4. Liver and retroperitoneal adipose tissue weights on day 120 (mean ± SD, n = 6)

Group	Liver weight (g)	Adipose tissue weight (g)
Normal control (NC)	11.9 ± 1.8	7.2 ± 3.8
Disease control (DC)	18.78 ± 6.58*	16.67 ± 8.51*
Positive control (PC)	17.46 ± 3.46	15.75 ± 5.68
Test formulation (TF)	18.17 ± 4.08	15.56 ± 4.50*

*P < 0.05 vs. NC (two-way ANOVA with Dunnett's multiple comparison test).

Histopathological findings

On gross examination at sacrifice, NC livers were dark red with smooth, sharp edges, DC livers were pale and enlarged with irregular surfaces, and PC and TF treatment alleviated these gross changes. H&E-stained sections from NC animals showed normal lobular architecture, with hepatocytes arranged in cords radiating from central veins and no lipid vacuolation (Figure 2). DC animals showed extensive macrovesicular and microvesicular steatosis, hepatocellular

hypertrophy and focal inflammatory infiltration, features consistent with steatohepatitis. Both PC and TF animals showed a mild decrease in macrovesicular steatosis relative to the DC group.

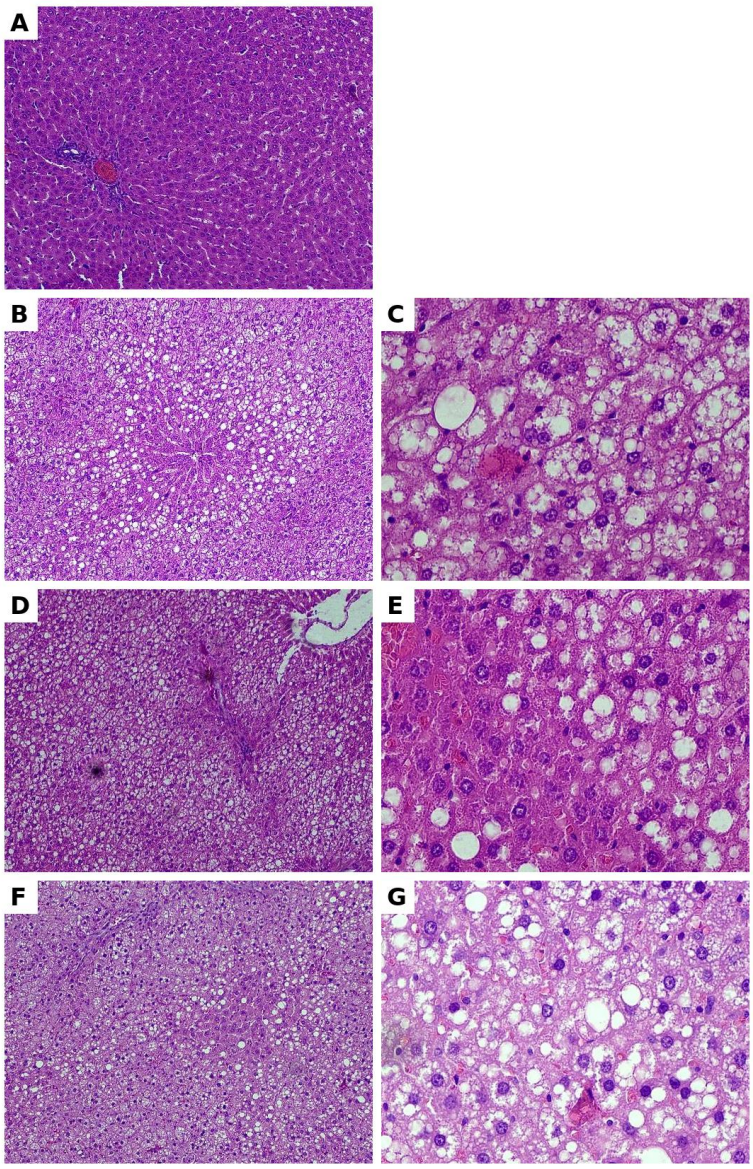

Figure 2. Representative photomicrographs of H&E-stained liver sections. (A) NC, 10×: normal lobular architecture; (B, C) DC, 10× and 40×: macrovesicular and microvesicular steatosis with hepatocellular hypertrophy and inflammation; (D, E) PC (saroglitazar), 10× and 40×, and (F, G) TF (GA proniosomes), 10× and 40×: mild decrease in macrovesicular steatosis.

DISCUSSION

GA proniosomes prepared with Span 60 improved glycemic and lipid parameters and mildly reduced macrovesicular steatosis in rats fed an HFD for 120 days. In the DC group, the diet produced fasting hyperglycemia, hypertriglyceridemia, lower HDL cholesterol, higher liver and retroperitoneal adipose tissue weights, and hepatic steatosis with hepatocellular hypertrophy and focal inflammation, which is in line with the use of HFD feeding as a model of human metabolic disease (Buettner et al., 2007). Total cholesterol and serum insulin were not significantly altered by the diet, so the model reproduced the glycemic, triglyceride and hepatic changes more clearly than changes in cholesterol or insulin.

The reduction in fasting glucose with GA proniosomes was similar to that with saroglitazar. AMPK activation limits SREBP activity and hepatic steatosis in insulin-resistant mice (Li et al., 2011), and this is one pathway through which a polyphenol such as GA might act, although signaling was not examined here. Span 60 niosomes can enhance intestinal permeation and protect entrapped compounds from enzymatic degradation (Uchegbu and Florence, 1995). However, because the study had no free GA group, the effects observed cannot be attributed to improved bioavailability.

Triglycerides in the TF group returned to the NC range, whereas saroglitazar lowered them to a smaller, non-significant extent. Triglycerides were numerically lower with GA proniosomes than with saroglitazar, but superiority cannot be claimed without a direct statistical comparison .GA has been reported to reduce hepatic lipogenesis by downregulating SREBP-1c and its target enzymes and to promote fatty acid β-oxidation (Locatelli et al., 2013; Huang et al., 2016). This would fit the lower triglycerides and mild reduction in steatosis seen here, but hepatic gene expression was not measured, so it remains a hypothesis. Because GA showed weak predicted binding to PPAR-α and PPAR-γ in our earlier docking study (Padher et al., 2025), the lipid effects seen here may act through other routes, such as AMPK and

SREBP-1c signaling, or through changes in receptor expression rather than direct ligand binding; measuring expression of Ppara, Pparg and Srebf1 would test this. The higher HDL cholesterol in the TF group, above the NC level, suggests a favorable shift in the lipid profile, and its mechanism was not examined.

Histology showed a mild decrease in macrovesicular steatosis in TF animals, in line with the biochemical findings, although visible steatosis remained (Figure 2). Liver weight was not significantly reduced, and retroperitoneal adipose tissue weight remained above that of NC animals, so the mild histological change was not accompanied by a measurable change in these gross weights. Lower circulating triglycerides would reduce the substrate available for hepatic uptake and esterification, and GA may also act through antioxidant defenses, including superoxide dismutase, catalase and glutathione peroxidase (Nabavi et al., 2013); these markers were not quantified in this study.

Proniosomes are dry, stable systems that avoid the aggregation and hydrolysis associated with liquid niosome preparations and form niosomes on hydration (Rajera et al., 2011; Moghassemi and Hadjizadeh, 2014). The high phase transition temperature of Span 60 gives a rigid bilayer that can limit drug leakage and improve entrapment.

This study has limitations. There was no free GA or blank proniosome group, so the benefit of encapsulation over free GA cannot be established, and no pharmacokinetic or tissue distribution data were collected. Liver enzymes and hepatic triglyceride content were not measured, and histological grading was semi-quantitative.Only one dose was tested, in male rats with six animals per group. Oxidative stress markers (malondialdehyde, reduced glutathione and superoxide dismutase) were collected but remain to be estimated in stored tissue, and expression of genes regulating lipid metabolism and inflammation (Pparg, Ppara, Srebf1, Tnf and Il6) is planned. Dose-response studies, inclusion of female animals and a free GA comparator are needed before clinical evaluation.

CONCLUSION

GA-loaded proniosomes prepared by solvent evaporation with Span 60 lowered fasting glucose and triglycerides, raised HDL cholesterol and mildly reduced macrovesicular steatosis in HFD-fed rats, with an effect on glucose like that of saroglitazar. Body weight, insulin, total cholesterol and liver weight were not significantly changed. These results support GA proniosomes as a candidate phyto-nanoformulation for NAFLD and justify comparison with free GA, together with pharmacokinetic and gene expression studies to define the mechanism.

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