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Psidium guajava Leaf Extract Attenuates High-Fat Diet–Induced Nonalcoholic Fatty Liver Disease in Wistar Rats

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Abstract

Background and Aim: Nonalcoholic fatty liver disease represents the most prevalent chronic liver condition globally, despite the scarcity of approved drug treatments. Leaves of *Psidium guajava* L. (guava) are abundant in polyphenolic compounds known for their hepatoprotective, lipid-lowering, and insulin-sensitizing properties. This study aimed to assess the therapeutic potential of a standardized ethanolic *P. guajava* leaf extract against NAFLD induced by a high-fat diet combined with fructose in Wistar rats.

Materials and methods: Adult Wistar rats were fed a high-fat diet (60%) supplemented with 10% fructose in drinking water, or normal chow, for 16 weeks. From Week 9 to Week 16, high-fat-fed rats received daily oral doses of *P. guajava* leaf extract (PGE250; 250 mg/kg) or pioglitazone (PIO20; 20 mg/kg) as the reference insulin sensitizer. The extract was standardized by HPLC for ellagic acid, quercetin, and rutin. At Week 16, body weight, food intake, fasting glucose, insulin and HOMA-IR, plasma lipid profile, liver function markers, hepatic oxidative stress and antioxidant status, pro-inflammatory cytokines, and liver histopathology were assessed.

Results: High-fat and fructose feeding significantly increased body weight, fasting glucose, insulin, HOMA-IR, triglycerides, total cholesterol, LDL-C, ALT, AST, ALP, TNF-α, IL-1β, IL-6, MDA, absolute liver weight, and hepatosomatic index, while significantly reducing HDL-C, total protein, catalase, SOD, and GSH. Treatment with PGE250 significantly reversed all of these alterations ($p < 0.001$ to $p < 0.0001$), restored normal hepatic architecture — no abnormality detected on histopathology, versus vacuolar degeneration with inflammatory cell infiltration (grade < 2) in the disease control — and produced effects comparable to those of pioglitazone.

Conclusion: *P. guajava* leaf extract ameliorates high-fat diet and fructose-induced NAFLD in Wistar rats through coordinated insulin-sensitizing, hypolipidemic, antioxidant, and anti-inflammatory mechanisms, supporting its potential as a safe, plant-based therapeutic candidate for NAFLD.

Keywords: Nonalcoholic fatty liver disease; *Psidium guajava*; insulin resistance; hepatic triglyceride; high fat diet; fructose; cytokines; steatosis.

Introduction

NAFLD is diagnosed when hepatic fat accumulates in the absence of alternative etiologies such as significant alcohol consumption, viral hepatitis, or drug-induced injury [1]. Its clinical spectrum extends from uncomplicated steatosis through steatohepatitis to fibrosis, cirrhosis, and hepatocellular carcinoma [2]. Pooled data from more than eight million individuals place the global prevalence near 24–25%, establishing NAFLD as the leading chronic hepatic condition of the present era [3]. Geographically, the Middle East (~32%) and South America (~31%) carry the heaviest burden, followed by Asia, the USA, and Europe (~24–27%), with Africa at the lower end (~14%) [3]. Notably, biopsy-based series indicate that roughly 60% of affected patients harbor the inflammatory NASH phenotype, emphasizing the clinical weight of the progressive form of the disease [4]. NAFLD is now broadly regarded as the hepatic expression of the metabolic syndrome, sharing an almost identical risk factor profile — obesity, type 2 diabetes mellitus, and dyslipidemia [5]. Approximately 43% of NAFLD patients meet metabolic syndrome criteria [6]. Its pathogenesis is multifactorial, arising from the

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convergence of insulin resistance, lipotoxic injury, oxidative stress, and persistent low-grade inflammation [7]. As obesity and diabetes continue to expand worldwide, NAFLD incidence will rise accordingly, and the resulting cirrhosis and terminal liver disease will place mounting strain on healthcare systems and economies [8].

The Western dietary pattern, overloaded with fat and fructose, is among the most powerful drivers of the NAFLD epidemic [9]. Fructose is metabolized almost exclusively within the liver, where it bypasses the normal glycolytic checkpoints and funnels directly into fatty acid synthesis through induction of the lipogenic transcription factors SREBP-1c and ChREBP [9]. Sustained fructose exposure promotes insulin resistance, dyslipidemia, and fatty liver, and can provoke hepatic necroinflammation even in the absence of obesity [10]. Experimental comparisons in rats indicate that dietary fat stimulates NAFLD more potently than fructose alone [11], and combining the two insults — a high-fat diet plus 10% fructose — produced severe steatosis reaching 78.33%, accompanied by steatohepatitis, dyslipidemia, and elevations in alkaline phosphatase and uric acid, in non-obese Wistar rats [12]. This combined regimen also generates higher steatosis grades and more pronounced ballooning degeneration than cholesterol- or sucrose-based diets [13], and extended feeding drives the lesion toward fibrosis and macrophage infiltration [14]. The HFHF model therefore recapitulates the metabolic and histological hallmarks of human NAFLD, making it well suited for testing candidate therapies [15].

Against this backdrop, treatment options remain strikingly limited: no pharmacological agent has been licensed specifically for NAFLD or NASH [16,17]. Lifestyle measures, chiefly weight reduction and physical activity, remain the standard of care and can confer histological benefit, but long-term adherence is difficult to sustain [18]. Only a minority of patients achieve the degree of weight loss required to reverse steatohepatitis, and lifestyle modification alone is unlikely to halt advanced disease [18]. Although pioglitazone and vitamin E have demonstrated histological efficacy in randomized trials, both remain off-label, with pioglitazone's utility tempered by weight gain and unresolved safety concerns [19,20]. The need for safe, effective, and affordable therapies is therefore urgent.

Psidium guajava L. (guava), a widely cultivated tropical fruit tree, merits particular attention in this regard. Its leaves feature prominently in folk medicine for antioxidant, hepatoprotective, antiallergic, antimicrobial, antidiabetic, and anti-inflammatory indications, and guava-leaf tea remains a traditional remedy for gastroenteritis, dysentery, and childhood diarrhea [21,22]. The leaves are densely populated with polyphenols, including gallic acid, catechin, epicatechin, quercetin, and rutin; HPLC analysis of the ethanolic extract used in the present study identified ellagic acid (51.89 ppm), quercetin (31.33 ppm), and rutin (26.24 ppm) as the principal marker compounds. Preclinical work has repeatedly confirmed the hepatoprotective potential of guava leaf extracts, which lower serum transaminases and alkaline phosphatase, counter carbon tetrachloride-induced oxidative damage, and restore hepatic architecture in toxin-based models [23,24]. A triterpenoid-standardized guava leaf preparation similarly improved transaminase levels after acetaminophen challenge [25], and dietary guava leaf powder mitigated steatosis and dyslipidemia induced by a high-carbohydrate, high-fat regimen [21]. Most compellingly, a hydroethanolic guava leaf extract suppressed fructose-evoked NAFLD in growing rats by restoring insulin signaling (Akt/FOXO1/SREBP-1c), quenching hypoxia–inflammation cascades (HIF-1α/TNF-α), preserving mitochondrial function, and curtailing de novo lipogenesis [26]. Hypolipidemic effects of guava leaf extracts against diet- and toxin-induced hyperlipidemia have likewise been documented [27].

Taken together, these observations indicate that guava leaves carry pleiotropic bioactivities well matched to the multi-pronged pathophysiology of NAFLD [26]. Even so, the efficacy of a chemically standardized ethanolic guava leaf extract against NAFLD evoked by combined high-fat, high-fructose feeding has not been formally evaluated in adult rats. Accordingly, the present study assessed whether daily oral administration of *P. guajava* leaf extract (PGE250; 250 mg/kg) for eight weeks (Weeks 9–16) improves High fat diet (60%) and high

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fructose (10%) -induced NAFLD in Wistar rats, with pioglitazone (20 mg/kg) employed as the active comparator. Outcome measures comprised body weight and food intake, glycemic and insulinemic parameters, plasma lipid profile, liver function markers, hepatic oxidant–antioxidant status, pro-inflammatory cytokines, and liver histopathology.

Materials and Methods

Chemicals and Reagents

ELISA kits for measuring rat TNF-α, IL-1β, IL-6, and insulin (GENLISA™ brand) were obtained from Krishgen Biosystems Private Limited. Kits for triglycerides, direct LDL cholesterol, ALT, AST, ALP, and glucose were sourced from Transasia Bio-Medicals Ltd. (Erba Mannheim). Total cholesterol and total protein kits came from ARKRAY Healthcare Pvt. Ltd., while the direct HDL-C kit was supplied by Pathozyme Diagnostics. The high-fat diet (VRKHFD 60) and standard chow pellets were obtained from VRK Nutritional Solutions (Pune, Maharashtra). Pioglitazone hydrochloride was sourced from Dr. Reddy's Laboratories, and the reference compounds aegeline, quercetin, and umbelliferone were purchased from Yucca Enterprises, Mumbai.

Plant Material and Extraction

Healthy, leaves of Psidium guajava were collected from local areas of Nashik, Maharashtra. The plant material was authenticated by the Western Regional Centre, Botanical Survey of India (BSI), Pune, and a voucher specimen (BSI/WRC/Tech./2025/ PSB-110/ Specimen No. 03) was deposited in the herbarium of the Western Regional Centre, Botanical Survey of India, Pune.

The harvested leaves were rinsed thoroughly under running tap water and then washed again with distilled water to eliminate dirt, soil, and surface debris. They were then dried in the shade. Once dry, the leaves were ground into a coarse, even powder using an electric mixer grinder. This process yielded roughly 500 g of dried leaf powder, which was transferred to airtight glass containers and kept at room temperature, away from sunlight, until extraction began. For the sequential Soxhlet extraction, about 300 g of the dried, powdered *Psidium guajava* leaf material was precisely weighed and packed evenly into a cellulose thimble, which was then loaded into the Soxhlet apparatus. A 500 mL round-bottom flask containing 350–400 mL of the appropriate solvent was attached. Extraction proceeded sequentially with solvents of increasing polarity — petroleum ether, ethyl acetate, and ethanol. The powdered material was first extracted with petroleum ether over 18–24 hours, until the solvent visible in the siphon tube ran clear. The remaining plant residue (marc) was then separated and air-dried at room temperature to eliminate any leftover non-polar solvent before moving to the next solvent stage.

This dried marc was next extracted using 350–400 mL of ethyl acetate over another 18–24-hour Soxhlet cycle. Once finished, the residue was air-dried again and then extracted with 350–400 mL of ethanol for a further 18–24 hours. Using petroleum ether, ethyl acetate, and ethanol in sequence allowed for the stepwise recovery of non-polar constituents (lipids/fatty acids), moderately polar compounds, and polar phytoconstituents (alkaloids, flavonoids, tannins, and carbohydrates/pectins), respectively [28]. Each solvent extract produced through Soxhlet extraction was passed through Whatman No. 1 filter paper to remove any remaining particulates. The filtrates were then concentrated under reduced pressure via rotary evaporation, maintaining a temperature range of 40–45°C to avoid degrading heat-sensitive compounds [29]. Further drying of the concentrated extracts produced the final crude extracts. Each dried extract was accurately weighed, and the percentage yield was calculated. Sequential Soxhlet extraction of 300 g of dried, powdered *Psidium guajava* leaves produced petroleum ether, ethyl acetate, and ethanolic extracts weighing 12 g, 13.5 g, and 30.3 g, corresponding to percentage yields of 4%, 4.5%, and 10.1%, respectively. All crude extracts were placed into separate, clean, airtight glass containers, properly labeled, and stored at 2–8°C, shielded from light, moisture, and contamination, pending further phytochemical screening, HPLC analysis and pharmacological testing.

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HPLC Standardization

Standardization of the ethanolic leaf extract of *Psidium guajava* was carried out by HPLC, using rutin, ellagic acid, and quercetin as marker compounds — three constituents chosen for their known biological relevance to the extract's expected hepatometabolic activity [30]. The analysis employed an HPLC 3000 Series system fitted with a binary-gradient pump (P-3000M), a UV-3000M detector, a manual injector, and HPLC Workstation software. Chromatographic separation was performed on a Cosmosil C18 column (250 × 4.6 mm, 5 µm), with methanol: water (70:30, v/v) serving as the mobile phase at a flow rate of 0.8 mL/min and an injection volume of 20 µL.

Detection wavelengths were set at 257 nm for rutin, 254 nm for ellagic acid, and 256 nm for quercetin, and these were compared against the *P. guajava* extract profile recorded at 258 nm. Stock solutions of each standard (1000 ppm) were prepared by dissolving 10 mg in 10 mL of mobile phase, and working standards at 50 ppm were obtained by diluting 0.5 mL of stock solution to a final volume of 10 mL. The extract sample was similarly dissolved in methanol: water (70:30), sonicated, diluted to the desired concentration, and filtered prior to injection. Quantification was carried out by comparing the peak areas of the sample against those of the standards.

Animal Models and Experimental Design

Wistar rats of either sex, weighing between 150–180 g, were selected for the in vivo portion of this study. This strain is one of the most commonly used outbred rat models in nutrition and liver-related research [31], given that strain background, dietary composition, and feeding duration are all known to influence the development of NAFLD in this species. Twenty-four animals in total were included and allowed a one-week acclimatization period before the study began. Animals were maintained under standard laboratory conditions (25 ± 0.5 °C, 12:12 h light–dark cycle) with unrestricted access to food and water unless otherwise noted.

All procedures adhered to the guidelines set by the Committee for the Control and Supervision of Experiments on Animals, Government of India, and received approval from the Institutional Animal Ethics Committee (approval no. IAEC/CPD/415/11). The research was carried out at the Animal House of Smt. Sharadchandrika Suresh Patil College of Pharmacy, Chopda, Jalgaon, Maharashtra, India.

After acclimatization, animals were assigned to four groups (n = 6 per group). The normal control group was maintained on standard laboratory chow (3.365 kcal/g; VRK Nutritional Solutions, Pune, India), while the remaining three groups were fed a high-fat diet (VRKHFD 60; VRK Nutritional Solutions, Pune, India) supplying 60% of energy from fat, 25.6% from carbohydrate, and 14.4% from protein, with an estimated caloric density of 5.00 kcal/g. Diets of this type are well documented to promote obesity, dyslipidemia, insulin resistance, and hepatic steatosis in rodents when administered over prolonged periods [32]. All diets included suitable vitamin and mineral supplementation. Additionally, animals on the HFD received 10% w/v fructose in their drinking water continuously from Day 1 through the end of week 16; combining fructose with a high-saturated-fat diet is known to accelerate hepatic de novo lipogenesis and worsen insulin resistance, producing a rodent hepatic phenotype that closely mirrors human NAFLD [14].

Following 8 weeks of dietary induction, treatment administration began and continued for an additional 8 weeks (weeks 9–16). Group assignments and the treatment schedule are outlined in Table 1. Immediately before dosing, the ethanolic leaf extract of *Psidium guajava* and pioglitazone hydrochloride were each suspended separately in 0.5% CMC prepared in distilled water. These treatments were administered once daily via oral gavage across weeks 9–16. The normal control group received the vehicle alone (0.5% CMC, 10 mL/kg body weight, p.o.). Body weight, food intake, and water consumption were tracked weekly throughout the study to evaluate the metabolic effects of the intervention.

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Table 1. Experimental Design and Treatment Schedule

Group	Experimental groups (n=6)	Dietary Regimen and Treatment	Dose and Route	Treatment Period
I	Normal Control (ND)	Normal chow diet + 0.5% CMC	10 mL/kg, p.o.	Weeks 1–16
II	Disease Control (HFD)	HFD + 10% w/v fructose in drinking water	—	Weeks 1–16
III	*Psidum guajava* Extract (AME250)	HFD + 10% w/v fructose in drinking water + ethanolic extract of *Psidum guajava* leaves	250 mg/kg, p.o.	Weeks 9–16
IV	Positive Control (PIO20)	HFD + 10% w/v fructose in drinking water + pioglitazone HCl	20 mg/kg, p.o.	Weeks 9–16

The disease-control group remained on the HFD with 10% w/v fructose in drinking water for the full 16-week study period and did not receive any test treatment; the fructose solution was delivered exclusively via drinking-water bottles rather than by gavage. Food intake, body weight, and fasting blood glucose were measured at fixed intervals — at the end of weeks 1, 8, and 16.

Blood Collection, Plasma Separation, and Biochemical Analysis

At the conclusion of the 16-week study, Wistar rats were fasted for 8–12 hours and then anesthetized using isoflurane. Blood samples were drawn from the dorsal aorta into EDTA-containing Vacutainer tubes. These were centrifuged at 3,000 × g for 15 minutes at 25 °C, and the resulting plasma was stored at 2–8 °C pending analysis. Plasma samples were used to determine fasting glucose and insulin levels for HOMA-IR calculation, as well as liver function markers (total protein, ALT, AST, and ALP) and lipid profile parameters (triglycerides, total cholesterol, HDL, and LDL). All measurements were carried out using commercial diagnostic kits on an ERBA XL-180 automated clinical chemistry analyzer.

Absolute Liver Weight and Hepatosomatic Index

At the end of the study period, animals were euthanized following the approved ethics protocol. Livers were excised, rinsed with ice-cold PBS (pH 7.4), blotted, and weighed without delay. The hepatosomatic index (relative liver weight) was determined as a percentage of final body weight, using the formula: $hepatosomatic\ index\ (\%) = (absolute\ liver\ weight\ /\ final\ body\ weight) \times 100$. This measure is a commonly used marker of diet-induced hepatic lipid buildup [33], since enlarged liver size and increased mass are typical outcomes of high-fat feeding [21] and are associated with hepatic triglyceride accumulation [34].

Tissue Homogenate Preparation

Following euthanasia, liver tissues were collected, rinsed in ice-cold PBS (pH 7.4), and homogenized in chilled PBS containing protease inhibitors to yield a 10% (w/v) homogenate. Homogenates were then centrifuged at 10,000 × g for 15 minutes at 4 °C, and the supernatants were collected, divided into aliquots, and stored at −80 °C until further use. Prior to testing, samples were diluted as needed and brought to room temperature; all standards and samples were run in triplicate.

Supernatants were evaluated for antioxidant markers (total protein, CAT, SOD, GSH, and lipid peroxidation) as well as inflammatory cytokines (IL-1β, IL-6, and TNF-α). All measurements were carried out using commercial diagnostic kits on an ERBA XL-180 automated clinical chemistry analyzer. Additionally, specific segments of the hepatic tissue were fixed in 10% neutral buffered formalin for 24 hours and subsequently processed for paraffin embedding to facilitate histopathological assessment of lipid infiltration and inflammatory cellular architecture [35].

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Antioxidant Activity

Catalase Estimation

Catalase activity was measured spectrophotometrically following the method of Aebi (1984), which tracks the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen. Absorbance at 240 nm was recorded every minute over a 3-minute period. The reaction mixture consisted of 3.0 mL of 30 mM H_2O_2, 0.5 mL of 50 mM phosphate buffer (pH 7.0), and 0.5 mL of the 10% tissue homogenate supernatant. Enzyme activity was derived from the rate of absorbance decline, applying the molar extinction coefficient of H_2O_2 ($\varepsilon = 43.6\ M^{-1}\ cm^{-1}$), and reported as µmol H_2O_2 decomposed per minute per mg protein [36].

Superoxide Dismutase (SOD) Estimation

SOD activity was assessed using the Kono method (1978), which relies on the enzyme's ability to inhibit nitroblue tetrazolium (NBT) reduction by superoxide radicals generated through hydroxylamine autoxidation. The assay mixture included sodium carbonate buffer (50 mM, pH 10.2), EDTA (0.1 mM), NBT (24 µM), hydroxylamine hydrochloride (1 mM), Triton X-100 (0.03%), and tissue homogenate. Following a 20-minute incubation at 37°C, absorbance was read at 560 nm against a reagent blank. SOD activity was calculated based on the percentage inhibition of NBT reduction compared to control, with one unit defined as the enzyme quantity needed to achieve 50% inhibition under the given assay conditions. Results were expressed as U/mL of homogenate, and, where relevant, as U/mg protein [37].

Reduced Glutathione (GSH) Estimation

GSH levels were quantified using the method of Moron et al. (1979), based on the reaction between GSH sulfhydryl groups and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), which produces a yellow product measurable at 412 nm. In brief, 0.5 mL of the 10% tissue homogenate supernatant was deproteinized using 0.5 mL of 4% sulfosalicylic acid, followed by the addition of 4.0 mL of 0.01 M DTNB in PBS (pH 7.4). Absorbance was then read at 412 nm against a reagent blank using a UV–Visible spectrophotometer. GSH concentration was derived from the absorbance readings and expressed as µmol per mL of tissue homogenate. Where applicable, values were normalized against protein concentration (determined via a BSA standard curve) and expressed as µmol per mg protein [38].

Lipid Peroxidation (LPO) Estimation

Lipid peroxidation was assessed using the thiobarbituric acid reactive substances (TBARS) assay, as outlined by Ohkawa et al. (1979). This method is based on the reaction between malondialdehyde (MDA) — a byproduct of lipid peroxidation — and thiobarbituric acid (TBA), which generates a pink MDA–TBA complex measurable at 532 nm. In brief, 0.1 mL of 10% tissue homogenate was combined with 0.2 mL of 8% sodium lauryl sulfate, 1.5 mL of 20% acetic acid (pH 3.5), and 1.5 mL of 0.8% TBA. This mixture was heated at 95°C for 60 minutes, allowed to cool, extracted using n-butanol:pyridine (15:1), and centrifuged at 2200 × g for 5 minutes. Absorbance of the resulting organic layer was measured at 532 nm against a reagent blank. MDA concentration was calculated using the molar extinction coefficient of the MDA–TBA complex ($1.56 \times 10^5\ M^{-1}\ cm^{-1}$) and reported as nmol MDA per mL of tissue homogenate. LPO values were also normalized to protein content and expressed as nmol MDA per mg protein [39].

Histopathological Study

After euthanasia, whole livers were removed, rinsed in normal saline, blotted dry with filter paper, weighed, and examined grossly. Representative samples from the right hepatic lobes were fixed in 10% formalin for 24 hours, dehydrated through a graded ethanol series, cleared in xylene, embedded in paraffin, sectioned at 3–5 µm thickness, and stained with hematoxylin and eosin (H&E). Histological features across treatment groups were

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then compared to those of the control group. Microscopic evaluation of steatosis and hepatocellular hypertrophy was performed at 40× magnification. Lesions were graded according to the extent of tissue involvement, using the following scale: 0 = no abnormality/within normal limits, 1 = minimal (0–10%), 2 = mild (11–20%), 3 = moderate (21–40%), and 4 = severe (41–100%). Additional characterization included organ-specific location, pattern of distribution (focal, multifocal, or diffuse), lesion type, and chronicity [40].

Statistical Analysis

Results are presented as mean ± standard error of the mean. Group differences were assessed using one-way analysis of variance, followed by Dunnett's post-hoc test for multiple comparisons. A p-value below 0.05 was considered statistically significant. All statistical analyses and graph generation were performed using GraphPad Prism (version 11.1.0).

Results

HPLC analysis

The quantitative analysis of the ethanolic leaf extract of *Psidium guajava* was successfully performed using an optimized reversed-phase HPLC-UV method. Three key bio-active polyphenolic marker compounds—quercetin, ellagic acid, and rutin—were separated and quantified using an isocratic elution of methanol: water (70:30, v/v). Chromatographic peaks were evaluated at their respective maximum absorbance wavelengths for standard compounds (rutin at 257 nm, ellagic acid at 254 nm, and quercetin at 256 nm) and compared against the extract profile detected at 258 nm. Standard stock solutions and working solutions (50 ppm) yielded clear chromatographic separation, enabling quantification via comparative peak area analysis. (Figure 1 to 4) The concentration of each phytoconstituent in the prepared extract sample was determined as follows:

Table 2: Quantitative Analysis of Selected Marker Compounds in the *P. guajava* extract

***P. guajava* Ethanolic Extract**			
Standard	**Standard Area**	**Sample Area**	**Concentration (ppm)**
Quercetin	1134089	710566	31.32761185
Ellagic Acid	168144	174506	51.89183081
Rutin	806836	423369	26.2363727

Ellagic acid emerged as the predominant marker compound among the three quantified constituents, followed by quercetin and rutin. The simultaneous identification and quantification of these markers establish a reliable analytical profile for quality control and standardization of *P. guajava* leaf extracts, supporting its prospective evaluation in hepatometabolic applications.

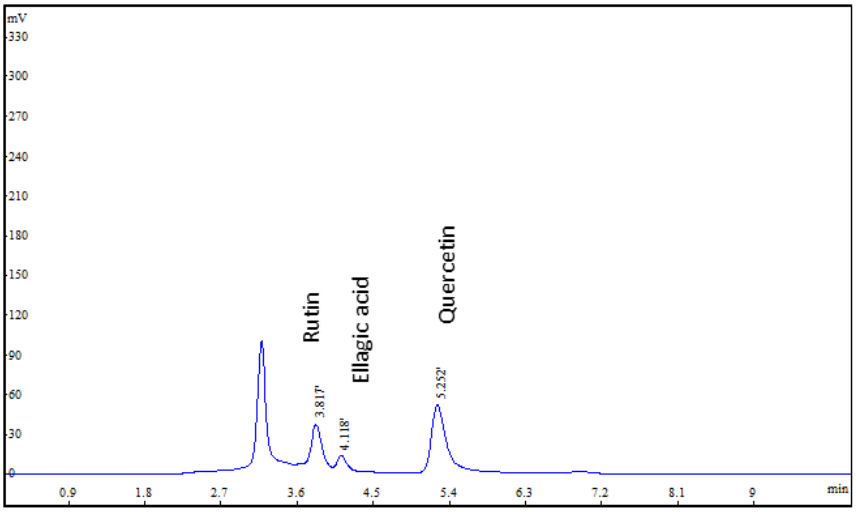

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Figure 1. Reversed-phase HPLC chromatographic profiling of *P. guajava* ethanolic extract

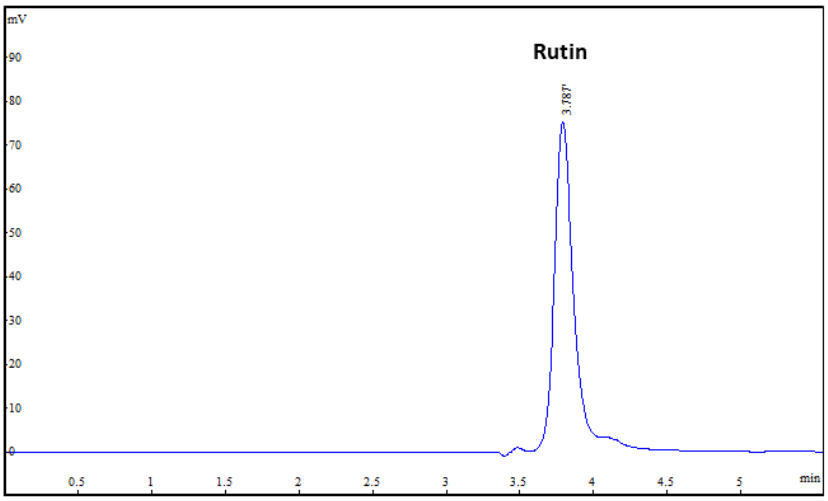

Figure 2. Reversed-phase HPLC chromatogram of the rutin standard

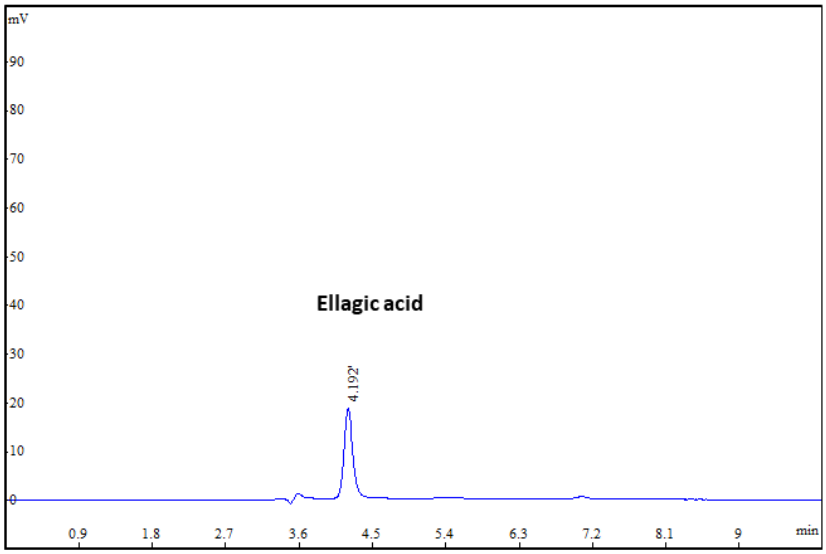

Figure 3. Reversed-phase HPLC chromatogram of the ellagic acid standard

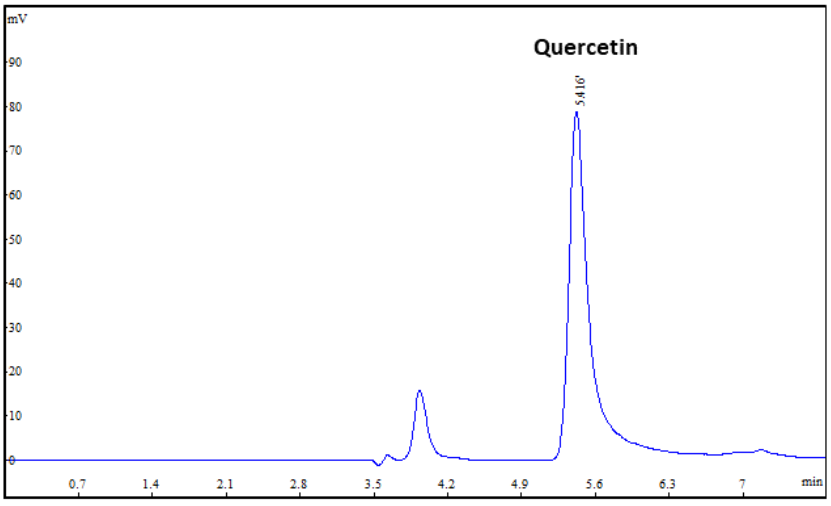

Figure 4. Reversed-phase HPLC chromatogram of the quercetin standard

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Effect of *P. guajava* extract on Food Intake on Wistar rats fed on High Fat Diet

Food intake was significantly higher in the ND group than in the HFD group at Weeks 1, 8, and 16 ($p < 0.0001$). At Week 16, treatment with *P. guajava* extract (PGE250; 250 mg/kg) or pioglitazone (PIO20; 20 mg/kg) did not significantly alter food intake compared with the HFD group (ns, $p > 0.05$). Thus, PGE250 had no significant effect on food consumption in HFD/fructose-induced NAFLD rats. (Figure 5)

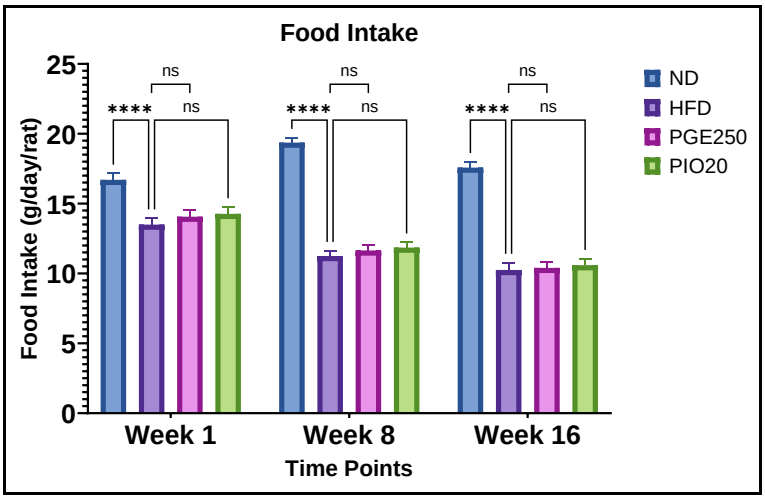

Figure 5. Effect of *P. guajava* extract on food intake in High Fat Diet induced NAFLD in Wistar rats at different time points. Food intake (g/day/rat) was recorded across experimental groups at Weeks 1, 8, and 16. Groups: normal chow diet in normal control (ND), high-fat diet + 10% fructose in disease control (HFD), *Psidium guajava* extract-treated (PGE250, 250 mg/kg p.o.), and pioglitazone-treated (PIO20, 20 mg/kg p.o.) Wistar rats. Treatments were administered daily from Week 9 to Week 16. Data are presented as Mean ± SEM. Statistical analysis was performed using ordinary two-way ANOVA followed by Dunnett's multiple-comparisons test. **** $p < 0.0001$, and ns = non-significant ($p > 0.05$).

Effect of *Psidium guajava* extract on Body Weight on Wistar rats fed on High Fat Diet

Body weight was comparable among all groups at Week 1 (ns, $p > 0.05$). By Week 8, HFD rats showed a significant increase in body weight compared with ND rats ($p < 0.0001$). Treatment with PGE250 and PIO20 significantly reduced body weight compared with the HFD group ($p < 0.001$ and $p < 0.0001$, respectively). At Week 16, HFD rats exhibited a further significant increase in body weight compared with ND rats ($p < 0.0001$), while both PGE250 and PIO20 significantly attenuated this increase ($p < 0.0001$). Thus, *P. guajava* extract significantly reduced HFD-associated body-weight gain, with an effect comparable to the reference treatment. (Figure 6)

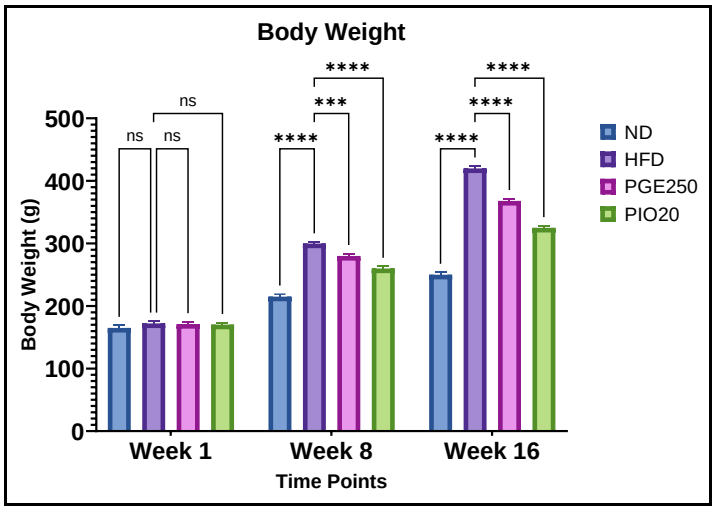

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Figure 6. Effect of *P. guajava* extract on body weight in High Fat Diet induced NAFLD in Wistar rats at different time points. Progression of body weight (g) across experimental groups at Weeks 1, 8, and 16. Groups: Normal chow diet in normal control (ND), High-Fat Diet + 10% fructose in disease control (HFD), *Psidium guajava* extract (PGE250, 250 mg/kg p.o.), and Pioglitazone (PIO20, 20 mg/kg p.o.). Treatments were administered daily from Week 9 through Week 16. Values represent Mean ± SEM. Statistical analysis was performed using ordinary two-way ANOVA with Dunnett's post-hoc multiple-comparisons test. *** $p < 0.001$, **** $p < 0.0001$, ns = non-significant ($p > 0.05$).

Effect of *P. guajava* extract on Fasting Blood Glucose on Wistar rats fed on High Fat Diet

Fasting blood glucose was comparable among all groups at Week 1 (ns, $p > 0.05$). At Week 8, the HFD group exhibited a significant increase in fasting blood glucose compared with the ND group ($p < 0.0001$), whereas PGE250 and PIO20 did not differ significantly from the HFD group. By Week 16, HFD rats showed a marked elevation in fasting blood glucose compared with ND rats ($p < 0.0001$). Treatment with PGE250 and PIO20 significantly reduced fasting blood glucose compared with the HFD group ($p < 0.0001$). Thus, *P. guajava* extract significantly attenuated HFD/fructose-induced hyperglycemia. (Figure 7)

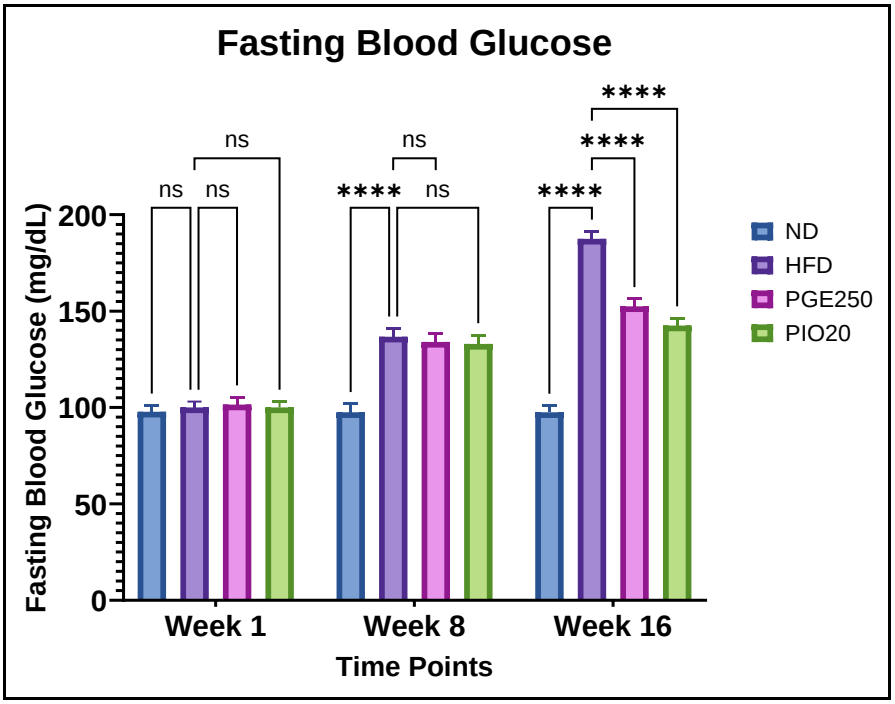

Figure 7. Effect of *P. guajava* extract on fasting blood glucose in High Fat Diet induced NAFLD in Wistar rats at different time points. Fasting blood glucose was recorded across experimental groups at Weeks 1, 8, and 16. Groups: normal chow diet in normal control (ND), high-fat diet + 10% fructose in disease control (HFD), *Psidium guajava* extract-treated (PGE250, 250 mg/kg p.o.), and pioglitazone-treated (PIO20, 20 mg/kg p.o.) Wistar rats. Treatments were administered daily from Week 9 to Week 16. Data are presented as Mean ± SEM. Statistical analysis was performed using ordinary two-way ANOVA followed by Dunnett's multiple-comparisons test. **** $p < 0.0001$, and ns = non-significant ($p > 0.05$).

Effect of *P. guajava* extract on Insulin and HOMA-IR on Wistar rats fed on High Fat Diet

At Week 16, the HFD group showed a marked increase in HOMA-IR and fasting plasma insulin compared with the ND group ($p < 0.0001$), indicating pronounced insulin resistance. Treatment with PGE250 significantly reduced HOMA-IR and fasting plasma insulin compared with the HFD group ($p < 0.0001$). PIO20 also produced a significant reduction in both parameters ($p < 0.0001$). Overall, *P. guajava* extract significantly improved HFD/fructose-induced hyperinsulinemia and insulin resistance. (Figure 8)

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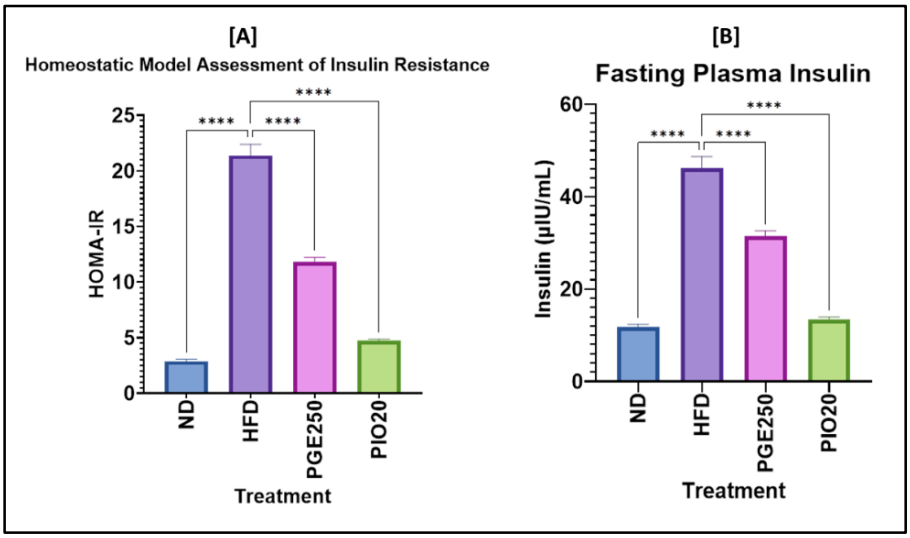

Figure 8. Effect of *P. guajava* extract on fasting plasma insulin levels and HOMA-IR in High Fat Diet induced NAFLD in Wistar rats at week 16. Fasting insulin and HOMA-IR were recorded across experimental groups at Weeks 1, 8, and 16. Groups: normal chow diet in normal control (ND), high-fat diet + 10% fructose in disease control (HFD), *Psidium guajava* extract-treated (PGE250, 250 mg/kg p.o.), and pioglitazone-treated (PIO20, 20 mg/kg p.o.) Wistar rats. Treatments were administered daily from Week 9 to Week 16. Data are presented as Mean ± SEM. Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple-comparisons test. **** $p < 0.0001$, and ns = non-significant ($p > 0.05$).

Effect of *P. guajava* extract on absolute liver weight and hepatosomatic index on Wistar rats fed on High Fat Diet

At Week 16, HFD rats exhibited a significant increase in absolute liver weight and hepatosomatic index compared with the ND group ($p < 0.0001$). Treatment with PGE250 and PIO20 significantly reduced both absolute liver weight and hepatosomatic index compared with the HFD group ($p < 0.0001$). Thus, *P. guajava* extract significantly attenuated HFD/fructose-induced liver enlargement and hepatosomatic index elevation. (Figure 9)

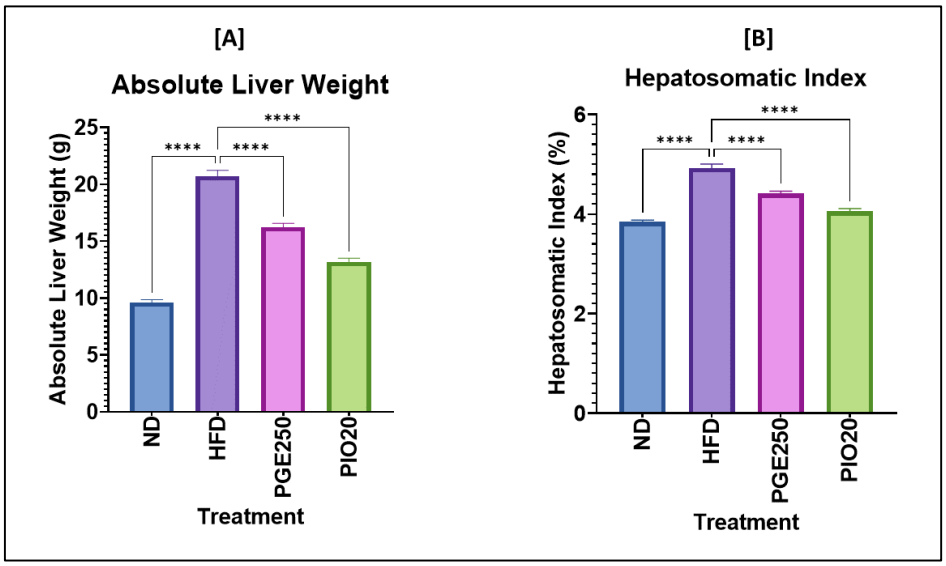

Figure 9. Effect of *P. guajava* extract on absolute liver weight and hepatosomatic index in High Fat Diet induced NAFLD in Wistar rats at week 16. The absolute liver weight and hepatosomatic index were recorded across experimental groups at Weeks 1, 8, and 16. Groups: normal chow diet in normal control (ND), high-fat diet + 10% fructose in disease control (HFD), *Psidium guajava* extract-treated (PGE250, 250 mg/kg p.o.), and pioglitazone-treated (PIO20, 20 mg/kg p.o.) Wistar rats. Treatments were administered daily from Week 9 to Week 16. Data are presented as Mean ± SEM. Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple-comparisons test. **** $p < 0.0001$, and ns = non-significant ($p > 0.05$).

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Effect of *P. guajava* extract on Lipid Profile on Wistar rats fed on High Fat Diet

At Week 16, the HFD group showed a significant increase in triglycerides, total cholesterol, and LDL-C**,** along with a significant reduction in HDL-C**,** compared with the ND group ($p < 0.0001$). Treatment with PGE250 significantly reduced triglycerides, total cholesterol, and LDL-C compared with HFD ($p < 0.0001$) and significantly increased HDL-C ($p < 0.001$). PIO20 produced a similar improvement in the lipid profile, with significant reductions in triglycerides, total cholesterol, and LDL-C ($p < 0.0001$). Overall, *P. guajava* extract markedly improved HFD/fructose-induced dyslipidemia**.** (Figure 10)

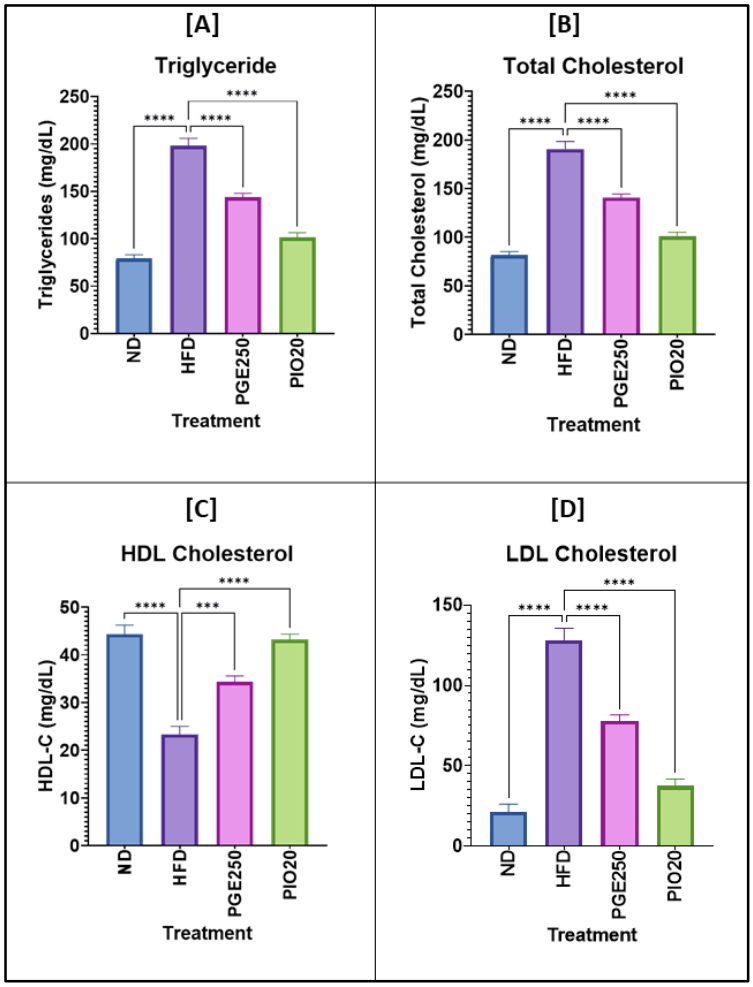

Figure 10. Effect of *P. guajava* extract on plasma triglycerides, total cholesterol, HDL cholesterol, and LDL cholesterol in HFD- and fructose-induced NAFLD in Wistar rats at week 16. The lipid profile was recorded across experimental groups at Weeks 1, 8, and 16. Groups: normal chow diet in normal control (ND), high-fat diet + 10% fructose in disease control (HFD), *Psidium guajava* extract-treated (PGE250, 250 mg/kg p.o.), and pioglitazone-treated (PIO20, 20 mg/kg p.o.) Wistar rats. Treatments were administered daily from Week 9 to Week 16. Data are presented as Mean ± SEM. Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple-comparisons test. **** $p < 0.0001$, and ns = non-significant ($p > 0.05$).

Effect of *P. guajava* extract on Liver Function Test on Wistar rats fed on High Fat Diet

At Week 16, the HFD group showed a significant reduction in plasma total protein and marked increases in ALT, AST, and ALP compared with the ND group ($p < 0.001$; $p < 0.0001$). Treatment with PGE250 significantly increased total protein and reduced ALT, AST, and ALP levels compared with the HFD group ($p < 0.001$; $p < 0.0001$). PIO20 produced comparable improvements in these liver function parameters. Overall, *P. guajava* extract significantly improved the HFD/fructose-induced alterations in liver function markers**,** indicating a hepatoprotective effect. (Figure 11)

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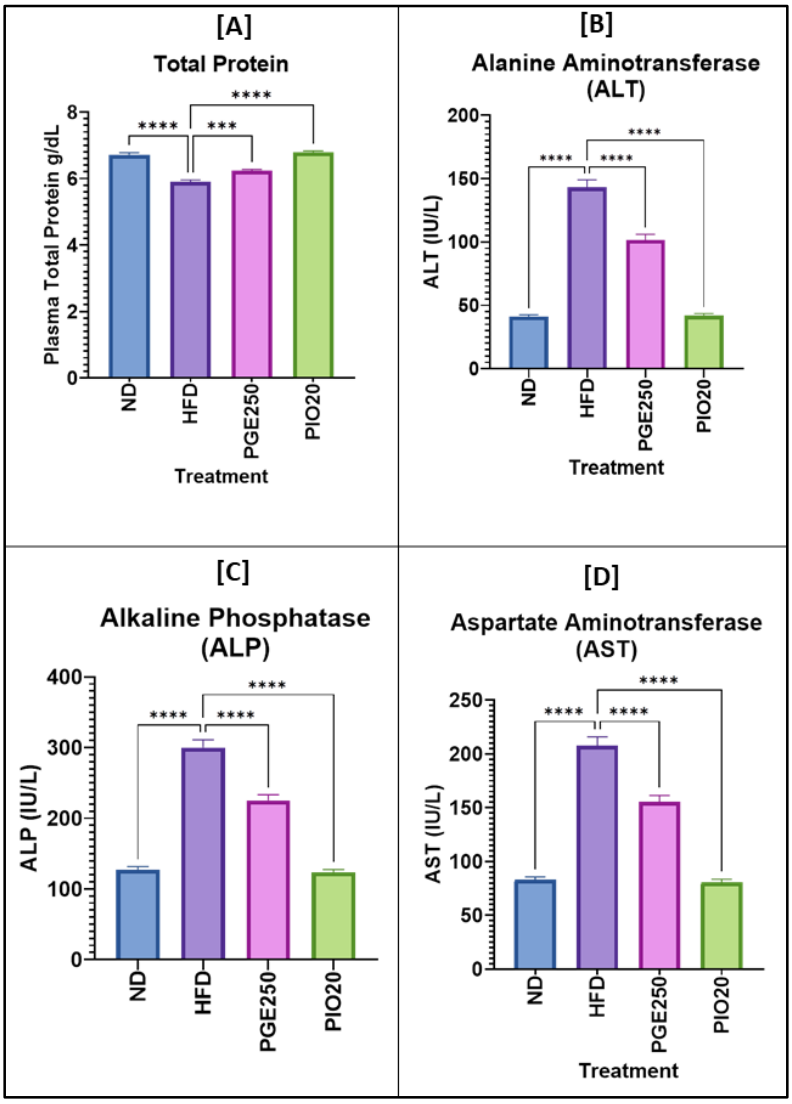

Figure 11. Effects of *P. guajava* extract on plasma total protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) levels in HFD- and fructose-induced NAFLD in Wistar rat at week 16. The liver function test was recorded across experimental groups at Weeks 1, 8, and 16. Groups: normal chow diet in normal control (ND), high-fat diet + 10% fructose in disease control (HFD), *Psidium guajava* extract-treated (PGE250, 250 mg/kg p.o.), and pioglitazone-treated (PIO20, 20 mg/kg p.o.) Wistar rats. Treatments were administered daily from Week 9 to Week 16. Data are presented as Mean ± SEM. Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple-comparisons test. **** $p < 0.0001$, and ns = non-significant ($p > 0.05$).

Effect of *P. guajava* extract on Hepatic Pro-Inflammatory Cytokines on Wistar rats fed on High Fat Diet

At Week 16, HFD rats exhibited markedly elevated hepatic TNF-α, IL-1β, and IL-6 levels compared with the ND group ($p < 0.0001$). Treatment with PGE250 significantly reduced the levels of all three pro-inflammatory cytokines compared with the HFD group ($p < 0.0001$). PIO20 produced a comparable reduction in hepatic cytokine levels. Overall, *P. guajava* extract significantly attenuated HFD/fructose-induced hepatic inflammation**,** as evidenced by reduced TNF-α, IL-1β, and IL-6 levels. (Figure 12)

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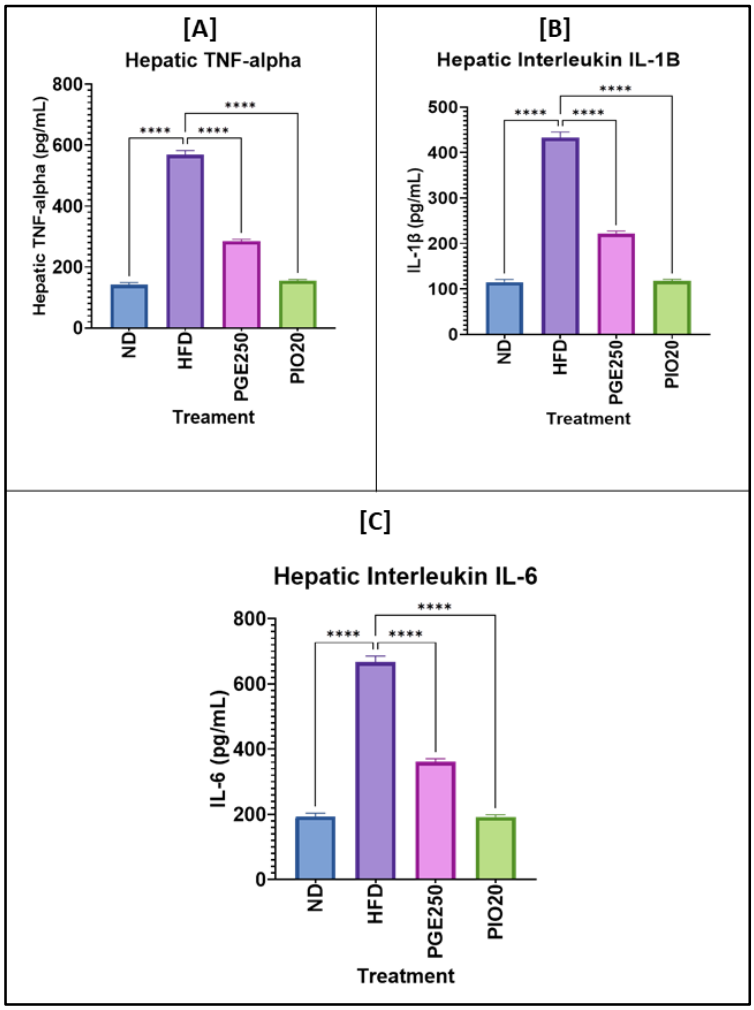

Figure 12. Effect of *P. guajava* extract on hepatic tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) levels in HFD- and fructose-induced NAFLD in Wistar rats at week 16. The liver proinflammatoy cytokines was recorded across experimental groups at Weeks 1, 8, and 16. Groups: normal chow diet in normal control (ND), high-fat diet + 10% fructose in disease control (HFD), *Psidium guajava* extract-treated (PGE250, 250 mg/kg p.o.), and pioglitazone-treated (PIO20, 20 mg/kg p.o.) Wistar rats. Treatments were administered daily from Week 9 to Week 16. Data are presented as Mean ± SEM. Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple-comparisons test. **** $p < 0.0001$, and ns = non-significant ($p > 0.05$).

Effect of *P. guajava* extract on Hepatic Antioxidants on Wistar rats fed on High Fat Diet

At Week 16, the HFD group showed a significant reduction in hepatic **catalase, SOD, and GSH** levels, accompanied by a marked increase in **lipid peroxidation (MDA)** compared with the ND group ($p < 0.001$; $p < 0.0001$). Treatment with PGE250 significantly increased catalase, SOD, and GSH levels and reduced MDA levels compared with the HFD group ($p < 0.001$; $p < 0.0001$). PIO20 produced a comparable improvement in hepatic antioxidant status. Overall, *P. guajava* extract significantly attenuated HFD/fructose-induced oxidative stress and restored hepatic antioxidant defenses. (Figure 13)

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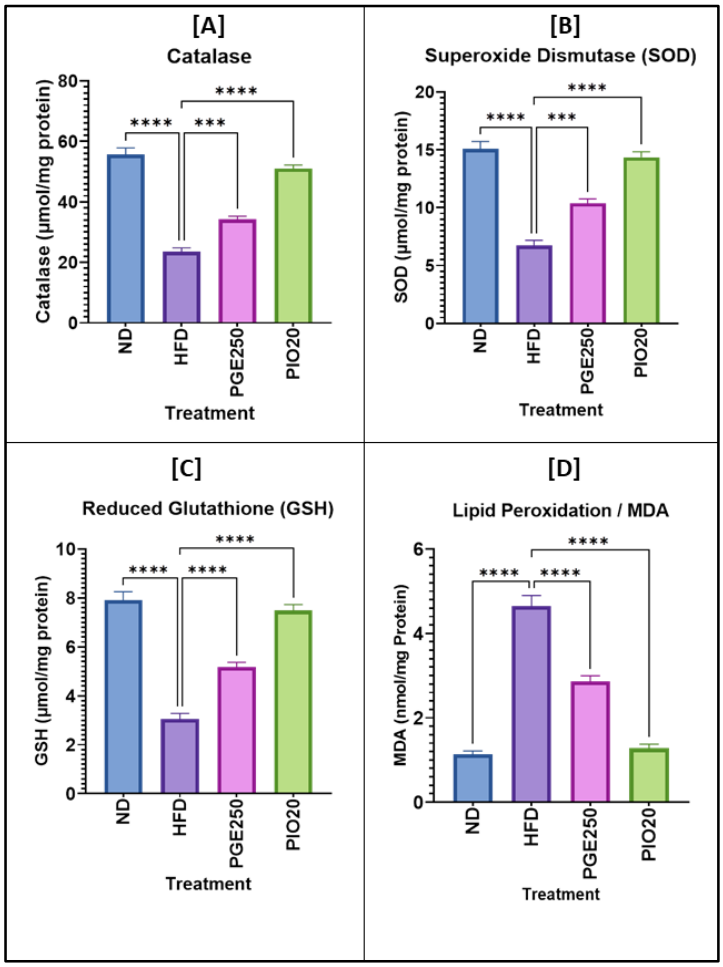

Figure 13. Effect of *P. guajava* extract on hepatic catalase, superoxide dismutase, reduced glutathione (GSH), and lipid peroxidation in HFD- and fructose-induced NAFLD in Wistar rats at week 16. The hepatic antioxidant activity was recorded across experimental groups at Weeks 1, 8, and 16. Groups: normal chow diet in normal control (ND), high-fat diet + 10% fructose in disease control (HFD), *Psidium guajava* extract-treated (PGE250, 250 mg/kg p.o.), and pioglitazone-treated (PIO20, 20 mg/kg p.o.) Wistar rats. Treatments were administered daily from Week 9 to Week 16. Data are presented as Mean ± SEM. Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple-comparisons test. **** $p < 0.0001$, and ns = non-significant ($p > 0.05$).

Effect of *P. guajava* extract on Gross Morphology and Histopathology of liver tissue

Gross morphological examination at Week 16 revealed marked differences among the experimental groups. The ND group showed a normal reddish-brown liver with well-defined margins, whereas the HFD + fructose group exhibited pale discoloration, hepatomegaly, and blunted margins**,** consistent with hepatic steatosis. Treatment with PGE250 markedly improved the gross hepatic appearance, with restoration of a more normal color and morphology. PIO20 showed a similar preservation of gross liver morphology. Overall, *P. guajava* extract attenuated the HFD/fructose-induced gross morphological alterations of the liver. (Figure 14)

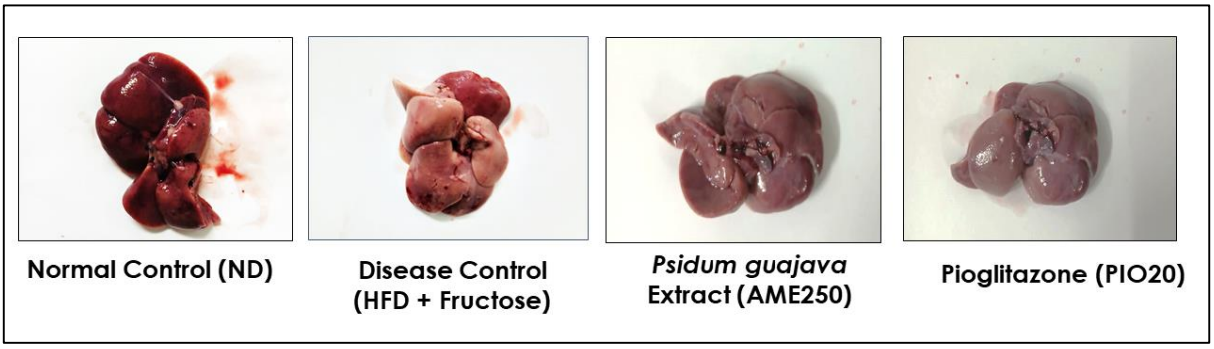

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Figure 14. Gross morphology of liver tissues in HFD- and fructose-induced NAFLD in Wistar rats at week 16. Representative gross anatomical photographs of harvested livers across experimental groups: (**A**) Normal Control (ND), displaying normal reddish-brown color and sharp anatomical margins; (**B**) Disease Control (HFD+ Fructose), displaying severe yellowish-pale discoloration, organ enlargement, and blunted borders indicative of steatosis and cellular swelling; (**C**) *P. guajava* extract (PGE250, 250 mg/kg p.o.), showing restoration of normal gross hepatic morphology and color; and (**D**) Pioglitazone (PIO20, 20 mg/kg p.o.), showing complete preservation of healthy liver architecture.

Histopathological examination at Week 16 revealed normal hepatic architecture with no abnormality detected (NAD) in the ND group. In contrast, the HFD + fructose group exhibited hepatocellular vacuolar degeneration with inflammatory cell infiltration**,** corresponding to a mild histopathological grade (<2). Treatment with PGE250 restored hepatic morphology, with no abnormality detected, comparable to the PIO20 group. Overall, *P. guajava* extract markedly attenuated HFD/fructose-induced hepatic histopathological alterations**,** demonstrating a protective effect on liver tissue. (Figure 15)

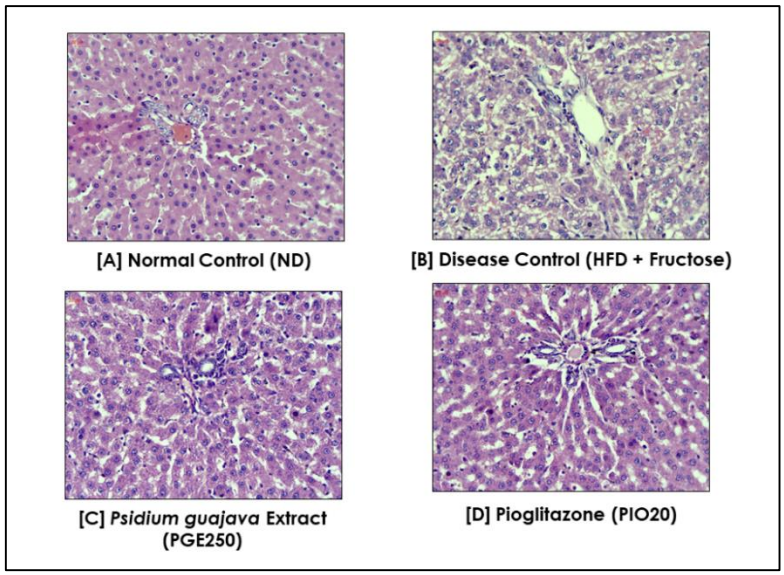

Figure 15. Histopathological evaluation of liver tissues in experimental groups at week 16. Histopathological grading confirmed no abnormality detected (NAD) in ND, PGE250, and PIO20 groups, compared to hepatocellular vacuolar degeneration with inflammatory cell infiltration (Grade <2) in the HFD + Fructose group (n = 6 per group).

Discussion

The present study demonstrates that eight weeks of oral treatment with *Psidium guajava* leaf extract (PGE250, 250 mg/kg) markedly ameliorated high-fat diet -induced NAFLD in Wistar rats, attenuating body-weight gain, hyperglycemia, hyperinsulinemia, insulin resistance, dyslipidemia, hepatomegaly, and liver injury while restoring hepatic antioxidant defenses and suppressing hepatic inflammation. Histopathological examination confirmed the biochemical improvements, with restoration of normal hepatic architecture in extract-treated animals. Notably, the effects of PGE250 were comparable to those of the reference insulin sensitizer pioglitazone (20 mg/kg), which is currently used in clinical practice for biopsy-proven NASH [19], supporting the therapeutic potential of guava leaf as a natural alternative.

The HFHF dietary model employed here reproduces key features of human NAFLD. Excessive fructose consumption is both a substrate and an inducer of hepatic de novo lipogenesis: unlike glucose, fructose is taken up almost entirely by the liver and is directed toward fatty acid synthesis [41], stimulating the master lipogenic transcription factor SREBP-1c and generating reactive oxygen species through ATP depletion [9]. Combined high-fat, high-fructose feeding has been shown to produce severe hepatic steatosis with dyslipidemia, elevated liver enzymes, and hyperuricemia even in non-obese rats [12], and dietary fat promotes NAFLD development more potently than fructose alone [11]. Consistent with these reports, the disease-control group in the present

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study exhibited significant increases in body weight, fasting glucose, insulin, HOMA-IR, triglycerides, total cholesterol, LDL-C, ALT, AST, ALP, TNF-α, IL-1β, and IL-6, together with reduced HDL-C, total protein, catalase, SOD, and GSH recapitulating the metabolic, inflammatory, and oxidative abnormalities central to human NAFLD [42].

An important observation was that PGE250 reduced body weight and body mass index-related weight gain without altering food intake, which remained comparable to the HFHF group throughout treatment (ns, $p > 0.05$). This indicates that the metabolic benefits of the extract are not attributable to appetite suppression or reduced caloric intake, but rather to genuine improvements in energy metabolism. Similar weight-reducing effects independent of energy intake have been reported for rutin, one of the marker polyphenols identified in our extract, which lowered body weight in high-fat diet-fed rats despite equivalent food consumption, through AMPK activation and enhanced mitochondrial biogenesis [43]. This profile is particularly attractive when contrasted with pioglitazone, whose clinical utility is limited by weight gain — averaging approximately 4% — and increased adiposity in treated patients [20].

The improvement in glucose homeostasis observed in PGE250-treated rats significant reductions in fasting blood glucose, fasting plasma insulin, and HOMA-IR at Week 16 ($p < 0.0001$) indicates that the extract exerts insulin-sensitizing effects analogous to those of pioglitazone, which improves hepatic and peripheral insulin sensitivity and lowers liver fat in NASH patients [44]. This finding is consistent with prior reports that guava leaf extract suppresses fructose-mediated NAFLD by modulating hepatic insulin signaling, including the Akt/FOXO1/SREBP-1c pathway, and that it outperformed pioglitazone and metformin in reversing hepatic insulin resistance in fructose-treated hepatocytes [26]. The restoration of glycemic control is mechanistically relevant because insulin resistance is regarded as the central driver linking NAFLD to the metabolic syndrome [45], and it likely contributed to the amelioration of hepatic steatosis in our model.

The marked improvement in the plasma lipid profile significant reductions in triglycerides, total cholesterol, and LDL-C with a significant rise in HDL-C ($p < 0.0001$; HDL-C $p < 0.001$) — corroborates the established hypolipidemic activity of *P. guajava* leaf [27] and is supported by earlier work showing that guava leaf powder supplementation reduces dyslipidemia and hepatic fat deposition in high-carbohydrate, high-fat diet-fed rats [21]. The polyphenolic constituents quantified in our extract plausibly mediate these effects. Ellagic acid, the predominant marker compound (51.89 ppm), has been shown to increase HDL-C while reducing total cholesterol and LDL-C in mice through modulation of lipogenesis, lipolysis, and fatty acid oxidation [46], and to alleviate hepatic steatosis via inhibition of PPARγ signaling [47]. Quercetin (31.33 ppm) and rutin (26.24 ppm) suppress lipogenic gene expression, including SREBP-1c and fatty acid synthase, through AMPK pathway activation [48], and rutin consistently reduces hepatic triglyceride and cholesterol content in dietary models [49]. The co-occurrence of these three compounds in a single extract may therefore confer synergistic lipid-lowering action.

PGE250 also exerted clear hepatoprotection, significantly increasing plasma total protein and reducing ALT, AST, and ALP ($p < 0.001$; $p < 0.0001$), alongside significant reductions in absolute liver weight and hepatosomatic index ($p < 0.0001$). These results extend earlier demonstrations of the hepatoprotective activity of *P. guajava* leaf extracts against carbon tetrachloride-, paracetamol-, and thioacetamide-induced liver injury, in which the extract reduced serum transaminases and alkaline phosphatase and prevented hepatomegaly [23]. Standardized triterpenoid-enriched guava leaf extracts have likewise restored hepatic architecture and improved serum ALT and AST in acetaminophen-treated mice [25]. The attenuation of hepatomegaly and enzyme leakage in our study indicates protection of hepatocellular integrity, consistent with the histopathological restitution observed.

Oxidative stress is a critical amplifier of the transition from simple steatosis to steatohepatitis [9,50], and the restoration of hepatic catalase, SOD, and GSH with a parallel reduction in MDA after PGE250 treatment ($p <$

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0.001; $p < 0.0001$) points to potent antioxidant activity. Guava leaf supplementation has previously been shown to re-establish the antioxidant enzyme system and reduce lipid peroxidation products in high-fat diet-fed rats [21]. Among the quantified markers, ellagic acid scavenges reactive oxygen species and enhances endogenous antioxidant defenses through Nrf2/ARE activation, and its gut-microbial metabolite urolithin exerts similar effects [51,52]; quercetin protects against hepatic steatosis through AMPK activation and lipotoxicity blockade [53,54]. Because oxidative stress and lipid peroxidation are primary drivers of hepatocyte injury in NAFLD [2], this antioxidant restoration is likely a key mechanism underlying the extract's overall hepatoprotective effect.

The significant suppression of hepatic pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 ($p < 0.0001$) demonstrates an anti-inflammatory action that is central to halting NASH progression [18]. The reduction in IL-1β is especially relevant, as fructose-induced hepatic insulin resistance and steatohepatitis are propagated through NLRP3 inflammasome activation and the downstream maturation of IL-1β [55]. Guava leaf extract has been reported to suppress hypoxia-inflammation signaling, including HIF-1α and TNF-α, in fructose-fed growing rats [26], and ellagic acid inhibits NF-κB-driven TNF-α and IL-6 expression [51]. The combined antioxidant and anti-inflammatory profile of PGE250 is therefore coherent with the histopathological finding that treated livers showed no abnormality detectable, whereas HFHF-fed livers exhibited hepatocellular vacuolar degeneration with inflammatory cell infiltration (grade < 2). These observations mirror the reduced inflammatory-cell infiltration and collagen deposition reported after guava leaf supplementation in diet-induced hepatic steatosis [21], and corroborate the hepatoprotective histology seen with *P. guajava* extracts in toxic liver injury models [23].

The efficacy of PGE250 was comparable to that of pioglitazone across nearly all endpoints. Pioglitazone is among the few agents with randomized-trial evidence of histological improvement in NASH, including reductions in steatosis, lobular inflammation, and NAFLD activity score [56], yet it remains unapproved for this indication and is associated with weight gain and concerns regarding long-term safety [20]. The present finding that PGE250 achieved parallel improvements including weight reduction without altered food intake positions guava leaf extract as a promising, well-tolerated candidate for NAFLD management, consistent with the call for accessible natural therapies to address the escalating global NAFLD burden [16].

Conclusion

The present study demonstrates that *Psidium guajava* leaf extract — standardized for ellagic acid, quercetin, and rutin content — effectively ameliorates HFHF diet-induced NAFLD in Wistar rats through coordinated improvements in insulin sensitivity, lipid metabolism, oxidative stress, and hepatic inflammation, with histological restitution comparable to pioglitazone. These findings provide a strong experimental basis for further preclinical development and eventual clinical evaluation of guava leaf extract as a safe, plant-based therapeutic option for NAFLD, a disease for which approved pharmacological therapies remain lacking. Future investigations should focus on elucidating the specific molecular pathways by which these bioactive compounds regulate mitochondrial bioenergetics and lipid trafficking within the hepatocyte.

Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this research.

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