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Research Paper

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Modulatory Effect of Guava Leaf Extract on Liver Tissue in Hypercholesterolemic Rats (*Rattus norvegicus*)

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Abstract

Background: Hypercholesterolemia is a global health issue with high prevalence, contributing to conditions such as Non-Alcoholic Fatty Liver Disease (NAFLD) & Non-Alcoholic Steatohepatitis (NASH). Conventional therapies, although effective, have side effects. Guava leaf extract (*Psidium guajava*) is known for its hepatoprotective & antioxidant effects, but research on its impact on liver histopathology in the context of hypercholesterolemia remains limited. **Objectives:** To investigate the effect of *Psidium guajava* leaf extract on the histopathological picture of the liver in Wistar rats (*Rattus norvegicus*) with a hypercholesterolemia model. **Methods:** This experimental study used a post-test only control design. The experiment utilized a completely randomized design with four groups. Data analysis was performed univariately &bivariately. The research sample consisted of 28 Wistar rats, divided into 4 groups of 7 rats each. The rats were induced with a high-fat diet for 2 weeks to induce hypercholesterolemia, followed by treatment with guava leaf extract via gavage for 2 weeks. After termination, histopathological scoring of the liver was performed. Bivariate analysis was conducted using One-Way ANOVA & post hoc LSD tests. **Key findings:** Univariate analysis showed the highest mean liver damage score in the sick control group (4.00 ± 0.632) &the lowest in the group receiving guava leaf extract before hypercholesterolemia induction (1.33 ± 1.366). Bivariate analysis revealed significant differences between the healthy control &sick control groups (p = 0.00), as well as between the sick control & the guava leaf extract treatment group (p = 0.00). Guava leaf extract was found to reduce liver damage due to hypercholesterolemia, supporting its potential as an ameliorative & hepatoprotective agent. **Conclusions:** The administration of *Psidium guajava* leaf extract significantly influenced the histopathological score of the liver in Wistar rats induced with hypercholesterolemia. This result is supported by significant statistical differences in the histopathological liver scores across the four groups overall.

Keywords: High fat diet, hypercholesterolemia, liver histopathology, *Psidium guajava*.

Introduction

Hypercholesterolemia is a global health issue with an increasing prevalence, particularly in developing countries [1]. According to the World Health Organization (WHO, 2008), 39% of the population aged over 25 years suffers from hypercholesterolemia, with the highest prevalence reported in Europe (54%), America (48%), and Asia (22.6%). This trend has been observed to increase among high socioeconomic groups, and from 1980 to 2017, there has been no significant decline in its prevalence, with some indications of further increases in developing nations. Hypercholesterolemia contributes to 4.4 million deaths due to hyperlipidemia worldwide in 2019 [2]. The prevalence of hypercholesterolemia associated with Non-Alcoholic Fatty Liver Disease (NAFLD) reaches 69%, while that linked to Non-Alcoholic Steatohepatitis (NASH) reaches 72% [3]. In Indonesia, the prevalence of hypercholesterolemia is as high as 36% among individuals aged over 25 years [4]. High-fat and carbohydrate diets are primary risk factors for hyperlipidemia and metabolic syndrome, which elevate the risk of cardiovascular diseases and NAFLD [5].

Hypercholesterolemia, as a component of hyperlipidemia, is also associated with increased levels of liver enzymes such as gamma-glutamyl transferase (GGT), which are crucial for monitoring liver function. If left untreated, this condition can progress to NAFLD, NASH, and eventually cirrhosis [6]. The management of hypercholesterolemia typically involves the use of statins as the primary therapy, which effectively reduce blood lipid levels by 22-50% [7].

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However, statins have side effects, including myopathy, hepatotoxicity, and an increased risk of type 2 diabetes. High doses of statins may also cause liver damage [8]. Meanwhile, pharmacological approaches for NAFLD and NASH are still under investigation, although certain drugs like metformin, pioglitazone, and gemfibrozil have shown potential in improving liver function [9]. Beyond pharmacological therapies, herbal treatments have gained popularity due to their minimal side effects [10]. Guava leaf extract (*Psidium guajava*) has been extensively studied and demonstrated hepatoprotecti-ve, antioxidant, and lipid-lowering properties, specifically reducing LDL cholesterol and increasing HDL cholesterol [11]. Traditionally, guava leaf extract has been used to treat various ailments, including gastrointestinal disorders [12]. However, research on its impact on liver histopathology in hyoercholesterole-mic conditions remains limited. Histopathological studies of NAFLD/NASH primarily focus on three key aspects: steatosis (liver fat accumulation), lobular inflammation, and ballooning degeneration. Steatosis is the primary manifestation in hypercholesterolemia, and its reduction can prevent progression to NASH and fibrosis [13]. Therefore, this study aims to evaluate the effect of *Psidium guajava* leaf extract on the histopatho- logical characteristics of the liver in Wistar rats induced with a high-fat diet. Wistar rats (*Rattus norvegicus*) were chosen as the experimental model due to their physiological similarities to humans and sensitivity to high-fat diets.

This study investigates whether the administration of *Psidium guajava* leaf extract influences the histopathological picture of the liver in a hypercholest-erolemic rat model. The objectives of the study include analyzing the histopathological differences between hypercholesterolemic rats without treatment and control rats, as well as comparing the effects of guava leaf extract administered before and after inducing hypercholesterolemia. Theoretically, this study is expected to contribute to the fields of phytopharmacol- ogy and anatomical pathology. Practically, the findings could serve as a foundation for further research, provi- de academic data, and enhance public understanding of the potential of guava leaf extract in preventing and treating hypercholesterolemia.

Materials and Methods

The design of this research is True Experimental. True Experimental design is an experimental design that has a control group that is equivalent to the treatment group [14]. In this design, the research uses the Post Test Only Control Design model. To achieve equivalence between the control group and the treatment group, randomization is performed.

Tools and Materials

This study used male white rats of the *Rattus norvegicus* Wistar strain as test animals, obtained from the Animal Testing Laboratory of the Faculty of Medicine, Jenderal Soedirman University. The rats were selected based on inclusion criteria such as being healthy and active without any abnormalities, and exclusion criteria such as experiencing weight loss. The age of the rats used is 2-3 months, considering that at this age growth begins to slow down, thus minimizing the influence of hormonal interventions and weight changes. To determine the required sample size, the Federer formula was used and a 20% drop-out correction was applied, resulting in a minimum of 7 rats per group with a total of 28 rats. The test animals were divided into three treatment groups, as well as one positive control group and one negative control group. In this study, various materials were used such as Psidium guajava leaves, ethanol of various concentrations, 10% formalin, Giemsa solution, lard, and propylthiouracil, which were used for the induction of hypercholesterol-emia and the histopathological analysis process. In addition, this research also utilizes various laboratory equipment such as stomach tubes, microscopes, rotary evaporators, grinders, flasks, and surgical instrument sets to support the research procedures and sample analysis.

The Progress of the Research

The sequence of work in this research begins with the submission of a proposal to be reviewed by the Research Ethics Code section of the Faculty of Medicine, Jenderal Soedirman University (UNSOED) to obtain ethical clearance. After that, plant determination was carried out to ensure that the species used was guava (Psidium guajava L.), which was conducted at the Tawangmangu Traditional Health Service Functional Unit. The test animals used were male Wistar strain white rats (*Rattus norvegicus*), obtained from the Animal Laboratory of FK UNSOED, totaling 28 individuals aged 2-3 months and weighing 200 g, and in healthy condition. These rats were acclimatized for 7 days in a controlled cage environment and were given standard feed with temperature and humidity maintained according to regulations. After acclimatization, hypercholesterolemia was induced in the rats by administering a high-fat diet (HFD) and propylthioura- cil (PTU) for 14 days. Simple randomization was performed in the process of allocating rats into experimental groups using a completely randomized design (CRD), which divided the samples into five groups randomly with the same conditions for all test animals, including cages measuring 375 cm^2 × 15 cm containing one rat, a temperature of 22±3°C, humidity of 30%-70%, and light-dark illumination every 12 hours.

The experimental group design is as follows: Group 1 (C-): 7 rats were given standard feed ad libitum and aqua destilled sonde without HFD induction and without extract

as a healthy control; Group 2 (C+): 7 rats were given PTU 2 mg/200 g in duck egg yolk for 2 weeks along with HFD feed, then given HFD feed without extract as a sick control; Group 3 (P1): 7 rats were given PTU and HFD feed for 2 weeks, then given HFD feed and guava leaf extract 150 mg/kgBW/day for 2 weeks; Group 4 (P2): 7 rats were given guava leaf extract 150 mg/kgBW/day along with HFD feed for 2 weeks, then given HFD feed for the next 2 weeks. The extraction of guava leaves was performed using the maceration method with 96% ethanol, resulting in a concentrated extract that was then administered to the rats via intragastric intubation, with the dosage calculated based on the rats' body weight. After the treatment is completed, termination is performed using the decapitation and necropsy methods to obtain rat liver samples. Histopathological examination was conducted through a graded dehydration process, clearing, and impregnation in liquid paraffin, followed by hematoxylin-eosin (HE) staining. The resulting preparations were observed using a light microscope at 40x magnification to identify histological changes, such as steatosis, inflammatory foci, and cell enlargement (ballooning). The observation scores were obtained using the NAS CRN scoring system, and documentation was carried out using LAS-EZ software.

Data Analysis

In this study, the independent variable is the dosage of guava leaf extract, while the dependent variable is the histopathological score of the rat liver. After being treated with HFD induction for 2 weeks and guava leaf extract for 2 weeks, the rats were then terminated and the liver organs were collected for histopathological examination using the paraffin method. The dosage of the extract was confirmed using a balance. Histopathol- ogical score calculation was performed using the NAS scoring system. The collected data were then processed and analyzed using SPSS software version 22.0 for Windows. Univariate analysis was conducted by determining the mean, median, minimu-um, maximum values, and testing the normality of each data group. The normality test was conducted using the Shapiro-Wilk method because the sample size was less than 50. Next, the homogeneity test was conducted using the Levene test. Since the analysis results showed that the data were normally and homogeneously distributed, the analysis continued with bivariate testing using One-Way ANOVA. Then, a post hoc analysis was conducted using the Least Significant Difference (LSD) test.

Results

This research was conducted at the Animal Laboratory of the Faculty of Medicine, Jenderal Soedirman University, from October to November 2024. This research has received ethical approval from the Medical Research Ethics Committee of the Faculty of Medicine, Jenderal Soedirman University, with reference number 087/KEPK/PE/X/2024, approved on October 10, 2024. During the course of the research, there were incidents of death among the test animals, with one animal dying in each of groups 1, 2, and 3. Histopathological analysis of rat liver was conducted using a Leica microscope equipped with the LAS-EZ application, which facilitates the observation and assessment of tissues. The data obtained from this observation were then analyzed using the IBM SPSS Statistic Base 25.00 software to calculate various statistical parameters, such as minimum, maximum, median, mean, and standard deviation for each experimental group. The analysis results show that group 2 experienced the most severe liver damage, followed by group 3, group 1, and the least damage was in group 4. Significant differences in liver tissue damage levels among these experimental groups can be seen in Table 1 and Table 2.

Data that has been analyzed univariately is then tested for normality and homogeneity. The normality test using the Shapiro-Wilk method shows that the data is normally distributed, with p values > 0.05 for all groups: group 1 (0.093), group 2 (0.101), group 3 (0.212), and group 4 (0.093). The homogeneity test using Levene's test resulted in a p value of 0.093, indicating that the variances between groups are homogeneous or similar. further analysis was conducted using the One-Way ANOVA method as shown in Table 2.

Table 1 Univariate analysis of NAS scores in each group

Groups	n	Steatosis (%) (Mean ± SD)	Lobular Inflammation (Mean ± SD)	Ballooning Hepatosit (Mean ± SD)	NAS Score (Mean ± SD)	Interpretation
Healthy control	6	6,83 ± 1,53	0,30 ± 0,19	0,60 ± 0,21	1,67 ± 1,36	Minimal damage/None
Sick control	6	39,66 ± 6,13	0,73 ± 0,21	0.83 ± 0.15	4,00 ± 0,63	Moderate damage
Treatment 1	6	11,50 ± 2,86	0,30 ± 0,10	0.43 ± 0.15	2,17± 0,73	Minimal damage/None
Treatmen 2	7	6,28 ± 0,96	0,28 ± 0,20	0.45 ± 0.36	1,33 ± 1,36	Minimal damage/None

The results of the One-Way ANOVA analysis show a p-value of 0.00 ($p < 0.05$), indicating a significant difference between groups. To determine which pairs of groups differ significantly, further analysis was conducted using the Post Hoc LSD test. This test revealed that some pairs of groups showed significant differences in NAS scores, while others did not. The complete results of the Post Hoc LSD test can be seen in Table 3, which details the comparisons between groups.

Table 2 Bivariate analysis one-way ANOVA of NAS scores of all groups

Variable	Variance Source	p-value	Conclusion
NAS Score	Between groups	0.001	Significant

Table 3 Post hoc bivariate analysis of NAS scores between groups

Groups	1	2	3	4
1	-	0.00*	0.42	0.53
2	0.00*	-	0.00*	0.00*
3	0.42	0.00*	-	0.15
4	0.53	0.00*	0.15	-

Note: *There is a significant difference

The table shows that Group 1 (healthy control) and Group 2 (sick control induced by hypercholesterolem-ia) have a significant difference ($p = 0.00$), indicating that hypercholesterolemia induction causes histopath- ological damage to the liver. The comparison between Group 2 and Group 3 (treatment 1) is also significant ($p = 0.00$), indicating that the administration of guava leaf extract after hypercholesterolemia induction can help improve liver condition. Similarly, Group 2 and Group 4 (treatment 2) show a significant difference ($p = 0.00$), indicating that guava leaf extract before induction can protect the liver from damage caused by hypercholest-erolemia. On the other hand, comparisons between Group 1 and Group 3 ($p = 0.42$), Group 1 and Group 4 ($p = 0.53$), as well as Group 3 and Group 4 ($p = 0.15$) did not show significant differences, indicating that guava leaf extract is capable of preventing liver damage due to hypercholesterolemia, whether administered before or after induction.

Discussion

The obtained histopathological images show significant differences between the healthy control group (K-), the sick control group (K+), and the treatment groups (P1 & P2) as shown in Figure 1. The image of the K- group shows cells of normal size without any changes and inflammation, and mild steatosis is found. The K+ group shows significant changes in the form of macrovesicul- ar steatosis accompanied by inflammatory cell infiltra-tion and noticeable ballooning. Meanwhile, between the P1 and P2 groups, there were no significa- nt differences observed.

Mild steatosis can be caused by several factors, such as protein malnutrition, environmental toxins, oxidative stress, or individual genetic variations. Although mild steatosis does not always indicate significant liver function damage, this condition still needs to be monitored [15]. The provision of poultry feed such as BR2-Comfeed ad libitum to rats in this study contributed to the emergence of mild steatosis in the healthy control group. Although formulated for poultry, this feed may not fully meet the nutritional needs of rats, which can lead to metabolic imbalances and lipid accumulation in hepatocytes. Ad libitum feeding also allows for excessive calorie consumption, increasing the risk of hepatic steatosis. Previous research has shown that a high-fat and high-sugar diet can cause morphological changes in liver cells in rats, including damage and fat accumulation in hepatocytes [16]. In the sick control group, histopathological examination showed significant changes in the livers of Wistar rats, where most hepatocytes exhibited microvesicular and macrovesicular steatosis. Microvesicular steatosis is characterized by small lipid vacuoles in the hepatocyte cytoplasm, while macrovesicular steatosis shows large lipid vacuoles pressing against the cell nucleus. In addition to steatosis, there is infiltration of inflammatory cells such as neutrophils, macrophages, and lymphocytes forming inflammatory foci in the liver tissue. Cellular degeneration is also clearly visible with some hepatocytes showing ballooning degeneration, indicating damage and dysfunction of hepatocytes due to a high-fat diet.

Hypercholesterolemia induced by a high-fat diet, such as the administration of Comfeed feed and pig fat via a probe in Wistar rats, causes an increase in blood lipid levels, including cholesterol and free fatty acids. These excess lipids accumulate in circulation and enter the liver through several pathophysiological mechanisms, contributing to the formation of fatty liver [17]. This process begins with an increase in free fatty acid levels in the blood, which the liver takes up for processing. However, when the amount of fatty acids entering exceeds the liver's oxidation capacity, triglyceride accumulation occurs in hepatocytes. Furthermore, the disruption of fatty

acid oxidation caused by the administration of propylthiouracil (PTU) at a dose of 2 mg inhibits the conversion of thyroxine (T4) to triiodothyronine (T3), reduces the basal metabolic rate, and exacerbates steatosis by increasing triglyceride storage in hepatocytes [18].

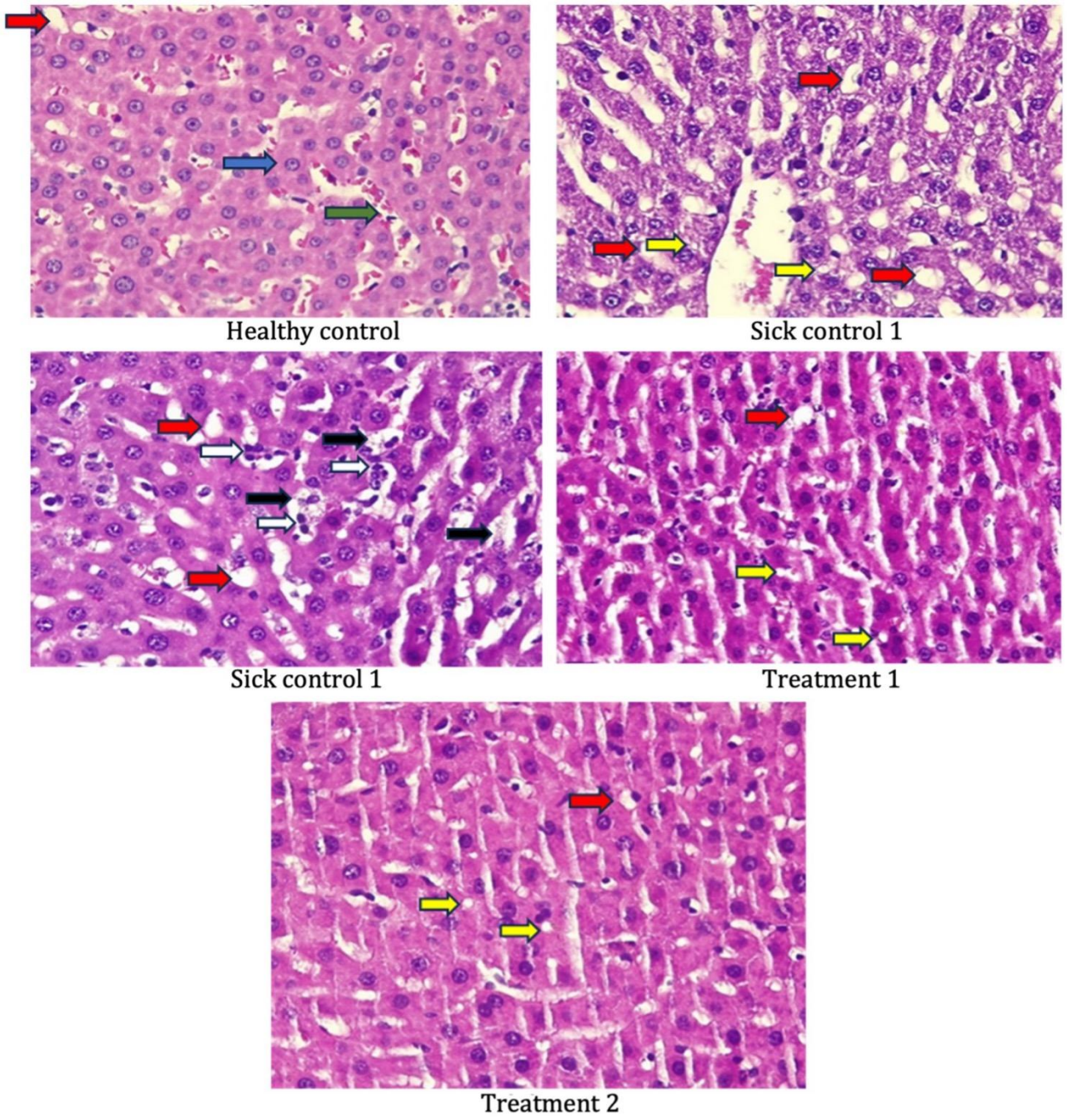

Figure 1 Histopathological appearance of the four experimental groups. Note: ⇨ = Example of normal hepatocytes; ⇨ = Perisinusoidal space; ⇨= Macrovesicular steatosis; ⇨ = Microvesicular steatosis; ⇨ = Lobular inflammatory foci; ⇨ = Ballooning

Furthermore, hypercholesterolemia stimulates pro-inflammatory pathways and the production of reactive oxygen species (ROS), leading to oxidative stress in hepatocytes. This oxidative stress damages cell membranes and mitochondria, inhibits lipid oxidation processes, and exacerbates fat accumulation in the liver. Histopathologically, steatosis appears as large or small lipid vacuoles in the hepatocyte cytoplasm, which are clearly visible in the sick control group. The combination of increased lipid intake, impaired fatty acid oxidation due to PTU, and oxidative stress explains how diet-induced hypercholesterolemia can lead to steatosis in Wistar rats [19]. The addition of PTU in this model not only reduces lipid metabolism through its hypothyroid effect but also exacerbates triglyceride accumulation in the livered liver,

making it an important contributing factor in creating a non-alcoholic fatty liver disease .

Steatosis due to hypercholesterolemia can develop into inflammation with the infiltration of inflammatory cells, forming inflammatory foci in liver tissue. The accumulation of triglycerides in hepatocytes due to excessive lipid intake causes steatosis, while hypercholesterolemia stimulates the production of reactive oxygen species (ROS), which triggers oxidative stress. Oxidative stress damages cell membranes and organelles such as mitochondria, which can lead to apoptosis or cell death. Hepatocyte damage triggers the release of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, which recruit inflammatory cells such as neutrophils, macrophages (including Kupffer cells), and T lymphocytes. The infiltration of these inflammatory cells forms inflammatory foci around degenerating or necrotic hepatocytes. One of the manifestations of this damage is ballooning degeneration, characterized by enlarged hepatocytes and clear cytoplasm due to organelle loss, as shown in image 3. If the damage continues, some hepatocytes may undergo necrosis, characterized by the loss of cell membrane integrity and the release of cellular contents, worsening the inflammatory response and potentially leading to fibrosis, an early stage towards cirrhosis [20,21]. In the treatment group 1, the histopathological picture showed significant improvement compared to the sick control group in figure 4. Steatosis appears less, with a dominance of hepatocytes that look normal without excessive lipid accumulation. Inflammatory foci are also less frequently found, reflecting a reduction in the inflammatory response in the liver. Additionally, hepatocyte degeneration, such as ballooning degeneration, is almost undetectable, indicating that guava leaf extract provides a therapeutic effect that repairs cellular damage caused by a high-fat diet. These changes indicate that guava leaf extract can improve liver conditions by reducing steatosis, inflammation, and cell degeneration in Wistar rats.

Guava leaf extract (*Psidium guajava*), which is rich in phenolic and flavonoid compounds such as quercetin, kaempferol, myricetin, and gallic acid, provides strong antioxidant and anti-inflammatory effects, protecting the livers of Wistar rats from steatosis induced by a high-fat diet. These compounds neutralize reactive oxygen species (ROS) and reduce oxidative stress, which contributes to the protection of hepatocytes from oxidative damage.

This extract also enhances the activity of endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, which help reduce the accumulation of ROS in liver cells, maintain cellular integrity, and prevent membrane and intracellular organelle damage that triggers inflammation and hepatocyte degeneration [22]. The mechanism of cholesterol and fat reduction by guava leaf extract involves the inhibition of the HMG-CoA reductase enzyme, a key enzyme in cholesterol biosynthesis. Compounds such as quercetin and gallic acid interact with this pathway, reducing cholesterol synthesis in the liver and increasing LDL receptor expression, which enhances cholesterol uptake from the blood.

The decrease in cholesterol and triglyceride levels in the blood reduces lipid accumulation in hepatocytes. The combination of these mechanisms results in a liver histopathology profile showing less steatosis and no signs of inflammation or degeneration, significantly different from the sick control group [23].Flavonoids such as quercetin and kaempferol in guava leaf extract play a role in modulating the inflammatory response by inhibiting pro-inflammatory signaling pathways such as NF-κB and MAPK, which reduces the production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β. This prevents the infiltration of inflammatory cells into liver tissue and reduces the formation of inflammatory foci. This extract also functions as an immunomodulator that balances the body's immune response, preventing chronic inflammation that often occurs in steatohepatitis. In the prevention of steatosis, gallic acid and myricetin enhance fatty acid oxidation through the activation of the PPAR-α pathway, reduce triglyceride synthesis, and decrease the activity of lipogenic enzymes such as fatty acid synthase (FAS) [23,24].

The treatment group 2 showed a striking histopathological picture compared to the sick control group, although there was lipid accumulation in the hepatocyte cells, the dominant steatosis was of the microvesicular type, with small lipid vacuoles in the hepatocyte cytoplasm without significantly affecting the cell nucleus (Figure 4.5). The mechanism of action of guava leaf extract in the treatment group 2 focuses on preventing lipid accumulation and cell damage from the outset. This extract reduces oxidative stress through the antioxidant activity of phenols and flavonoids, which prevents the excessive formation of ROS before cell damage occurs. Additionally, the extract inhibits lipogenic enzymes and enhances PPAR-α activity, accelerating fatty acid oxidation and preventing triglyceride accumulation in the liver. By suppressing inflammatory pathways such as NF-κB, this extract prevents inflammation before the infiltration of inflammatory cells occurs. This mechanism is different from the curative mechanism that repairs damage after it occurs (Li et al., 2021).

This study shows that a high-fat diet causes liver steatosis in the sick control group, while treatment with guava leaf extract significantly reduces liver steatosis and inflammation. Guava leaf extract, rich in flavonoids and phenols, provides a protective effect against liver damage caused by a high-fat diet. These findings support guava leaf extract as a potential agent for preventing and improving non-alcoholic fatty liver disease (NAFLD), which is impo-

rtant in the development of natural therapies for this condition.

Conclusion

This study aims to determine the effect of administering Psidium guajava leaf extract on the histopathological picture of the liver in a hypercholest- erolemia model of *Rattus norvegicus* rats. Based on the results obtained, it can be concluded that the administr- ation of *Psidium guajava* leaf extract has a significant effect on the histopathological picture of the liver in Wistar rats induced with hypercholesterolemia. Additi- onally, hypercholesterolemia induction has been proven to affect the histopathological picture of the liver in Wistar rats. However, the guava leaf extract did not show a significant difference in effect between administration before and after hypercholesterolemia induction.

Supplementary Material

None

Author Contributions

MAL : Conceptualization, Methodology, Writing– Original Draft. **HS** : Conceptualization, Methodology, Data Curation, Formal Analysis, Visualization. **W** : Conceptualization, Methodology, Supervision, Writing – Review & Editing. **FWP** : Conceptualization, Methodology, Supervision, Writing – Review & Editing. All authors should have approved the final version of the manuscript and agree to be accountable for their contributions.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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