

Evaluation the Therapeutic Effects of Iraqi Hibiscus tiliaceus Ethanolic Leaf Extract in Cerulein-Induced Acute Pancreatitis: An Experimental Rat Model Study

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ABSTRACT

Background: Acute pancreatitis is caused by enzyme self-digestion, oxidative stress, and apoptotic cell death. Cytokines produced exuberantly and excessive reactive oxygen species trigger cellular injury and systemic inflammation. The antioxidant and anti-inflammatory phytochemicals present in *Hibiscus tiliaceus* L. make it useful for protecting and treating the pancreas. **Method:** *H. tiliaceus* leaves were defatted with hexane, and the leaves were extracted with ethanol using a Soxhlet apparatus. Forty female Wistar rats were divided into five groups: controls, cerulein-induced pancreatitis, and three other groups given 125, 250, or 500mg/kg body weight of the *H. tiliaceus* leaf extracts for seven days. ELISA measured serum enzymes, MDA, and GSH, while TNF- α , IL-6, and caspase-3 were measured by RT-PCR and Western blot, respectively. **Results:** Phytochemical screening showed that there are alkaloids, flavonoids, polyphenols, and sterols in the plant. HPLC profiling confirmed the presence of bioactive compounds in the plant, including quercetin, rutin, kaempferol, gallic acid, and scopatine. Cerulein induction markedly elevated serum enzyme levels, oxidative stress markers, and pro-inflammatory cytokines, while *H. tiliaceus* extract treated rats significantly and dose-dependently reduced these parameters. The extract at 500 mg/kg restored near-normal levels of amylase, lipase, MDA, GSH, TNF- α , IL-6, and caspase-3. **Conclusion:** *H. tiliaceus* leaves' ethanolic extract showed a substantial therapeutic effect against cerulein-induced acute pancreatitis. Iraq *H. tiliaceus* ethanolic leaves' extract, hence providing potential evidence that could be a natural remedy or treatment approach against acute pancreatitis.

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1. Introduction

Acute Pancreatitis (AP), is an acute inflammatory condition involving the pancreas, marked by the early intracellular activation of digestive enzymes in acinar cells, leading to autodigestion, consequently resulting in an inflammatory response, both localized and systemic (Mederos et al., 2021). It is a serious condition, as the estimated prevalence incidence is as high as 13-45 cases per 100,000 population, and is among the leading causes leading to hospital admission due to gastrointestinal distress (Mihoc et al., 2024). However, while mild and self-limiting, 20% cases progress to severe conditions, predisposed to systemic inflammatory response syndrome, organ failure, and mortality rates estimated at 30% (Ozbeyli et al., 2020).

The mechanism underlying AP is complex, and the pathways involved are multifactorial. The primary injury, whether biliary obstruction or alcohol, results in the trypsinogen end-point activated intra-acinar cell injury due to trypsin production, which further activates other zymogens (Kim, 2008). The pathways culminate in cellular injury, damage-associated molecular patterns, and the resultant promotion of an upheaval of an exaggerated, predominant, and prominent inflammatory response, expressed as the release of excessive amounts of tumor necrosis factor- α and other pro-inflammatory cytokines, including IL-6, due to the stimulation by transcription nuclear factor κ -B (Mederos et al., 2021). Concurrently, oxidative stress plays a pivotal role: the generation of reactive oxygen species (ROS) overwhelms cellular antioxidant defences, leading to lipid peroxidation, protein denaturation, and DNA damage, thereby amplifying cellular injury and inflammation. These interconnected pathways of inflammation and oxidative stress ultimately lead to acinar cell death through apoptosis pathways, with caspase activation, modulation of Bcl-2 family proteins, and cytochrome c release acting as a key executioner protease in the apoptotic pathway (Mihoc et al., 2024).

The prevalent model used to study this signalling is cerulein-induced pancreatitis, as repeated cerulein stimulation recapitulates the biochemical and histopathological features seen in human acute pancreatitis. Excessive stimulation of the acinar cells by cerulein, acting as an analog of cholecystokinin, triggers an intracellular calcium surge, initiating signaling pathways including, but not limited to, the NF- κ B, MAPK, and JAK/STAT pathways, leading to an excessive production of cytokines, oxidative stress, and apoptosis due to cellular injury (Kim, 2008; Ozbeyli et al., 2020; Watanabe et al., 1984).

Although substantial progress has been made in the management of ancillary care, fluid replacement, pain control, nutritional support, and management of complications, to date, a targeted pharmacologic approach toward the molecular pathophysiological mechanisms of AP has not been established (Abdul-Awal et al., 2016; Rosa et al., 2006). The pressing therapeutic void has, hence, driven exploratory research initiatives toward innovative agents capable of regulating the principal pathophysiological processes: oxidative stress, inflammation, and apoptosis.

H. tiliaceus L. (Malvaceae), commonly known as the sea hibiscus, is a tropical plant with a well-established history in traditional medicine for treating fever (Maleki et al., 2019; Raina et al., 2014), inflammation, and digestive disorders (Lim, 2014; Vinh et al., 2021). Modern phytochemical investigations have revealed that *H. tiliaceus* leaves are rich in flavonoids (e.g., quercetin, rutin, kaempferol), phenolic acids (e.g., gallic, vanillic, syringic acid), and coumarin derivatives (Abdul-Awal et al., 2016; Rosa et al., 2006). Critically, these compounds are individually known to modulate pathways relevant to AP: flavonoids are potent activators of the Nrf2 antioxidant pathway and inhibitors of the NF- κ B inflammatory signaling cascade, while phenolic acids and coumarins exhibit strong free-radical scavenging and anti-apoptotic activities (Borive Amani et al., 2025; Jeffery & Richardson, 2021). Aqueous and ethanolic extracts of *H. tiliaceus* exhibit considerable antioxidant capacity, evidenced by low IC₅₀ values in DPPH and ABTS experiments, as well as elevated total phenolic and flavonoid concentrations. Both in vitro and in vivo studies have substantiated its anti-inflammatory activity by inhibiting proinflammatory mediators and downregulating NF- κ B signaling (Abdul-Azeez & Mutlag, 2023; Samsudin et al., 2019). Pharmacological evaluations have reported that *H. tiliaceus* exhibits a broad range of biological activities, including anti-inflammatory, antibacterial, antioxidant, and anthelmintic properties (Abdul-Awal et al., 2016). These findings support its potential application as a therapeutic agent (Rawool & Parulekar, 2019).

Despite its traditional use and rich phytochemical profile, the potential therapeutic efficacy of the Iraqi variant of *H. tiliaceus* leaf extract in acute pancreatitis remains underexplored. Therefore, this study aims to systematically evaluate the therapeutic effects of an ethanolic extract of Iraqi *H. tiliaceus* leaves in a well-established rat model of cerulein-induced acute pancreatitis. We hypothesise that the extract will ameliorate AP severity by mitigating oxidative stress, suppressing inflammation, and inhibiting apoptosis through the concerted action of its constituent bioactive compounds.

2. Methods and Materials

2.1 Chemicals and Reagents

The reference standard, cerulein (CAS No. 17650-98-5), was purchased from Sigma-Aldrich (Germany). The anaesthetics xylazine hydrochloride (XYL-M2, VMD Livestock Pharma, Belgium) and ketamine (10%, produced by Alfasan Nederland BV, Netherlands) were used. The biochemical kits for the measurement of amylase, lipase, and malondialdehyde (MDA; E-EL-

Reagents and materials used for molecular studies included TransZol reagent, a reagent kit specifically designed for RNA, a TransScript First-Strand cDNA Synthesis SuperMix kit by TransGen Biotech (China), and primers designed by Macrogen Inc. (Korea) targeting genes encoding TNF- α and IL-6. Primary antibodies designed by Elabscience (USA).

2.2 Plant Material and Authentication

Fresh leaves of *H. tiliaceus* L. were harvested on the 23rd of November, 2024, from Master of Regents City, Kirkuk-Baghdad Road, Iraq. Prof. Dr Ibrahim Al-Jubori identified the plant species at the Al-Razi Centre for Alternative Medicine, and a voucher specimen was given an accession number (205), dated 23rd November 2024.

Based on research by Iraq-based researchers, as well as environmental laws and guidelines, the plant material was collected on agricultural land, and this involved plants that are not endangered or protected. The norm is that if the collection involves any protected or endangered plants, a special permit is required, but this was not the case, as all work was carried out in accordance with Iraq Quarantine Law No. 76/2012.

2.3 Extraction of Plant Material

Leaves were air-dried at ambient temperature and subsequently ground into a fine powder. Sixty grams of the powder substance were defatted with hexane for 72 hours to eliminate lipophilic components. The residue was extracted using a Soxhlet device with 80% ethanol for 20 hours. The extract was concentrated at 40 °C under decreased pressure using a rotary evaporator and then dried to yield a stable solid residue, which was stored in airtight containers at 4 °C until required (Mahire & Patel, 2020).

2.4 Phytochemical Screening

A qualitative phytochemical screening was performed on the ethanolic extract, following standard procedures to identify major active phytochemicals, including flavonoids, coumarins, tannins, alkaloids, and terpenoids. For alkaloids, a 0.5 mL aliquot of each extract was loaded into a test tube, and a few drops of Dragendorff's reagent were added, then the mixture was shaken well. The appearance of an orange precipitate indicated a positive result for alkaloids.

The Ferric chloride test can be used to evaluate phenolic compounds, in which 0.5 mL aliquots of each extract are mixed with 2 mL of 5% ferric chloride solution. A 0.5 mL aliquot of each extract was loaded into a test tube, a few drops of Dragendorff's reagent solution were added, and the mixture was well shaken. The appearance of an orange precipitate indicated a positive result. To assess flavonoids, 0.5 mL of extract was introduced into a test tube, followed by the addition of few drops of weak NaOH solution.

The emergence of a strong yellow colour in the test tube, followed by the addition of a few drops of weak HCl, which produces a colourless solution, is indicative of the presence of flavonoids. For sterol detection, use the Liebermann-Burchard test: combine 2 ml of acetic anhydride, 0.5 ml of plant extract, and 2 ml of H₂SO₄. The color transition from violet to blue or green in samples signifies the presence of steroids (Spangenberg et al., 2011; Sticher, 2008; Waksmundzka-Hajnos et al., 2008).

2.5 HPLC Conditions of *H. Tiliaceus* Leave Ethanolic Extract

- Mobile phase: Isocratic: Methanol: D.W =80:20
- Column: SYKAMN LC: C18 -ODS (25cm x 4.6 mm, 5 µm particle size).
- Sample: ethanol (E1).
- Standard: Gallic acid, Kampefrol, Quercetin, Rutin, Syringic acid, vanillic acid, scopaltine and Coumarin by using four concentrations (5,10,15,20 µg/ml).
- Flow rate: 1 ml/min.
- Injection volume: 100 µL.
- Injection concentration: 1 mg /ml.
- Detection: UV Detector at λ 280 nm.

2.5.1 Isolation of Phenolic Compounds from *H. tiliaceus* L. Ethanolic Extract by HPLC

The phenolic components present in the ethanolic extract of **H. tiliaceus** were identified by reversed-phase HPLC on a system (SYKAM, Germany) equipped with a UV detector. The C18-OSD column, which measured 25 cm by 4.6 mm, was held at 30°C, and a mobile phase composed of eluent A, methanol, and eluent B, 1% solution of formic acid, v/v, was applied to the column at a gradient program: 0-6 minutes, 40% B; 6-15 minutes, 50% B, at a constant flow rate of 1.0 mL/min.

The sample and standard solutions (100 µL each) were automatically injected, and detection was performed at 280 nm. The prominent components identified were gallic acid, kaempferol, quercetin, rutin, syringic acid, vanillic acid, scopoletin, and coumarin. The purified fractions were achieved by recrystallization with hot methanol after gradient elution, which took around 13 minutes (Cavalcante et al., 2022).

2.6 Preparation of Cerulein Solution for the Induction of Acute Pancreatitis

Lyophilised cerulein acetate was dissolved in sterile distilled water to prepare a 1 mg/mL stock solution, consistent with its aqueous solubility. Aliquots were freshly prepared before each administration to ensure stability. The cerulein solution was prepared at a concentration of 100 µg/mL. Rats weighing 200–250 g received 50 µg/kg of cerulein intraperitoneally (IP), administered twice at 1-hour intervals. The corresponding injection volumes ranged from 0.1–0.125 mL per dose, depending on the individual rat's body weight, to induce acute pancreatitis (Lerch & Gorelick, 2013).

2.7 Experimental Animals and Study Design

Forty healthy female Wistar albino rats (8 weeks old, 200–250 g) (Miyasaka et al., 1992) were obtained from the Animal House Facility, College of Pharmacy, University of Baghdad. Animals were maintained under controlled environmental conditions (23 ± 2 °C, 50 ± 5% humidity, 12-h light/dark cycle) with free access to food and water. After a two-week acclimatisation period, rats were randomly allocated. The University of Baghdad's College of Pharmacy Research Ethics Committee gave the project the go-ahead with Approval No. Rec022476A on October 20, 2024. The Office International' animal ethical guidelines say that the study must follow these rules. The methods used on the animals fully comply with all local and international laws governing the ethical treatment and use of laboratory animals. The authors

followed the ARRIVE 2.0 guidelines criteria (Percie du Sert et al., 2020).

Five experimental groups (n = 8 per group):

- Group I (Negative control): Rats received an oral daily dose of 1 ml/kg of distilled water for 7 days.
- Group II (Induction group): Rats received an oral daily dose of 1 ml/kg of distilled water for 6 days, and on day 7, they were injected intraperitoneally with cerulein at 50 µg/kg twice at a 1-hour interval. Rats were sacrificed 24 hours after the last injection.
- Group III (Treatment 1): Rats injected with cerulein at 50 µg/kg twice at a 1-hour interval, then treated orally with 125 mg/kg of *H. tiliaceus* leaf extract 24 hours after the last cerulein dose. Treatment was administered once daily for 7 days.
- Group IV (Treatment 2): Rats injected with cerulein at 50 µg/kg twice at a 1-hour interval, then treated orally with 250 mg/kg of *H. tiliaceus* leaf extract 24 hours after the last cerulein dose. Treatment was administered once daily for 7 days.
- Group V (Treatment 3): Rats injected with cerulein at 50 µg/kg twice at a 1-hour interval, then treated orally with 500 mg/kg of *H. tiliaceus* leaf extract 24 hours after the last cerulein dose. Treatment was administered once daily for 7 days (Lugea et al., 2006).

All treatments were administered by oral gavage. Animals were monitored daily for clinical signs and potential toxicity. No mortality or adverse effects were observed throughout the experimental period.

2.8 Sample Collection and Euthanasia

Twenty-four hours following the last treatment, the rats were anaesthetized with an intraperitoneal injection of a ketamine and xylazine mixture (50mg/kg + 10mg/kg). Blood was harvested by cardiac puncture and allowed to clot before centrifugation at 3,000 rpm for 10 minutes. Serum was stored at -80°C. The animals were exsanguinated, and their humane sacrifice involved cervical dislocation, following AVMA procedures. The pancreatic tissues were immediately harvested for biochemical, molecular, and histopathological studies.

2.9 Biochemical and Molecular Assessments

2.9.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Serum levels of amylase, lipase, MDA, and GSH were quantified using rat-specific ELISA kits according to manufacturer instructions. Optical density was measured at 450 nm using a calibrated microplate reader (Shimadzu Model AA200) using specific assay kits from ELK Biotechnology (USA): Mouse AMY1 (Cat. ELK9837), Mouse LIPA (Cat. ELK7101), MDA (Cat. E-EL-0060), and GSH (Cat. E-EL-0026).

2.9.2 Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using GENEzol reagent using the SV Total RNA Extraction Kit (Promega, USA), and cDNA was synthesized via EasyScript® One-Step SuperMix. The relative expression of TNF-α and IL-6 mRNA was quantified using SYBR Green chemistry on a qPCR system. GAPDH served as the internal control (Livak & Schmittgen, 2001). Primer sequences were:

TNF-α: F 5'-TTAAGTGTGTGTCCTGAGGT-3', R 5'-CCAAGAGCAGCCCAACAAATC-3'

IL-6: F 5'-GCCACTGCCTTCCCTACTTC-3', R 5'-TCTGACAGTGCATCATCGCT-3'

GAPDH: F 5'-TGTGAACGGATTGGCCGTA-3', R 5'-TCCC GTTGATGACCAGCTTC-3'

Amplification cycles were: 94 °C for 10 s, followed by 40 cycles (61–64 °C for 15 s, 74 °C for 20 s). Relative expression was determined using the $2^{-\Delta\Delta C_t}$ method.

2.9.3 Western Blot Examination

Western blot analysis was performed to assess the levels of optimal protein markers in pancreatic tissues. The tissues were lysed with a protease/phosphatase inhibitor-containing RIPA cell lysis buffer, washed with cold phosphate-buffered saline, and finally processed for total protein quantification using the BCA Protein Assay Kit (Elabscience, USA). The absorbance was measured at 562 nm by a µ2 Microdigital Microspectrophotometer (Korea).

Equal amounts of protein were denatured by heating at 95°C for five minutes, separated by 5% SDS-PAGE gels, and electroblotted to polyvinylidene difluoride (PVDF) membranes utilizing the Bio-Rad Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, USA). The membranes were blocked, washed, and incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies. Bands were developed utilizing the Bio-Rad ChemiDoc Imaging System with an electrochemiluminescent substrate, and the optical densities were measured by ImageJ software, with parameters adjusted to internal controls (Atshan & Zalzal, 2024).

2.10 Histopathological Examination

The pancreatic tissues were fixed in 10% neutral-buffered formalin, dehydrated with graded alcohols, cleared with xylene, and embedded in paraffin wax. Sections, 5 μ m thick, were stained with H&E stain. The microscopic evaluation was conducted at 10x and 40x magnification by a histopathologist unaware of the sample's origin to assess acinar structure, oedema, infiltration, necrosis, and fibrosis.

2.11 Statistical Analysis

Data are expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using GraphPad Prism v10. Differences among groups were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Values of $p < 0.0001$ were considered statistically significant.

3. Results

3.1 Identification of Different Bioactive Constituents by High- Performance Liquid Chromatography (HPLC)

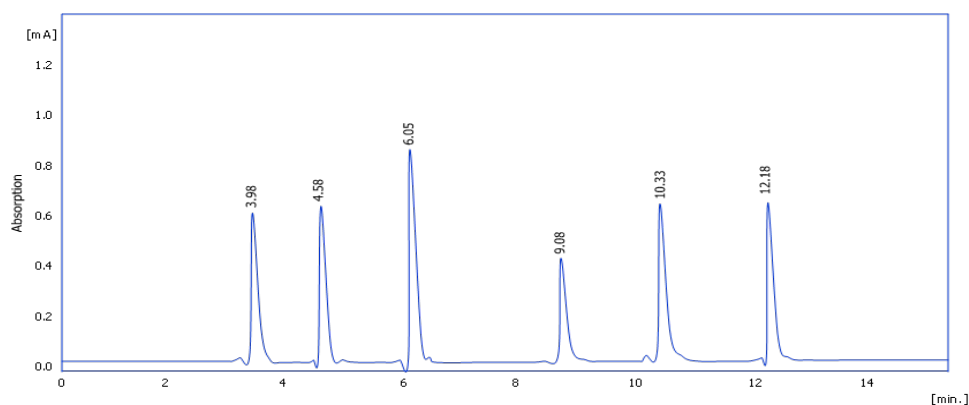
3.1.1 Qualitative Analysis by HPLC for Ethanol Extract (*H. Tiliaceus* L.)

Analytical HPLC analysis of *H. tiliaceus* ethanolic extract confirmed the presence of several bioactive phenolic and flavonoid compounds by matching retention times with reference standards (Table 1)

Prominent peaks were observed for gallic acid, kaempferol, quercetin, rutin, syringic acid, vanillic acid, scopoletin, and coumarin, indicating a rich phytochemical profile contributing to the extract's biological activity. As illustrated in Table 1 and Figure 1.

Table 1: The Concentration of the Proposed Compounds from the Extract of the Plant. With Retention Time and Peak Area of these Compounds

No.	Compounds	Conc (ppm)	Ret Time (Min)	Peak Area
1	Gallic acid	311.90	6.00	2874.47
2	Kaempferol	135.90	3.89	1257.09
3	Quercetin	262.80	12.33	3698.77
4	Rutin	290.70	10.36	2558.70
5	Syringic acid	187.60	4.58	1958.77
6	vanillic acid	95.5	6.00	1322.59
7	SCOPALTINE	190.20	5.90	3214.49
8	Coumarine	112.50	2058.99	2085.99



Result chromatography Table (Uncal - F:\ sample)						
No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]
1	3.98	4521.88	592.65	17.00	17.00	0.05
2	4.58	4211.41	591.14	17.00	17.00	0.05
3	6.05	6589.08	795.44	22.00	22.00	0.08
4	9.08	2145.87	399.85	10.00	10.00	0.03
5	10.33	4012.66	590.11	17.00	17.00	0.05
6	12.18	4157.94	592.65	17.00	17.00	0.05
Total		25638.19	3561.24	100.00	100.00	

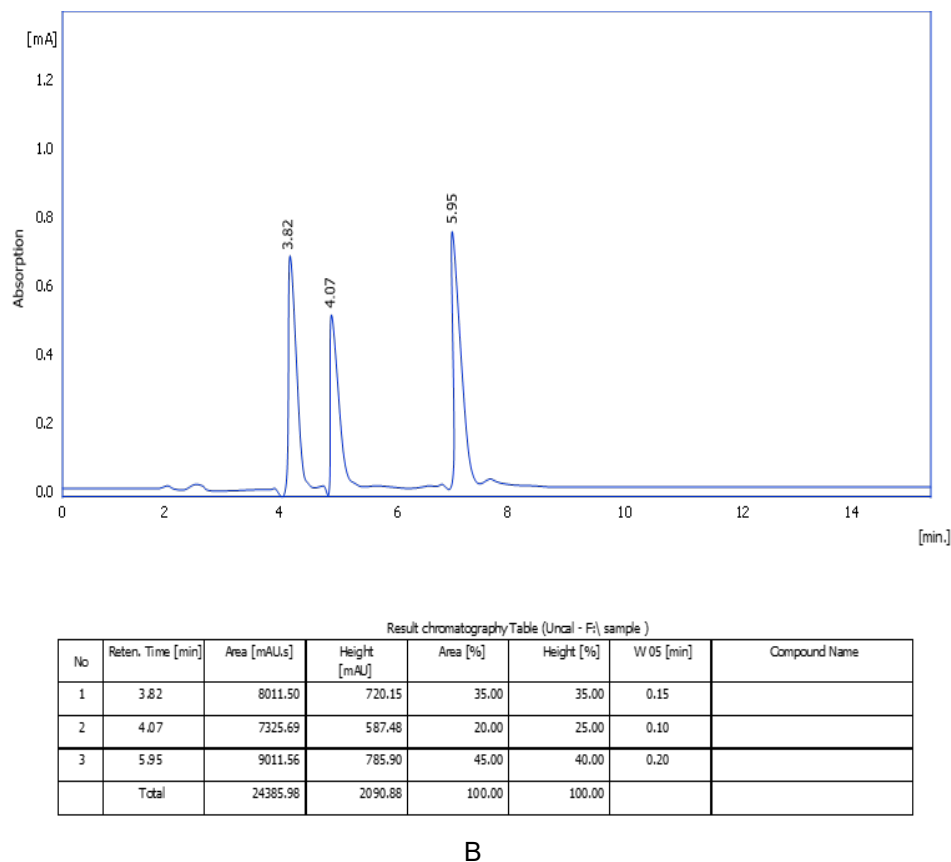
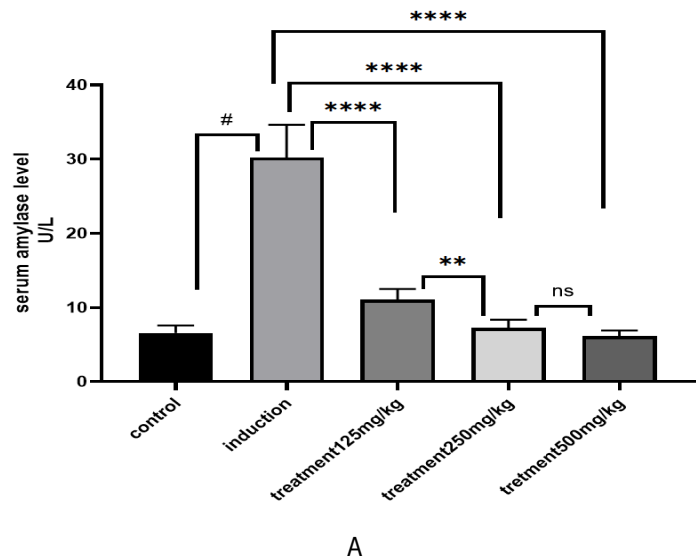


Figure 1: A (HPLC Chromotograhhy of Sample of Hibiscus Tiliaceus L. Ethanolic Extract). B (HPLC Chromotograhhy of Sample of Hibiscus Tiliaceus L. ethanolic extract).

3.2 Effects of H. Tiliaceus Extract on Blood Serum Amylase and Lipase Enzyme Levels

Figure 2 showed that amylase and lipase levels were significantly elevated in the induction group relative to the negative control group ($p<0.0001$). This marked increase in amylase and lipase levels was due to pancreatic injury and inflammation, and to the leakage of these enzymes into the bloodstream.

Treatment with 125mg/kg, 250mg/kg, and 500mg/kg H. tiliaceus leaf extract significantly reduced amylase and lipase levels when compared to the induction group ($p<0,0001$). The attenuation was more pronounced at 500 mg/kg. No statistically significant difference was detected between 250mg/kg extract and 500mg/kg extract and the negative control groups suggesting that the therapeutic threshold may be achieved at 250 mg/kg.



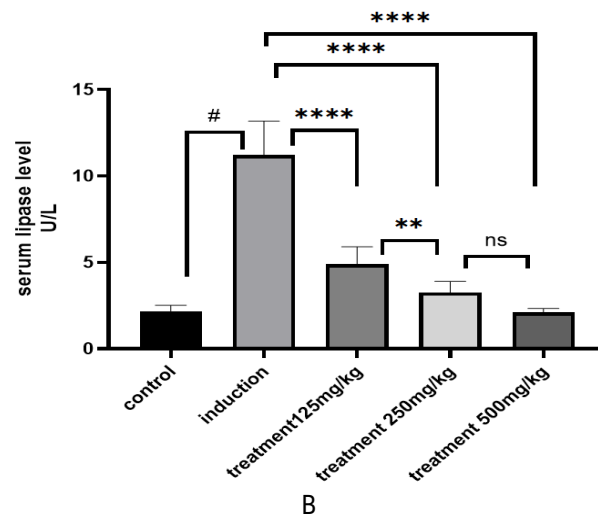


Figure 2: Effect of Hibiscus Tiliaceus Leaf Extract on Serum Amylase (A) and Lipase (B) Levels in Cerulein-induced Acute Pancreatitis in Rats.

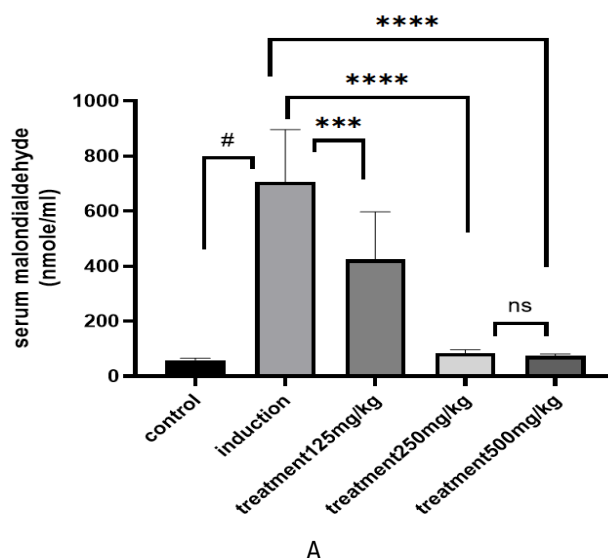
Data are presented as mean $\pm$ SD (n = 8 per group). Administration of *H. tiliaceus* extract significantly reduced serum amylase and lipase activities compared with the cerulein-induced group, indicating attenuation of pancreatic injury. # $p < 0.0001$ vs. negative control or induction group; **** $p < 0.0001$ the 125mg/kg and 250mg/kg and 500mg/kg and induction group. ** $p < 0.01$ significant difference 125mg/kg vs 250mg/kg. ns = non-significant. No significant differences were observed between the 250 mg/kg and 500 mg/kg treatment groups and the negative control, suggesting near-complete biochemical recovery at higher doses.

3.3 Effects of Three Different Doses of *H. Tiliaceus* Leave Extract on Selected Oxidative Stress (OS) Parameters

The level of MDA and GSH in serum in the induction group II /cerulein induced group rats was significantly increased ($p < 0.0001$) compared to such serum level in the negative control group. Figure 3.

Moreover, MDA serum levels in group treatment 2 (250mg/kg) and group treatment 3 (500mg/kg) were significantly reduced ($p < 0.0001$) compared to the induction group II/cerulein-induced group. Furthermore, no statistically significant difference was detected between the 250mg/kg extract and the 500mg/kg extract groups and the negative control group in serum MDA levels. Treatment with *H. tiliaceus* extract led to dose-dependent reduction in MDA level.

The group treated with 1 (125mg/kg) showed a significant decrease in MDA and GSH levels compared to the induction group II ($P < 0.001$). In contrast, group treatment 2 (250mg/kg) and treatment 3 (500mg/kg) exhibited a more pronounced reduction ($p < 0.0001$). Also, there was a significant difference between the treatment 2 group (250mg/kg plant extract) and the treatment 3 group (500mg/kg) in GSH levels ($p < 0.001$).



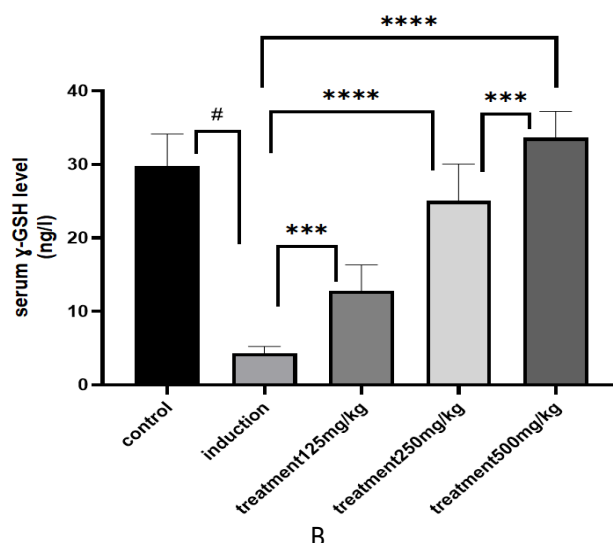
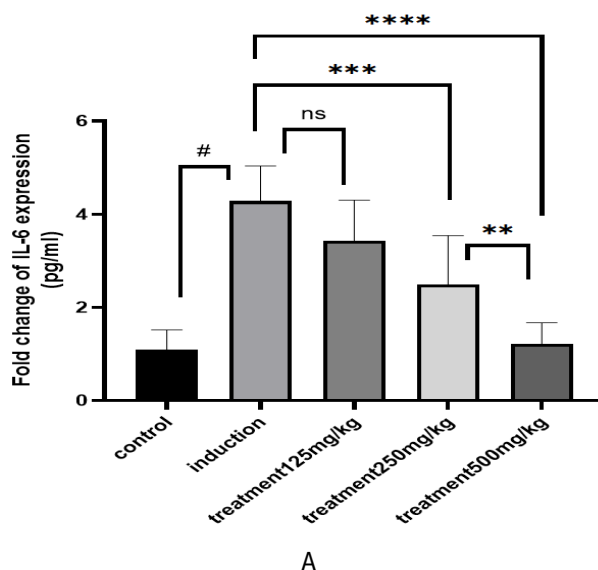


Figure 3: Effects of Hibiscus Tiliaceus Ethanolic Leaf Extract on Serum Oxidative Stress Biomarkers in Cerulein-Induced Acute Pancreatitis in Rats. (A) Serum malondialdehyde (MDA) concentrations. (B) Serum reduced glutathione (GSH) concentrations. Data are expressed as mean $\pm$ SD (n = 8 per group). Cerulein administration resulted in a marked increase in lipid peroxidation and depletion of endogenous antioxidant reserves compared with the negative control (# p < 0.0001), confirming the induction of oxidative injury. Oral treatment with H. tiliaceus extract produced a dose-dependent attenuation of oxidative stress, evidenced by significant reductions in MDA and restoration of GSH levels at 250 mg/kg and 500 mg/kg (****p < 0.0001 vs. induction group). ns indicates non-significant differences; *** p < 0.001; ****p < 0.0001. No significant variation was observed between the 250 mg/kg and 500 mg/kg treatment groups, or between the 500 mg/kg group and the negative control, in MDA levels, suggesting that H. tiliaceus at higher doses effectively restored the redox equilibrium disrupted by cerulein exposure.

3.4 Effects of Three Different Doses of H. Tiliaceus Leave Extract on Inflammatory Markers.

IL-6 and TNF- α levels were markedly elevated in the induction II group relative to the negative control group (p<0.0001), reflecting the pro-inflammatory state induced by acute pancreatitis. Figure 4.

Treatment with (250mg/kg) of H. tiliaceus extract resulted in a significant reduction in IL-6 and TNF- α levels compared to the induction group (p < 0.001), indicating the extract potential anti-inflammatory effects. The (500mg/kg) extract group demonstrated an even greater reduction in IL-6 levels, with a highly significant difference compared to the induction group II(p<0.0001). No statistically significant difference between treatment 1(125mg/kg) and the induction group I, suggesting that the higher dose of the extract offers anti-inflammatory benefits more than the other doses.



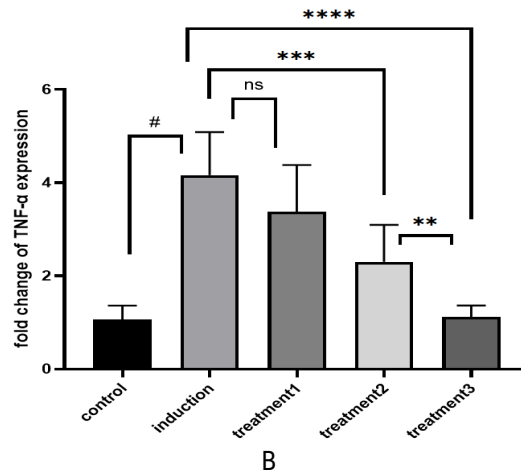


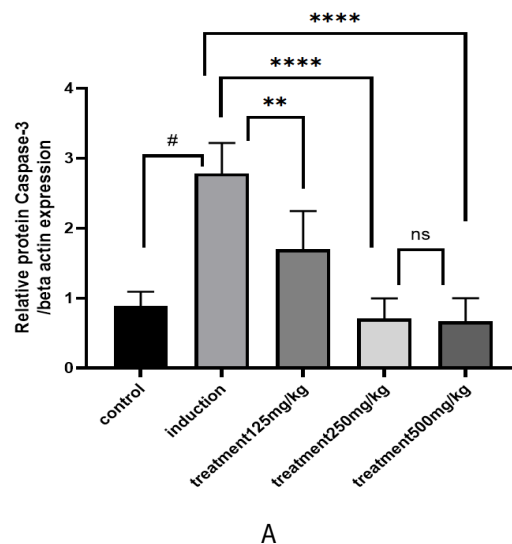
Figure 4: Effects of Hibiscus Tiliaceus Ethanolic Leaf Extract on Pro-Inflammatory Cytokine Expression in Cerulein-Induced Acute Pancreatitis in Rats.

(A) Fold change in interleukin-6 (IL-6) expression. (B) Fold change in tumor necrosis factor-alpha (TNF- α) expression. Data are expressed as mean $\pm$ SD (n = 8 per group). Cerulein administration caused a pronounced upregulation of IL-6 and TNF- α gene expression compared with the negative control group (# $p < 0.0001$), confirming induction of a strong inflammatory response. Oral treatment with *H. tiliaceus* extracts significantly and dose-dependently suppressed cytokine overexpression, with marked reductions observed at 250 and 500 mg/kg (** $p < 0.001$; **** $p < 0.0001$ vs. induction group). **significant difference between group (250mg/kg) and group(500mg/kg) ($p < 0.01$). ns indicates non-significant difference. At higher doses, *H. tiliaceus* restored cytokine profiles close to control levels, demonstrating its potent anti-inflammatory and immunomodulatory activity in mitigating cerulein-induced pancreatic inflammation.

3.5 Effects of Three Different Doses of *H. Tiliaceus* Leave Extract on the Apoptotic Markers Levels in Pancreatic Tissue

Figure 5 indicated the intensity of protein: Caspase-3 in rats pancreatic tissue homogenate by the use of PVDE membrane in Western Blot (W.B) Analysis. Caspase-3 levels were markedly elevated in the induction II group relative to the negative control group ($p < 0.0001$). Treatment with (125mg/kg) of *H. tiliaceus* extract resulted in a significant reduction in Caspase-3 levels compared to the induction group ($p < 0.01$).

Treatment with (250mg/kg) of *H. tiliaceus* extract resulted in a significant reduction in Caspase-3 levels compared to the induction group ($p < 0.0001$). The (500mg/kg) extract group demonstrated an even greater reduction in Caspase-3 levels, with a highly significant difference compared to the induction group II ($p < 0.0001$). No statistically significant difference between Treatment 2 (250mg/kg) and Treatment 3 (500mg/kg) and the negative control group I ($p < 0.0001$). Furthermore, the intensities of both proteins, Caspase-3 and Beta-actin, in pancreatic tissue, as determined by Western Blot (WB) analysis, are shown in Figure 5.



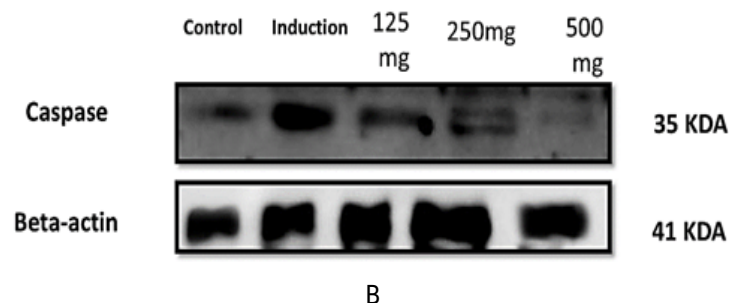
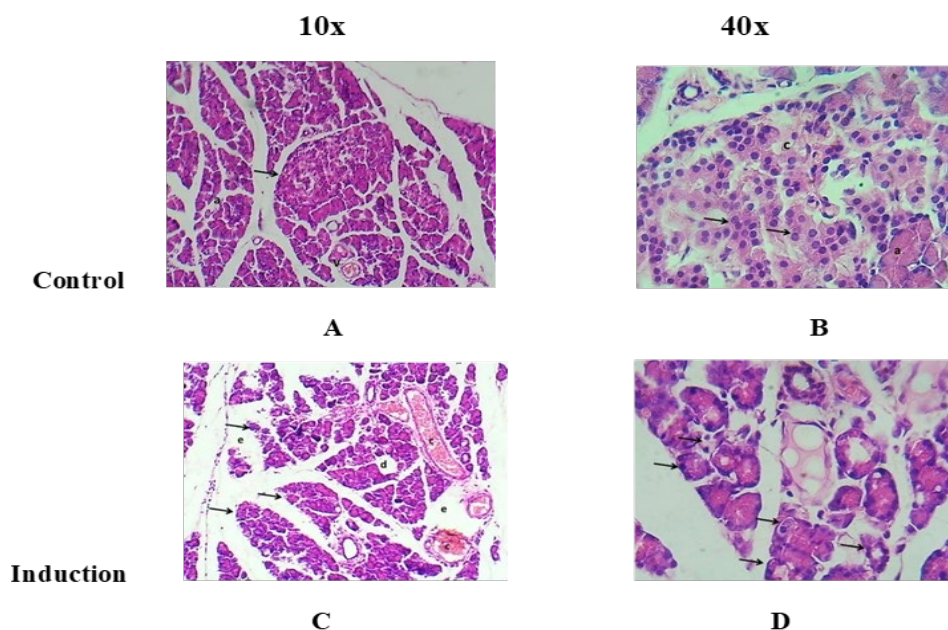


Figure 5: A: Effects of three different doses of Hibiscus tiliaceus leave extract on Caspase-3 tissue level in rat. # Represents a significant difference between the control group and the induction group ($p < 0.0001$), ****Significant difference between the induction group and treatment 3 (500mg/kg) ($p < 0.0001$) and treatment 2 (250mg/kg). ** Indicates a significant difference between treatment 1 (125mg/kg) and the induction group ($p < 0.01$). ns: represent non- significant difference by comparing between treatment3 (500mg/kg) and treatment 2(250mg/kg) and between negative control group. B: Western blot analysis of Caspase-3 expression in pancreatic tissue of cerulein-induced rats treated with Hibiscus tiliaceus extract.

3.6 Effects of Three Different Doses of H. Tiliaceus Leaf Extract on Pancreatic Tissue Histopathological Changes and Scores

The histopathological figures of the rats' pancreatic tissue in group I, the negative control, showed normal appearance of endocrine islet cells, normal acini and lobules with typical acinar lining cells, and normal interlobular blood vessels (Figure 6A, B). Additionally, the histopathological figures of pancreatic tissue in group II (the induction group) showed severe pancreatitis, characterised by marked thickening of the pancreatic capsule, infiltration by mononuclear leukocytes, interstitial oedema, and severe vascular congestion (Figure 6C). The acini exhibited severe degeneration with necrosis and atrophy, and the pancreatic islets showed tissue depletion (Figure 6D). All histopathological images of pancreatic tissue from treatment group 1 (125 mg/kg plant extract) showed normal islets, normal acinar lobules, and typical acinar lining cells. The interlobular connective tissue of the pancreas showed mild pancreatic fibrosis with periductal fibrosis (Figure 6 E), and other images revealed thickening of the interlobular connective tissue associated with leukocyte infiltration (Figure 6 F). All histopathological images of pancreatic tissue from treatment group 2 (250 mg/kg plant extract) showed normal islets and mild atrophy of some acini and acinar lining cells. The interlobular connective tissue exhibited normal thickness (Figures 6 G, H), similar to that of the control group, with normal lobules, acini, interlobular tissue, and islets. Pancreatic tissue from treatment group 3 (500 mg/kg plant extract) was similar to that of the control group, showing a normal appearance of endocrine islet cells, normal acini and lobules with typical acinar lining cells, and normal interlobular blood vessels (Figures 6 I, J).



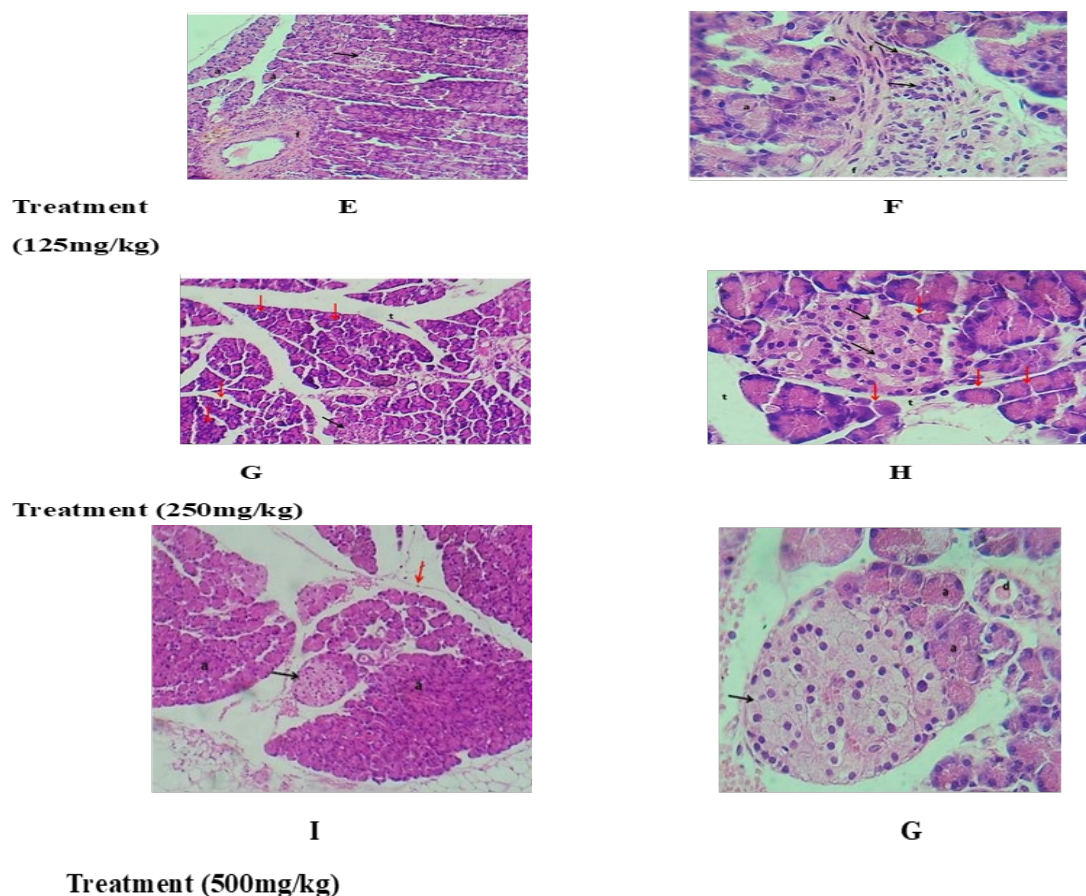


Figure 6: Representative histopathological sections of pancreatic tissue from cerulein-induced rats treated with Hibiscus tiliaceus ethanolic extract (H&E staining).

- A, B: Control group showing normal pancreatic architecture with intact acinar lobules (a), well-defined islets (arrow), and normal interlobular vasculature (V) (H&E, $\times 100$).
- C, D: Induction group displaying interstitial edema, marked leukocytic infiltration (e), degeneration and atrophy of acinar cells (black arrow), vascular congestion (C), and islet cell depletion (d) (H&E, $\times 100$, $\times 400$).
- E, F: Treatment group I (125 mg/kg) showing preservation of normal acini and islets (arrow) with mild periductal fibrosis (f) and limited leukocyte infiltration (H&E, $\times 100$).
- G, H: Treatment group II (250 mg/kg) exhibiting nearly normal acinar organization with mild acinar atrophy (red arrows) and normal islets (black arrow); interlobular tissue appears intact (H&E, $\times 100$, $\times 400$).
- I, J: Treatment group III (500 mg/kg) revealing restoration of normal pancreatic histoarchitecture, including well-preserved islets (black arrow), acinar lobules (a), and intercalated ducts (d), comparable to the control group (H&E, $\times 100$, $\times 400$).

4. Discussion

Natural products have garnered significant attention in recent years and are extensively researched for their possible antioxidant, anti-inflammatory, anti-apoptotic, anticancer, and antiviral properties (Tang et al., 2022). In this experimental study, the ethanolic leaf extract of *H. tiliaceus* was investigated for its potential therapeutic efficacy in a cerulein-induced model of acute pancreatitis (Lerch & Gorelick, 2013). Evidence shows that *H. tiliaceus* ethanolic L. extract is abundant in polyphenols and flavonoids, such as catechin, rutin, quercetin, and kaempferol derivatives, demonstrating significant antioxidant activity in standard in vitro assays (DPPH, ABTS), thereby affirming their ability to scavenge reactive oxygen species and mitigate lipid peroxidation (Hossain et al., 2015).

Such phytochemicals have demonstrated anti-inflammatory activity, including MPO inhibition and suppression of NF- κ B-induced cytokines, offering a viable molecular rationale for preventing inflammation-induced tissue injury. The toxicity studies appear to show a relative increase in the value for LD₅₀ (>2000-5000 mg/kg) with good tolerance at acceptable laboratory doses. However, extensive toxicity and pharmacokinetic studies are currently insufficient. The results

collectively support the choice of *H. tiliaceus* ethanolic leaf as a candidate antioxidant/anti-inflammatory agent in an animal model of cerulein-induced acute pancreatitis, among others (Borive Amani et al., 2025; Raina et al., 2014; Wong et al., 2016).

HPLC profiling of the ethanolic extract of *H. tiliaceus* leaves revealed the presence of several phenolic and flavonoid compounds, including gallic acid, syringic acid, vanillic acid, quercetin, kaempferol, rutin, scopoletin, and coumarin. These phytoconstituents are well-documented for their antioxidant, anti-inflammatory, and cytoprotective properties, which likely contribute to the extract's overall therapeutic effects observed in cerulein-induced acute pancreatitis (Hussein & Kadum, 2020; Hussien & Al-Hamashi, 2022; Lim et al., 2023).

The phenolic acids, such as gallic acid, syringic acid, and vanillic acid, as well as flavonoids, including quercetin, rutin, and kaempferol, present in *H. tiliaceus*, are antioxidants, anti-inflammatory, and exert their action by suppressing the pathways related to NF- κ B, MAPK, and Nrf-2, resulting in reduced production. Additionally, scopoletin and coumarin derivatives have been reported to exhibit anti-apoptotic and cell membrane-stabilising effects by inhibiting caspase-3 activation and mitigating mitochondrial dysfunction. The marked suppression of caspase-3 expression in the 250mg/kg and 500mg/kg treatment groups appears to validate this, implying that these coumarin derivatives are likely to protect acinar cells against apoptotic and necrotic cell death (Gao et al., 2024; Saadati et al., 2024).

Molecular Background in AP: Cerulein hyperstimulation leads to a massive generation of Reactive Oxygen Species (ROS) within pancreatic acinar cells. This oxidative stress causes lipid peroxidation (measured as MDA), depletes endogenous antioxidants like Glutathione (GSH), and directly damages cellular structures, exacerbating inflammation (Lerch & Gorelick, 2013).

The dose-dependent decrease in serum MDA levels and the improvement in GSH concentration in our study could well be attributed to the activation of the Nrf2 pathway by components of *H. tiliaceus*. Quercetin and kaempferol, two active components of *H. tiliaceus*, are well-known activators of the Nrf2 pathway, which induces GSH synthesis and phase II detoxifying enzymes. The resulting increase in antioxidant strength directly offsets the lipid peroxidation induced by the cerulein injection, as corroborated by the reduced MDA concentration in the treatment groups in our study. The role of gallic acid should also be noted as a primary antioxidant that scavenges free radicals and chelates metal ions, acting as the first line of defense against ROS (Borive Amani et al., 2025).

Treatment with *H. tiliaceus* extract (250-500mg/kg) significantly reduced serum amylase and lipase activities in AP-induced rats, indicating mitigation of pancreatic injury. IL-6 and TNF- α levels were dose-dependently reduced due to inhibition of the NF- κ B and MAPK pathways, demonstrating the extract's immunomodulatory effect. Higher MDA levels in cerulein-induced AP were significantly reduced by treatment, proving reduced oxidative stress due to the polyphenolic and flavonoid components present in the extract. The levels of caspase-3 were reduced, indicating resistance to apoptosis and mitochondrial membrane stabilisation.

Histopathological analysis disclosed the marked attenuation by *H. tiliaceus* L. ethanolic leaf extract of Pancreatic necrosis, congestion, and Leukocytic infiltration provoked by cerulein. The 500mg/kg/day *H. tiliaceus* L. ethanolic leaf extract dose, acting specifically in rats, exclusively protected the pancreatic cord structure and acinar surface integrity, consistent with the biochemical findings. Taken collectively, this battery of phytotoxic/phytomedicinals could cooperatively diminish oxidative injury, reduce mediator production, and resist apoptosis, ultimately culminating in a protected histopathology observed among the treated subjects. These observations align with previous research findings (Abdul-Azeez & Mutlag, 2023; Jiang et al., 2024; Zheng et al., 2024) who confirmed the structural preservation of *H. tiliaceus* L. ethanolic extract in a hepatotoxic model. Thus, *H. tiliaceus* extract acts not on a single target, but as a pleiotropic agent that concurrently modulates oxidative, inflammatory, and apoptotic axes to confer protection against cerulein-induced pancreatic injury (Hassan et al., 2024; Jazvinščak Jembrek et al., 2021; Mohammed & Mutlag, 2023; Salih & Mutlag, 2025; Younis & Mutlag, 2023). Collectively, these findings highlight the extract's potent antioxidant, anti-inflammatory, and anti-apoptotic properties, suggesting its therapeutic potential in mitigating pancreatic injury.

5. Limitation

The current study has limitations. It does not have a positive control group, which makes it impossible to compare effectiveness directly, and it includes only female rats, which could limit the applicability of the results due to potential sex-based differences. The next studies should include both sexes and a positive control, and examine the effects of a given dose and potential mechanisms to better elucidate the therapeutic potential of *H. tiliaceus* L. in acute pancreatitis and other inflammatory diseases.

6. Conclusion

The administration of *H. tiliaceus* extract attenuated the biochemical and histopathological alterations associated with

cerulein-induced acute pancreatitis in rats. *H. tiliaceus* L. ethanolic extract demonstrated potent antioxidant, anti-inflammatory, and anti-apoptotic effects, contributing to improved pancreatic tissue integrity. These findings indicate a potential therapeutic agent for alleviating acute pancreatitis and assess its translational relevance in clinical settings.

7 Ethical Approval

All animal procedures adhered to the ARRIVE 2.0 guidelines and were approved by the Ethical Committee of the College of Pharmacy, University of Baghdad (Approval No. Rec022476A). A voucher specimen of *H. tiliaceus* L. was collected from Baghdad, Iraq (33.3152° N, 44.3661° E) and deposited in the Herbarium of the University of Baghdad, Department of Biology (voucher no.205). The plant name was verified using The Plant List (<http://www.theplantlist.org>; accessed on 9 November 2024).

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