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The Potential Ability of Betulinic Acid to Prevent Experimentally Induced Acute Pancreatitis in Rats

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ABSTRACT

Acute pancreatitis (AP) is a serious pancreatic inflammatory disease that results in pancreatic enzyme activation and autodegradation. Betulinic acid (BA), a pentacyclic triterpene of natural origin that was isolated from several plants, has anti-inflammatory, immunomodulatory and antioxidant effects that can help with AP. With this study, we aimed to investigate the potential positive effects of BA on L-arginine-induced AP. A total of 24 male rats were divided into four groups (control, BA, AP and BA + AP). Animals in the BA group were given BA 50 mg/kg/day for 7 days. AP was induced by administering two doses of 250-mg/100-g L-arginine to animals in the AP group. The animals in the BA + AP group were administered 50-mg/kg/day BA (gavage) for 7 days and two doses of 250-mg/100-g L-arginine on the seventh day. BA pretreatment inhibited the increased lipase activity caused by AP and showed protective activity against oxidative damage to pancreatic tissue. It decreased the severity of inflammation by suppressing the release of pro-inflammatory cytokines while increasing the level of the anti-inflammatory cytokine IL-10. It showed a protective effect on pancreatic tissue by inhibiting tumour necrosis factor (TNF-α) and Bax expression. The findings of the study show that BA exhibits multifaceted protective activity in experimental AP induced with L-arginine.

1 | Introduction

Acute pancreatitis (AP) is a severe, progressive pancreatic inflammation associated with active pancreatic enzymes causing autodegradation locally and systemic inflammation [1]. Many factors such as gallstones; bile duct obstruction; alcohol; drugs; toxins; hypertriglyceridaemia; trauma; bacterial, parasitic and viral infections; food allergy; or idiopathic cases play a role in the pathogenesis of AP [2, 3]. Despite improvements in diagnosis and therapy, AP is still linked to high rates of morbidity and mortality [4, 5]. Even though it poses a serious risk to life, the AP process can be reversed [6]. The structure and function of the pancreas can revert to normal with the removal of the contributing causes. Even yet, AP has the potential to be a life-threatening illness [7]. A significant proportion of AP patients develop prediabetes or diabetes after the first attack of AP, and exocrine pancreatic insufficiency develops in one-quarter of the patients [8, 9]. Although research is mostly human oriented, AP is a critical problem in animals. Although the majority of AP cases are mild and self-limiting in cats and dogs, some do develop systemic problems that can be fatal. AP is a frequent disease in both species [10–12]. For instance, in 26.2% of dogs with AP, acute renal damage was found, and it was suggested that this condition may be a comorbidity of canine AP [13].

There is a rise in the production of cytokines and chemokines that promote inflammation and digestive enzymes like lipase and α-amylase in AP [14]. Extensive apoptotic acinar cell death

Summary

- Acute pancreatitis is a serious disease where the pancreas becomes inflamed. In this study, we tested whether a natural substance called betulinic acid could help in a model of pancreatitis caused by L-arginine.
- Our results showed that betulinic acid helped protect the pancreas by reducing inflammation, blocking harmful enzymes and supporting the immune system.
- These findings suggest that betulinic acid may have potential as a natural treatment option for acute pancreatitis.

is seen in mild AP, while widespread acinar cell necrosis is seen in severe AP [15]. Moreover, oxidative stress during AP is an influencing factor in the development of inflammation and complications. Free radicals and pro-inflammatory cytokines both set off reciprocal signal transduction pathways that conduct the inflammatory response and cause the upregulation of pro-inflammatory mediator genes, primarily via the activation of nuclear factor kappa B (NF-κB) [14].

Common in vivo models for AP in rodents include administering high doses of cholecystokinin, cerulein, muscarinic agonists, bile acids or L-arginine or feeding a choline-deficient diet [16]. One of the most widely used animal models is L-arginine, which has been experimentally shown to cause the development of severe AP in rodents [17]. The L-arginine-induced pancreatitis model is preferred for its ability to produce a more severe form of AP, closely mimicking the intense inflammatory response and complications seen in human cases. Its direct cellular toxicity and prolonged inflammation make it ideal for studying severe pancreatitis and testing potential therapeutic interventions [18]. L-Arginine permanently harms the pancreatic mitochondria, which are responsible for a number of adverse reactions such as hyperamylasaemia, trypsinogen activation, inflammation, vacuolization and necrosis [19].

Despite the global disease burden, effective therapeutic agents are currently unavailable to treat or prevent AP [3]. In recent years, interest in herbal medicines in order to prevent and treat diseases and related research to determine the potential of natural products has been revived and steadily increased. A pentacyclic triterpene of the lupane class, betulinic acid (BA) (3β-hydroxy-lup-20(29)-en-28-oic acid) is naturally isolated from various plants [20, 21]. BA is mainly obtained from the bark of white bark birch trees (*Betula* sp., Betulaceae). Native Americans employed white birch bark in tea and other drinks as a traditional treatment to cure gastrointestinal issues like diarrhoea and dysentery [20]. BA has a variety of biological and pharmacological characteristics, such as anti-HIV [20], antitumor [22], antibacterial [23], antiviral [24], antidiabetic [25], antioxidant and anti-inflammatory effects [26]. Birgani et al. [25] reported that BA treatment decreased blood sugar and α-amylase and improved insulin sensitivity and pancreatic histopathology in diabetic mice. It has been reported that BA depresses inflammation by controlling inflammatory cytokines and mediators, reduces oxidative stress by balancing the redox system and prevents paw oedema by suppressing MAPK-COX-2-PGE2 signalling pathway, in mice with λ-carrageenan-induced paw oedema [26]. In another study, it was reported that BA improved insulin sensitivity, high blood sugar, inflammation, dyslipidaemia and oxidative stress in metabolic syndrome induced by a high fructose diet in rats [27]. The most significant contributor to the pancreas' lipolytic activity, pancreatic triglyceride lipase (PNLIP), increases in adipose tissue during pancreatitis and enters adipocytes by various routes, hydrolysing adipose triglyceride and producing excess unesterified fatty acid (NEFA). Therefore, PNLIP can stimulate excessive visceral adipose tissue lipolysis independent of adipocyte-autonomous adipocyte triglyceride lipase during pancreatitis, leading to organ failure [28, 29]. Pharmacological lipase inhibition improves pancreatitis outcomes [28]. Studies show that BA dose-dependently inhibits pancreatic lipase activity [30, 31].

Previous research has demonstrated that BA supplementation has affected pro-inflammatory cytokines, hyperglycaemia, oxidant and antioxidant capacity and protects the structure of pancreatic β-cell. Given the limited research regarding the protective and therapeutic effects of BA in the context of AP, this study hypothesizes that BA pretreatment may confer protective effects against L-arginine-induced AP in rats. BA, through its established anti-inflammatory, antioxidant and pancreatic lipase inhibitory properties, is anticipated to attenuate critical pathogenic mechanisms in AP, including inflammatory cytokine production, oxidative stress and excessive pancreatic lipase activity. Consequently, BA pretreatment may reduce the severity of AP by preserving pancreatic tissue integrity, modulating inflammatory mediator release and maintaining redox homeostasis. This study aims to clarify BA's potential as a prophylactic agent in AP and to provide insights into its mechanisms of action in reducing AP-associated tissue damage and systemic complications.

2 | Materials and Methods

2.1 | Animals and Experiment Designs

A total of 24 male rats (Sprague Dawley), aged 3 months were used in the study obtained from Experimental Animal Research Institute, Erzurum, Turkey. The power analysis program was used to determine the number of animals in the group (G*Power 3.1.9.7. program). It was determined that at least six rats in each group and at least 24 rats in total were needed to obtain 99% working power (Type II error, β) with a 0.05 error (Type I, α). Data from a previous study were used for this analysis [32].

Our research was conducted at the Atatürk University Experimental Animal Research Center, Erzurum, Turkey. Animals were housed in polypropylene rat cages in a room at an ambient temperature of 21°C. The lights were set to a 12-h cycle, with lights on from 7:00 AM to 7:00 PM, followed by a 12-h dark period. The animals were given tap water and a standard rat diet ad libitum, with the diet obtained from Bayramoğlu Yem ve Sanayi Ticaret A.Ş. (Erzurum, Turkey). After acclimatization for 1 week, the animals were randomly

divided into four groups of six each (first group control, second group BA, third group L-arginine–AP and fourth group BA + AP). Animals in the control group did not receive any intervention. Animals in the BA group were given 50-mg/kg/day BA (95%, AB165465-CAS 472-15-1) (gavage) for 7 days and two doses of NaCl (ip) at 1-h intervals on the seventh day. BA was prepared for each animal by mixing it with saline based on body weight and was administered daily via gavage at 8:00 AM. AP was induced by administering two doses of 250-mg/100-g L-arginine (98%, BD30202-CAS 1119-34-2) (ip) 1 h apart on the seventh day of the study to animals in the AP group. L-Arginine was dissolved in saline. The animals in the BA + AP group were administered 50-mg/kg/day BA (gavage) for 7 days and two doses of 250-mg/100-g L-arginine (ip) at 1-h intervals on the seventh day. Blood samples were taken by cardiac route 24 h after the last L-arginine application. Before blood collection, the rats were anaesthetized with ketamine (80 mg/kg; Ketalar, 50 mg/mL, Eczacıbaşı, Istanbul, Turkey) and xylazine (10 mg/kg; Rompun, 2%, Bayer, Istanbul, Turkey). Following anaesthesia, the animals were euthanized by cervical dislocation; subsequently, intracardiac blood and tissue samples were collected. Serum samples were prepared by centrifuging the blood from the animals at 3500 rpm for 10 min. Serum samples were stored at −80°C until analyses were conducted. The study was carried out following ethical rules with the permission decision numbered E-75296309-050.01.04-2100107061, dated 12 April 2021 and numbered 73 by Atatürk University Animal Experiments Local Ethics Committee.

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies [33]. All experimental procedures were conducted following national and international ethical guidelines for animal research. Humane end-points were predefined to minimize distress and suffering. Animals were monitored daily for clinical signs of distress, including lethargy, dehydration, abnormal posture, respiratory difficulty and self-mutilation. A loss of more than 20% of initial body weight was also defined as a humane end-point. No animals met the criteria for humane end-points during the study. Body weights were regularly recorded throughout the experiment to monitor health status. The initial and final body weights of the animals are presented in Table 1.

2.2 | Enzymes Analysis

Serum lipase and amylase measurements were made using Cobas c 702 brand autoanalyser (Incorporated: Roche Diagnostics Turkey).

2.3 | Antioxidant and Oxidant Analysis

Preparation of tissues: Pancreatic tissue samples washed with phosphate-buffered solution (PBS) were lysed with Qiagen Tissue Lyser II for 30 Hz for 3 min by adding liquid nitrogen. Then, 0.1-g tissue sample was homogenized in Tissue Lyser II at 30 Hz for 30 s by adding homogenate buffers. Superoxide dismutase (SOD) enzyme activity was assayed essentially as described previously [34]. Tissue malondialdehyde level (MDA) measurement was performed as previously described [35]. The spectrophotometric method was used to measure the tissue glutathione (GSH) levels as previously described [36].

2.4 | Serum Cytokine Analysis

Analysis of serum cytokines was performed by solid-phase sandwich ELISA method in an ELISA reader device (BioTek, μQuant, USA) using rat-specific TNF-α (SunRed, Cat No: 201-11-0765), interleukin (IL)-6 (SunRed, Cat No: 201-11-0136), IL-10 (SunRed, Cat No: 201-11-0109), IL-1β (SunRed, Cat No: 201-11-0120) and CaspIII (SunRed, Cat No: 201-11-5114) kits.

2.5 | Histopathological Analysis

Following standard tissue follow-up procedures, tissue samples collected after the experiment were preserved in a 10% formaldehyde solution for 48 h before being embedded in paraffin blocks. Each block's preparations for histopathological evaluation were cut into sections of 4-μm thickness, stained with haematoxylin–eosin (H&E) and viewed under a light microscope (Olympus BX 51, Japan). According to their histological characteristics, sections were rated as absence (–), mild (+), moderate (++) and severe (+++).

2.6 | Immunohistochemical and Immunofluorescence Analysis

Tissues for immunohistochemical and immunofluorescence analysis were prepared as follows. Deparaffinization and dehydration were applied to tissue sections collected on the adhesive (poly-L-lysin) slide for immunoperoxidase examination. After 10 min in 3% H_2O_2, endogenous peroxidase was inactivated. Following that, the tissues were boiled in a 1% antigen retrieval (1% citrate buffer (pH + 6.1) 100×) solution and allowed to cool at room temperature. To avoid tissue background staining that was not specific, sections were treated with protein block for 5 min. For immunohistochemical analysis, the

TABLE 1 | Body weights of animals. Data were analysed using one-way ANOVA and Tukey post hoc test. The statistical significance level for all data was taken as $p < 0.05$.

	Groups				
Body weight (g)	**Control (*n*: 6)**	**BA (*n*: 6)**	**AP (*n*: 6)**	**BA + AP (*n*: 6)**	***p* value**
Initial body weight	255.13 ± 11.28	248.14 ± 11,20	251.994 ± 12.53	257.44 ± 9.80	0.495
Final body weight	265.33 ± 10.81	259.81 ± 11.52	262.55 ± 13.00	269.09 ± 9.76	0.503

Note: Values are given as mean ± SD.

tissues were incubated according to the manufacturer's instructions by dropping the primary antibody (TNF-α, Cat No: sc-52 746, dilution ratio: 1/100, USA). 3,3′-Diaminobenzidine (DAB) chromogen was used as chromogen in tissues. The stained sections were examined with a light microscope (Zeiss AXIO, Germany). For immunofluorescence analysis, the tissues were incubated according to the manufacturer's instructions by dropping the primary antibody (Bax, Cat No. sc-7480, dilution ratio: 1/100, USA). As a secondary marker (FITC, Cat No: ab6785, dilution ratio: 1/1000), an immunofluorescence secondary antibody was utilized and left in the dark for 45 min. The sections were kept in the dark for 5 min by dropping the DAPI with mounting medium (Cat No: D1306, dilution ratio: 1/200, UK) and then sealed with coverslips. Using a fluorescence microscope, the stained sections were analysed (Zeiss AXIO, Germany).

2.7 | Statistical Analysis

Data were analysed using one-way ANOVA and Tukey and Duncan post hoc tests in SPSS 20.00 statistical program. The statistical significance level for all data was taken as $p < 0.05$. The Kruskal–Wallis test was employed to assess differences between the groups of semiquantitative data from the histological examination, and the Mann–Whitney *U* test was used to compare the paired groups. Immunohistochemical and immunofluorescent staining produced images, and five randomly chosen locations from each image were assessed using the ZEISS ZEN Imaging Software to gauge the degree of positive staining. The statistical definition of a set of data is its mean and standard deviation (mean ± SD) for % area. Immunopositive stained locations and positive immunoreactive cells were compared to healthy controls using the one-way ANOVA and Tukey test in SPSS 20.00 statistical program. The results were presented as mean ± SD as a result of the test, and the $p < 0.05$ value was recognized as significant.

3 | Results

Experimental groups showed no significant differences in initial or final body weights ($p > 0.05$), and no weight loss was observed (Table 1), indicating stable health and welfare status throughout the study.

Our study observed that amylase and lipase activities increased significantly in the L-arginine-administered AP group compared to the other groups, while they were reduced to control levels in the AP + BA group, which received BA pretreatment (Table 2).

It was determined that the level of MDA increased significantly in the AP group, whereas the SOD activity and GSH levels decreased. BA pretreatment reduced the MDA level to the control level and increased the antioxidant level above the control in the AP + BA group. (Table 3).

IL-1β, IL-6 and TNF-α levels were significantly increased in the AP group, and BA pretreatment suppressed the release of proinflammatory cytokines and increased the IL-10 levels in the AP + BA group (Table 4).

In the histopathological examination of the pancreas tissues of the control and BA groups (Figure 1), both parenchyma tissue and serosa were in a normal histological structure was determined.

Oedema, inflammation, degeneration, necrosis in parenchymal cells, severe congestion and mononuclear cell increase were detected in the interlobular spaces in the pancreas tissues of the AP group (Figure 1).

In the pancreas tissues of the BA + AP group, mild oedema in the interlobular spaces and mild degeneration in the parenchymal vessels were observed (Figure 1). A statistically significant

TABLE 2 | Serum amylase and lipase activities. Data were analysed using one-way ANOVA and Tukey post hoc test. The statistical significance level for all data was taken as $p < 0.05$.

	Groups				
Parameters	**Control (*n*: 6)**	**BA (*n*: 6)**	**AP (*n*: 6)**	**BA + AP (*n*: 6)**	***p* value**
Lipase (U/L)	5.4 ± 0.55^{b}	5.6 ± 0.55^{b}	123.4 ± 10.36^{a}	5.8 ± 0.45^{b}	0.000
Amylase (U/L)	2263.4 ± 118.95^{b}	2427.75 ± 57.28^{b}	7627.8 ± 582.69^{a}	2306.8 ± 214.68^{b}	0.000

Note: Values are given as mean ± SD. [a, b]: Means in the same row with different superscripts differ significantly ($p < 0.05$).

TABLE 3 | Antioxidant and oxidant parameters of pancreatic tissue. Data were analysed using one-way ANOVA and Tukey post hoc test. The statistical significance level for all data was taken as $p < 0.05$.

	Groups				
Parameters	**Control (*n*: 6)**	**BA (*n*: 6)**	**AP (*n*: 6)**	**BA + AP (*n*: 6)**	***p* value**
MDA (μmol/g protein)	18.58 ± 4.25^{b}	18.53 ± 3.71^{b}	29.95 ± 3.47^{a}	20.16 ± 3.81^{b}	0.001
SOD (U/g protein)	4.60 ± 0.23^{b}	5.02 ± 0.26^{a}	3.44 ± 0.12^{c}	4.99 ± 0.14^{a}	0.000
GSH (μmol/g protein)	1.04 ± 0.04^{b}	1.19 ± 0.08^{a}	0.90 ± 0.11^{c}	1.23 ± 0.05^{a}	0.000

Note: Values are given as mean ± SD. [a, b, c]: Means in the same row with different superscripts differ significantly ($p < 0.05$).

TABLE 4 | The levels of caspase III and cytokines in serum. Data were analysed using one-way ANOVA and Tukey post hoc test. The statistical significance level for all data was taken as $p < 0.05$.

	Groups				
Parameters	**Control (*n*: 6)**	**BA (*n*: 6)**	**AP (*n*: 6)**	**BA + AP (*n*: 6)**	***p* value**
IL-1β (pg/L)	701.00 ± 24.24^{c}	802.25 ± 7.50^{b}	921.00 ± 62.75^{a}	797.80 ± 9.86^{b}	0.000
IL-6 (pg/mL)	20.46 ± 2.24^{d}	33.22 ± 3.23^{c}	65.59 ± 1.61^{a}	53.36 ± 2.62^{b}	0.000
IL-10 (ng/L)	52.26 ± 4.03^{a}	51.79 ± 2.72^{a}	25.31 ± 2.13^{b}	48.21 ± 8.17^{a}	0.000
TNF-α (ng/L)	96.74 ± 9.09^{b}	99.42 ± 3.54^{b}	114.65 ± 9.35^{a}	106.52 ± 11.64^{ab}	0.024
Caspase III (ng/mL)	2.72 ± 0.18^{b}	3.01 ± 0.06^{ab}	3.28 ± 0.33^{a}	3.20 ± 0.25^{a}	0.006

Note: Values are given as mean ± SD. $^{a, b, c}$: Means in the same row with different superscripts differ significantly ($p < 0.05$).

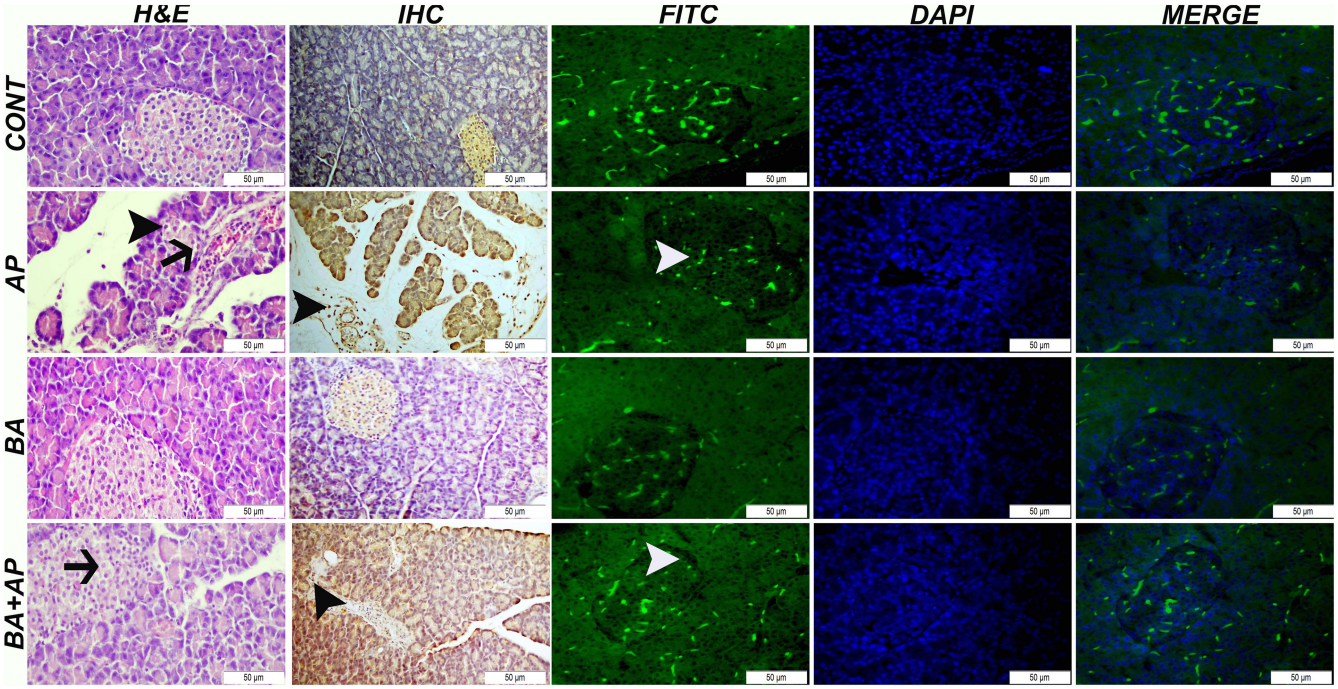

FIGURE 1 | Pancreatic tissue, degeneration (arrow) and necrosis (arrowhead) in islet cells. H&E, bar: 50 µm. TNF-α expressions in inflammatory cells (arrowhead), IHC-P, bar: 50 µm. Bax expression in islet cells (white arrowhead) (FITC), DAPI: nuclear stain, MERGE: the shape obtained by superimposing FITC and DAPI staining, IF, bar: 50 µm.

difference ($p < 0.05$) was determined when compared with the AP group. Histopathological findings are summarized in Table 5.

In the immunohistochemical and immunofluorescence examination of the pancreas tissues of the control and BA groups (Figure 1), TNF-α and Bax expressions were evaluated as negative.

In the AP group, severe TNF-α expressions were detected in inflammatory cells, interstitial spaces and around the vessels, and severe cytoplasmic Bax expressions were detected in parenchymal cells in immunofluorescent staining (Figure 1).

In the BA + AP group, mild TNF-α expressions were detected around the vessels in inflammatory cells, and mild cytoplasmic Bax expressions were detected in parenchymal cells (Figure 1). A statistically significant difference ($p < 0.05$) was found when compared with the AP group. Immunohistochemical and immunofluorescent findings are summarized in Table 6.

4 | Discussion

AP is severe pancreatic inflammation with local and systemic inflammation, that causes pancreatic enzymes to be activated and autodegraded [1]. Even with improvements in diagnosis and treatment, AP continues to relate to a considerable extent to morbidity and mortality [4, 5]. Although research is mostly human oriented, AP is a very important problem in animals. L-Arginine is one of the most often utilized animal models because it has been experimentally demonstrated to cause the development of severe AP in rodents [17]. The irreversible damage caused by L-arginine in pancreatic mitochondria results in the development of many pathological conditions such as trypsinogen activation, hyperamylasaemia, vacuolization, inflammation and necrosis

[19]. In the presented study, AP was induced with L-arginine. One of the best indicators for AP is increased levels of digestive enzymes such as α-amylase and lipase [14].

Blood amylase, one of the pancreatic enzymes, is the most widely used enzyme in the diagnosis of AP because it can be measured quickly. However, measurement of lipase is recommended because lipase is superior to amylase in sensitivity and specificity. Abnormal lipase activities in AP last longer than amylase, and if the amylase activity is normal, lipase measurement is more useful in diagnosing AP [37]. It was observed that amylase and lipase activities increased significantly ($p < 0.005$) in the L-arginine-administered AP group compared to the other groups (Table 2). Pharmacological lipase inhibition improves pancreatitis outcomes [28]. Studies have revealed the inhibitory effect of BA on pancreatic lipase activity [30, 31]. Our study observed that amylase and lipase activities were reduced to control levels in the AP + BA group, which received BA pretreatment (Table 2).

Oxidative stress is involved in the pathophysiology of AP, with high oxidant levels and reduced antioxidant defences, and is a factor that contributes to the development of inflammation. Free radical activities and lipid peroxide concentrations increase in plasma and tissue in AP [14, 38, 39]. In our study, it was determined that the level of MDA, a lipid peroxidation marker, increased significantly in the AP group ($p < 0.05$) (Table 3). Many studies are showing that BA reduces oxidative stress [26, 40, 41]. BA mitigates oxidative stress, a major contributor to cellular damage in conditions like pancreatitis, by enhancing the activity of antioxidant enzymes such as SOD and GSH which neutralize reactive oxygen species and reduce lipid peroxidation [42, 43]. Additionally, BA activates the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, a critical regulator of antioxidant gene expression. Nrf2 is a key transcription factor that controls the expression of genes involved in the body's antioxidant defence mechanisms. It regulates several antioxidative enzymes, including SOD, GSH, GSH peroxidase (GPx) and haem oxygenase-1 (HO-1), which enhance cellular defences against oxidative damage [44, 45]. In our study, we found that the levels of SOD and the activity of GSH, both antioxidant enzymes, were decreased in the AP group, while they increased significantly in the BA pretreatment group ($p < 0.05$) (Table 3). Study findings show that BA pretreatment reduces the MDA level to the control level and increases antioxidant levels above the control level, thereby defending pancreatic tissue against oxidative damage by reducing lipid peroxidation.

As it is known, AP is a condition characterized by interstitial oedema, inflammatory cell infiltration, bleeding, cellular apoptosis and necrosis [6]. In mild AP, there is extensive acinar cell death through apoptosis, whereas in severe AP, there is extensive acinar cell necrosis [15]. Our findings show that oedema, inflammation, degeneration and necrosis in parenchymal cells, severe congestion and mononuclear cell increase in the vessels occur in the pancreatic tissues of the AP group (Table 5, Figure 1).

In addition to oxidative stress, pro-inflammatory cytokines and chemokines such as IL-1β, IL-6, IL-8, IL-12, IL-17, IFN-γ and TNF-α are important factors in the pathophysiology of AP [14, 46, 47]. TNF-α and IL-1β are key regulators of the pro-inflammatory response in AP, initiating reactions to acinar cell injury and stimulating IL-6 production. IL-6 is crucial in advancing the systemic phase by promoting acute-phase protein synthesis [47]. In AP, the release of pro-inflammatory cytokines and chemokines is increased [14]. Notably, IL-1β and IL-6 show a significant increase in moderately severe and severe cases, with IL-6 identified in recent studies as an early predictor of AP severity [47, 48]. Cytokines have a significant impact on multi-organ dysfunction, which, in severe AP, causes death. TNF-α can be detected in the serum 1 h after the onset of pancreatitis and rises rapidly in the following 6 h. Similarly, the level of IL-1β increases in AP. A crucial function is played by TNF-α and IL-1β in stimulating their own and each other's production, as well

TABLE 5 | Scoring of histopathological findings observed in pancreatic tissues. The Kruskal–Wallis test assessed differences between the groups, and the Mann–Whitney *U* test was used to compare the paired groups.

	Groups			
Parameters	**Control (*n*: 6)**	**BA (*n*: 6)**	**AP (*n*: 6)**	**BA + AP (*n*: 6)**
Oedema in the interlobular spaces	−	−	+++	+
Degeneration in parenchyma cells	−	−	+++	+
Necrosis in parenchyma cells	−	−	+++	−
Inflammation	−	−	+++	+
Hyperaemia in the vessels	−	−	+++	++

TABLE 6 | The statistical representation of immunohistochemical and immunofluorescent findings observed in pancreatic tissues. Data were analysed using one-way ANOVA and Tukey post hoc test. The statistical significance level for all data was taken as $p < 0.05$.

	Groups			
Parameters	**Control (*n*: 6)**	**BA (*n*: 6)**	**AP (*n*: 6)**	**BA + AP (*n*: 6)**
TNF-α expression	20.45 ± 3.52^{a}	20.23 ± 4.78^{a}	80.42 ± 4.12^{b}	39.34 ± 3.86^{c}
Bax expression	20.27 ± 5.07^{a}	19.95 ± 4.43^{a}	82.14 ± 3.98^{b}	41.62 ± 4.61^{c}

Note: Values are given as mean ± SD. [a, b, c]: Means in the same row with different superscripts differ significantly ($p < 0.05$).

as inducing other regulatory genes. IL-1β and TNF-α stimulate the synthesis and release of IL-6, so IL-6 levels also increase in AP and its increase is associated with the severity of the disease [49]. Study findings show that IL-1β, IL-6 and TNF-α levels are significantly increased in the AP group (Table 4). Both free radicals and pro-inflammatory cytokines trigger reciprocal signal transduction pathways that direct the inflammatory process, leading to the overexpression of cytokines, chemokines and other pro-inflammatory mediator genes, mainly through NF-κB activation [14]. TNF-α expression is the primary response in AP. An invasion of TNF-α producing macrophages into the pancreas can be seen through immunohistochemical staining. The degree of pancreatic injury and inflammation is strongly correlated with the total increase in tissue and serum TNF-α concentrations [49]. Our findings show that there is severe TNF-α expression in pancreatic tissue in the group with AP (Table 6, Figure 1) whereas BA pretreatment inhibits TNF-α expression and reduces the severity of tissue damage and inflammation (Table 6, Figure 1).

Acinar cell death is the hallmark of AP. Acinar cell death in AP occurs through both necrosis and apoptosis. Several protease families, particularly caspase, are involved in apoptosis. Caspases are molecular implementers of apoptosis, and according to reports, one of the apoptosis pathways in AP may involve caspase activation. By activating caspase-3, which breaks down DNA repair proteins, oxidative stress in AP causes acinar cells to apoptosis [50]. It is observed that the caspase-3 level is significantly increased in the AP group compared to the control group (Table 4). Another functional element of the apoptosis pathway besides caspase is Bax, a member of the Bcl-2 gene family of proapoptotic. It has been reported that the increase in proapoptotic Bax gene expression in AP supports acinar cell apoptosis and may be partially responsible [50]. Our study findings showing the presence of severe Bax expression in the AP group support this view (Table 6 and Figure 1). BA pretreatment reduced tissue damage by inhibiting Bax expression (Table 6 and Figure 1).

In AP, IL-10 serves a protective function. IL-10 is an anti-inflammatory cytokine that modulates the expression of early pro-inflammatory cytokines and down-regulates TNF-α and IL-1. Furthermore, it promotes the synthesis of naturally occurring IL-1 receptor antagonists. IL-10 stops tissue deterioration by preventing the release of pro-inflammatory cytokines by monocytes and macrophages [49, 50]. Our study findings show that BA pretreatment suppresses the release of pro-inflammatory cytokines through the increase of IL-10 and thus prevents tissue damage (Table 4). Previous studies showed that BA has a strong anti-inflammatory activity [27, 51–53]. According to reports, the activity of BA is based on the suppression of protein kinase C, and this substance also inhibits the activation of both phospholipase A2 and NF-κB. In addition, the combination of cyclooxygenase-2 and matrix metalloproteinase-9 confers anti-inflammatory properties to BA due to its effect on NF-κB-dependent gene expressions [52]. Similarly, BA has been reported to exert prophylactic and therapeutic effects on cerulein-induced AP in mice by inhibiting the NF-κB signalling pathway [53]. It has been reported that BA exerts its effect via controlling macrophages' in vivo TNF-α production by an IL-10-dependent mechanism [51]. In another similar study, it was reported that BA decreases inflammation via modulating inflammatory cytokines and mediators and reduces oxidative stress by balancing the redox system [26]. Our study findings reveal that BA pretreatment significantly ($p < 0.05$) reduced the severity of AP-related inflammation findings and showed an anti-inflammatory effect (Table 5 and Figure 1).

5 | Conclusion

BA pretreatment inhibited the increased lipase activity caused by AP and showed a protective effect against oxidative damage to the pancreatic tissue by decreasing the oxidants levels while increasing the antioxidant level. In addition, it decreased the severity of inflammation by suppressing the release of pro-inflammatory cytokines while increasing the level of the anti-inflammatory cytokine IL-10 concerning the immune response. It showed a protective effect on pancreatic tissue by inhibiting TNF-α and Bax expression. In conclusion, the results of the current study show that BA exhibits multifaceted protective activity with its enzyme inhibitor, antioxidant and immunomodulatory properties in experimental AP induced with L-arginine.

Acknowledgements

The authors have nothing to report.

Ethics Statement

The study was carried out following ethical rules with the permission decision numbered E-75296309-050.01.04-2100107061, dated 12 April 2021 and numbered 73 by Atatürk University Animal Experiments Local Ethics Committee. All the experimental procedures were carried out following international guidelines for the care and use of laboratory animals. Animal handling and experimentation were performed in line with the Guide for the Care and Use of Laboratory Animals, 8th edition. The authors confirm that the study was performed in accordance with ARRIVE guidelines.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data underlying the results are available as part of the article, and no additional source data are required.

References

1. S. M. van Dijk, N. D. L. Hallensleben, H. C. van Santvoort, et al., "Acute Pancreatitis: Recent Advances Through Randomised Trials," *Gut* 66, no. 11 (2017): 2024–2032.

2. M. Manohar, A. K. Verma, S. Upparahalli Venkateshaiah, H. Goyal, and A. Mishra, "Food-Induced Acute Pancreatitis," *Digestive Diseases and Sciences* 62, no. 12 (2017): 3287–3297.

3. P. J. Lee and G. I. Papachristou, "New Insights Into Acute Pancreatitis," *Nature Reviews Gastroenterology & Hepatology* 16, no. 8 (2019): 479–496, https://doi.org/10.1038/s41575-019-0158-2.

4. S. J. Hashemi, R. Masjedizadeh, F. Mehran, and A. Tahmasebi, "The Relationship Between Etiology of Acute Pancreatitis and Its Clinical Features in Iranian Patients," *Biomedical and Pharmacology Journal* 10, no. 2 (2017): 875–880.

5. L. Boxhoorn, R. P. Voermans, S. A. Bouwense, et al., "Acute Pancreatitis," *Lancet* 396, no. 10252 (2020): 726–734, https://doi.org/10.1016/S0140-6736(20)31310-6.

6. P. Rawla, S. S. Bandaru, and A. R. Vellipuram, "Review of Infectious Etiology of Acute Pancreatitis," *Gastroenterology Research* 10, no. 3 (2017): 153–158.

7. M. Borran, M. Minaiyan, B. Zolfaghari, and P. Mahzouni, "Protective Effect of *Tribulus terrestris* Fruit Extract on Cerulein-Induced Acute Pancreatitis in Mice," *Avicenna Journal of Phytomedicine* 7, no. 3 (2017): 250–260.

8. S. L. M. Das, P. P. Singh, A. R. J. Phillips, R. Murphy, J. A. Windsor, and M. S. Petrov, "Newly Diagnosed Diabetes Mellitus After Acute Pancreatitis: A Systematic Review and Meta-Analysis," *Gut* 63, no. 5 (2014): 818–831.

9. R. A. Hollemans, N. D. L. Hallensleben, D. J. Mager, et al., "Pancreatic Exocrine Insufficiency Following Acute Pancreatitis: Systematic Review and Study Level Meta-Analysis," *Pancreatology* 18, no. 3 (2018): 253–262, https://doi.org/10.1016/j.pan.2018.02.009.

10. K. B. Jensen and D. L. Chan, "Nutritional Management of Acute Pancreatitis in Dogs and Cats," *Journal of Veterinary Emergency and Critical Care* 24, no. 3 (2014): 240–250.

11. C. Mansfield, "Acute Pancreatitis in Dogs: Advances in Understanding, Diagnostics, and Treatment," *Topics in Companion Animal Medicine* 27, no. 3 (2012): 123–132, https://doi.org/10.1053/j.tcam.2012.04.003.

12. A. Zoia and M. Drigo, "Association Between Pancreatitis and Immune-Mediated Haemolytic Anaemia in Cats: A Cross-Sectional Study," *Journal of Compaative Pathology* 156, no. 4 (2017): 384–388, https://doi.org/10.1016/j.jcpa.2017.02.003.

13. E. Gori, I. Lippi, G. Guidi, F. Perondi, A. Pierini, and V. Marchetti, "Acute Pancreatitis and Acute Kidney Injury in Dogs," *Veterinary Journal* 245 (2019): 77–81, https://doi.org/10.1016/j.tvjl.2019.01.002.

14. E. M. El Morsy and M. A. E. Ahmed, "Carvedilol Attenuates L-Arginine Induced Acute Pancreatitis in Rats Through Modulation of Oxidative Stress and Inflammatory Mediators," *Chemico-Biological Interactions* 327, no. June (2020): 109181, https://doi.org/10.1016/j.cbi.2020.109181.

15. M. Bhatia, "Apoptosis Versus Necrosis in Acute Pancreatitis," *American Journal of Physiology Gastrointestestinal and Liver Physiology* 286, no. 2 (2004): G189–G196.

16. A. Habtezion, A. S. Gukovskaya, and S. J. Pandol, "Acute Pancreatitis: A Multifaceted Set of Organelle and Cellular Interactions," *Gastroenterology* 156, no. 7 (2019): 1941–1950.

17. T. Mizunuma, S. Kawamura, and Y. Kishino, "Effects of Injecting Excess Arginine on Rat Pancreas," *Journal of Nutrition* 114, no. 3 (1984): 467–471.

18. K. H. Su, C. Cuthbertson, and C. Christophi, "Review of Experimental Animal Models of Acute Pancreatitis," *HPB* 8, no. 4 (2006): 264–286.

19. G. Biczo, E. T. Vegh, N. Shalbueva, et al., "Mitochondrial Dysfunction, Through Impaired Autophagy, Leads to Endoplasmic Reticulum Stress, Deregulated Lipid Metabolism, and Pancreatitis in Animal Models," *Gastroenterology* 154, no. 3 (2018): 689–703, https://doi.org/10.1053/j.gastro.2017.10.012.

20. P. Yogeeswari and D. Sriram, "Betulinic Acid and Its Derivatives: A Review on Their Biological Properties," *Current Medicinal Chemistry* 12, no. 6 (2010): 657–666.

21. A. Hordyjewska, A. Ostapiuk, A. Horecka, and J. Kurzepa, "Betulin and Betulinic Acid: Triterpenoids Derivatives With a Powerful Biological Potential," *Phytochemistry Reviews* 18, no. 3 (2019): 929–951, https://doi.org/10.1007/s11101-019-09623-1.

22. J. Zhao, R. Li, A. Pawlak, et al., "Antitumor Activity of Betulinic Acid and Betulin in Canine cancer Cell Lines," *In Vivo (Brooklyn)* 32, no. 5 (2018): 1081–1088.

23. H. O. B. Oloyede, H. O. Ajiboye, M. O. Salawu, and T. O. Ajiboye, "Influence of Oxidative Stress on the Antibacterial Activity of Betulin, Betulinic Acid and Ursolic Acid," *Microbial Pathogenesis* 111 (2017): 338–344, https://doi.org/10.1016/j.micpath.2017.08.012.

24. M. W. C. Loe, E. Hao, M. Chen, et al., "Betulinic Acid Exhibits Antiviral Effects Against Dengue Virus Infection," *Antiviral Research* 184 (2020): 104954, https://doi.org/10.1016/j.antiviral.2020.104954.

25. G. A. Birgani, A. Ahangarpour, L. Khorsandi, and H. F. Moghaddam, "Anti-Diabetic Effect of Betulinic Acid on Streptozotocin-Nicotinamide Induced Diabetic Male Mouse Model," *Brazilian Journal of Pharmaceutical Sciences* 54, no. 2 (2018): 1–7.

26. Z. Ou, J. Zhao, L. Zhu, et al., "Anti-Inflammatory Effect and Potential Mechanism of Betulinic Acid on λ-Carrageenan-Induced Paw Edema in Mice," *Biomedicine & Pharmacotheraphy* 118, no. June (2019): 109347, https://doi.org/10.1016/j.biopha.2019.109347.

27. R. A. Ajala-Lawal, N. O. Aliyu, and T. O. Ajiboye, "Betulinic Acid Improves Insulin Sensitivity, Hyperglycemia, Inflammation and Oxidative Stress in Metabolic Syndrome Rats via PI3K/Akt Pathways," *Archives of Physiology and Biochemistry* 126, no. 2 (2020): 107–115, https://doi.org/10.1080/13813455.2018.1498901.

28. K. Patel, R. N. Trivedi, C. Durgampudi, et al., "Lipolysis of Visceral Adipocyte Triglyceride by Pancreatic Lipases Converts Mild Acute Pancreatitis to Severe Pancreatitis Independent of Necrosis and Inflammation," *American Journal of Pathology* 185, no. 3 (2015): 808–819, https://doi.org/10.1016/j.ajpath.2014.11.019.

29. C. de Oliveira, B. Khatua, P. Noel, et al., "Pancreatic Triglyceride Lipase Mediates Lipotoxic Systemic Inflammation," *Journal of Clinical Investigation* 130, no. 4 (2020): 1931–1947.

30. D. S. Jang, G. Y. Lee, J. Kim, et al., "A new Pancreatic Lipase Inhibitor Isolated From the Roots of *Actinidia arguta*," *Archives of Pharmacal Research* 31, no. 5 (2008): 666–670.

31. J. Kim, Y. S. Lee, C. S. Kim, and J. S. Kim, "Betulinic Acid Has an Inhibitory Effect on Pancreatic Lipase and Induces Adipocyte Lipolysis," *Phytheraphy Research* 26, no. 7 (2012): 1103–1106.

32. I. I. Matalka, N. M. Mhaidat, and L. A. Fatlawi, "Antioxidant Activity of Simvastatin Prevents L-Arginine-Induced Acute Toxicity of Pancreas," *International Journal of Physiology, Pathophysiology and Pharmacology* 5, no. 2 (2013): 102–108.

33. P. Tveden-Nyborg, T. K. Bergmann, N. Jessen, U. Simonsen, and J. Lykkesfeldt, "BCPT 2023 Policy for Experimental and Clinical Studies," *Basic & Clinical Pharmacology & Toxicology* 133 (2023): 391–396.

34. Y. Sun, L. W. Oberley, and Y. Li, "A Simple Method for Clinical Assay of Superoxide Dismutase," *Clinical Chemistry* 34, no. 3 (1988): 497–500.

35. H. Ohkawa, N. Ohishi, and K. Yagi, "Assay for Lipid Peroxides in Animal Tissues by Thiobarbituric Acid Reaction," *Analytical Biochemistry* 95, no. 2 (1979): 351–358.

36. J. Sedlak and R. H. Lindsay, "Estimation of Total, Protein-Bound, and Nonprotein Sulfhydryl Groups in Tissue With Ellman's Reagent," *Analytical Biochemistry* 25 (1968): 192–205.

37. S. Kiriyama, T. Gabata, T. Takada, et al., "New Diagnostic Criteria of Acute Pancreatitis," *Journal of Hepato-Biliary-Pancreatic Sciences* 17, no. 1 (2010): 24–36.

38. J. A. Armstrong, N. Cash, P. M. G. Soares, M. H. L. P. Souza, R. Sutton, and D. N. Criddle, "Oxidative Stress in Acute Pancreatitis: Lost in Translation?," *Free Radical Research* 47, no. 11 (2013): 917–933.

39. S. Bansod, S. Chilvery, M. A. Saifi, T. J. Das, H. Tag, and C. Godugu, "Borneol Protects Against Cerulein-Induced Oxidative Stress and

Inflammation in Acute Pancreatitis Mice Model," *Environmental Toxicology* 36, no. 4 (2021): 530–539.

40. L. Kong, L. Zhu, X. Yi, et al., "Betulinic Acid Alleviates Spleen Oxidative Damage Induced by Acute Intraperitoneal Exposure to T-2 Toxin by Activating Nrf2 and Inhibiting MAPK Signaling Pathways," *Antioxidants* 10, no. 2 (2021): 1–17.

41. F. O. Osunsanmi, G. E. Zharare, R. A. Mosa, M. I. Ikhile, F. O. Shode, and A. R. Opoku, "Anti-Oxidant, Anti-Inflammatory and Anti-Acetylcholinesterase Activity of Betulinic Acid and 3β-Acetoxybetulinic Acid From *Melaleuca bracteata* 'Revolution Gold'," *Tropical Journal of Pharmaceutical Research* 18, no. 2 (2019): 303–309.

42. L. Zhu, C. Luo, C. Ma, et al., "Inhibition of the NF-κB Pathway and ERK-Mediated Mitochondrial Apoptotic Pathway Takes Part in the Mitigative Effect of Betulinic Acid on Inflammation and Oxidative Stress in Cyclophosphamide-Triggered Renal Damage of Mice," *Ecotoxicology and Environmental Safety* 246 (2022): 114150.

43. J. Yi, W. Xia, J. Wu, et al., "Betulinic Acid Prevents Alcohol-Induced Liver Damage by Improving the Antioxidant System in Mice," *Journal of Veterinary Science* 15, no. 1 (2014): 141–148.

44. L. Zhu, X. Yi, C. Ma, et al., "Betulinic Acid Attenuates Oxidative Stress in the Thymus Induced by Acute Exposure to T-2 Toxin via Regulation of the MAPK/Nrf2 Signaling Pathway," *Toxins (Basel)* 12, no. 9 (2020): 540.

45. Y.-Y. Bai, D. Yan, H.-Y. Zhou, et al., "Betulinic Acid Attenuates Lipopolysaccharide-Induced Vascular Hyporeactivity in the Rat Aorta by Modulating Nrf2 Antioxidative Function," *Inflammopharmacology* 28, no. 1 (2020): 165–174.

46. S. Rafaqat, D. Patoulias, A. H. Behnoush, S. Sharif, and A. Klisic, "Interleukins: Pathophysiological Role in Acute Pancreatitis," *Archives of Medical Science. AMS* 20, no. 1 (2024): 138–156.

47. H. Sternby, H. Hartman, H. Thorlacius, and S. Regnér, "The Initial Course of IL1β, IL-6, IL-8, IL-10, IL-12, IFN-γ and TNF-α With Regard to Severity Grade in Acute Pancreatitis," *Biomolecules* 11, no. 4 (2021): 591.

48. F. F. van den Berg, A. C. de Bruijn, H. C. van Santvoort, Y. Issa, and M. A. Boermeester, "Early Laboratory Biomarkers for Severity in Acute Pancreatitis; a Systematic Review and Meta-Analysis," *Pancreatology* 20, no. 7 (2020): 1302–1311.

49. M. Brady, S. Christmas, R. Sutton, J. Neoptolemos, and J. Slavin, "Cytokines and Acute Pancreatitis," *Bailliere's Best Practice in Clinical Gastroenterology* 13, no. 2 (1999): 265–289.

50. M. Bhatia, L. W. Fei, Y. Cao, et al., "Pathophysiology of Acute Pancreatitis," *Pancreatology* 5, no. 2–3 (2005): 132–144, https://doi.org/10.1159/000085265.

51. J. F. Oliveira Costa, J. M. Barbosa-Filho, G. L. De Azevedo Maia, et al., "Potent Anti-Inflammatory Activity of Betulinic Acid Treatment in a Model of Lethal Endotoxemia," *International Immunopharmacology* 23, no. 2 (2014): 469–474, https://doi.org/10.1016/j.intimp.2014.09.021.

52. J. L. Ríos and S. Máñez, "New Pharmacological Opportunities for Betulinic Acid," *Planta Medica* 84, no. 1 (2018): 8–19.

53. Z. Zhou, J.-W. Choi, J. Y. Shin, et al., "Betulinic Acid Ameliorates the Severity of Acute Pancreatitis via Inhibition of the NF-κB Signaling Pathway in Mice," *International Journal of Molecular Sciences* 22, no. 13 (2021): 6871.