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FULL ARTICLE

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Myrtus communis leaf extract protects against cerulein-induced acute pancreatitis in rats

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Abstract

In this study, the aim was to examine the potential protective effects of *Myrtus communis* subsp. *communis* leaf ethanol extract (MC) treatment against acute pancreatitis (AP) in rats. Thirty-two rats were grouped as the saline-pretreated control (C), MC-pretreated control (MC), saline-pretreated AP (AP), and MC-pretreated AP (MC + AP) groups. To induce AP, cerulein was administered (50 μg/kg) two times. The rats were given MC for 14 days before cerulein injection. Six hours after the final cerulein injection, the rats were sacrificed. Pancreatic damage was associated with an increase in the serum activity of lipase and amylase, the pancreatic activity of myeloperoxidase, and the pancreatic level of malondialdehyde, interleukin-1β, and interleukin-6. AP also led to a decrease in the pancreatic level of anti-inflammatory interleukin-10 and glutathione. Pretreatment with MC before the induction of AP significantly reduced the pancreatic damage observed during the histological examination as well as reversed the biochemical changes evoked by AP.

Practical applications

Acute pancreatitis is characterized by high mortality (average about 5%; severe cases may reach about 30%). The current treatment for acute pancreatitis is mainly symptomatic. The introduction of herbal drugs may lead to the development of a new strategy in the treatment of this disease. This study revealed that MC reduced pancreatic injury by decreasing pro-inflammatory cytokines, increasing antioxidant capacity and anti-inflammatory cytokine, IL-10. To the authors' knowledge, this research is the first report showing that MC inhibits the development of AP. This observation suggests that MC may be useful in the prevention and the treatment of AP in clinical settings.

KEYWORDS

acute pancreatitis, anti-inflammatory effect, antioxidant effect, IL-10, *Myrtus communis* subsp. *communis* leaf ethanol extract

1 | INTRODUCTION

Acute pancreatitis (AP) is a severe disease defined by acinar cell damage and local and systemic inflammation that occurs with different severities from mild pancreatic edema to necrosis, organ failure, and death (Goodchild, Chouhan, & Johnson, 2019). The early release of proteolytic enzymes causes an increase in reactive oxygen species (ROS) and inflammatory mediators. These events trigger acute inflammation and damage to acinar cells (Hammer, 2014; Pérez, Pereda, Sabater, & Sastre, 2015) and are clinically specified by elevated amylase and lipase

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levels (Goodchild et al., 2019). If the underlying causes are not treated effectively, acute recurrent pancreatitis (ARP), defined by recurrent episodes of AP, occurs (Steinberg & Tenner, 1994).

In the pathogenesis of AP, oxidative damage is an important pathogenic factor (Leung & Chan, 2009). For several reasons, the pathological activation of intra-acinar trypsinogen and other zymogen enzymes causes pancreatic injury and the release of cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) (Escobar et al., 2009). Subsequently, the activation of leukocytes leads to oxidative stress by producing huge amounts of ROS (Pereda et al., 2006). ROS also acts as an inflammatory mediator through leukocyte activation and increased pro-inflammatory cytokine expression (Pérez et al., 2015).

The current therapy strategies for AP are primarily symptomatic, and there is still no specific therapy for AP; however, antioxidants could be considered as promising alternative potential therapeutic agents due to their beneficial effects that have been demonstrated in various experimental AP models. Moreover, it has been shown that the administration of antioxidants, especially in the early phases of pancreatitis, reduces oxidant damage and prevents the recurrence of the disease (Uden et al., 1992).

Plants have been used for therapeutic purposes since ancient times. Currently, the aim of studies on herbal extracts is to determine new herbal remedies, which can be used in the treatment of a disease, and then to determine their indications, their comparative efficacy, and their potential side effect profiles (Falzon & Balabanova, 2017). Therefore, studies with plant extracts have gained importance.

Myrtus communis L. subsp. *communis* Ic: Sibth. & Sm. (Myrtaceae) is a traditional medicinal herb known for its use in alleviating various diseases, such as inflammation, ulcers, diarrhea, and respiratory and digestive system disorders (Aykac et al., 2019; Hennia, Miguel, & Nemmiche, 2018; Jabri, Marzouki, & Sebai, 2018). Antioxidants or the anti-inflammatory properties of *M. communis* leaves have been reported in previous experimental studies, such as liver fibrosis (Sen et al., 2016), ear edema and granuloma (Maxia, Frau, Falconieri, Karchuli, & Kasture, 2011), mouse paw edema (Rossi et al., 2009), pulmonary fibrosis (Samareh-Fekri et al., 2018), and colonic inflammation in rats (Sen et al., 2017). Nevertheless, the effect of *M. communis subsp. Communis* leaves on AP is unknown. In light of this information, it was expected that *M. communis* subsp. *communis* leaf ethanol extract (MC) may be a useful treatment strategy for protecting pancreatic tissue in AP. Therefore, the aim of this study was to determine the effect of MC on the development of experimental acute cerulein-induced pancreatitis.

2 | MATERIALS AND METHODS

2.1 | Animals and chemicals

Thirty-two Wistar albino rats (male, 200–250 g) were used. The rats were kept in cages (four rats per cage) under controlled temperature (22 ± 2°C) and moisture (63%–67%) levels for a 12-hours light/12-hours dark period. Feeding was done ad libitum. All study procedures were permitted by the Local Ethics Committee, Istanbul-Turkey (Marmara University; 62.2018.mar), and the Directive 2010/63/EU was followed. Animals were purchased from the Experimental Animals Application and Research Center, Breeding Unit (DEHAMER, Istanbul-Turkey).

Cerulein, 5-amino-2,3-dihydrophthalazine-1,4-dione (luminol), bis-N-methylacridium nitrate (lucigenin), dimethylsulfoxide (DMSO), 2-(4-(2-hydroxyethyl)piperazin-1-yl) ethanesulfonic acid (HEPES), hexadecyltrimethylammonium bromide (HETAB), N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid), trichloroacetic acid (TCA), thiobarbituric acid (TBA), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), o-Dianisidine, $Na_2HPO_4.2H_2O$, K_2HPO_4, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), potassium persulfate, indomethacine, ascorbic acid, trolox, lipoxidase from Glycine max (soybean), Folin-Ciocalteu reagent, linoleic acid, formaldehyde, methanol, and ethanol were obtained from Merck KGaA (Darmstadt, Germany). Sodium pentobarbital was purchased from IE Ulagay (Istanbul, Turkey). All other chemicals were of the purest grade commercially available.

2.2 | Plant material and extraction

The leaves of the plant were collected during flowering periods from the Aegean district (Turgutlu, Manisa) of Turkey. Voucher specimens were deposited at the Herbarium unit (School of Pharmacy, Marmara University, MARE No: 13006). *Myrtus communis* subsp. *communis* leaves were dried in the shade and powdered by a mechanical grinder. A total of 100 g of powdered material was extracted with ethanol (96%) using a Soxhlet device, and extraction was continued until the solution became colorless (up to 24 hr). The obtained ethanol extract was dried under a vacuum at 40°C. The powder ethanol extract (MC) obtained from the extraction of 100 g of powdered leaves was 28.56 g. This powder extract was used for activity tests and stored in the dark and refrigerated (4°C) until use.

2.3 | In vitro antioxidant and anti-inflammatory activity

The DPPH radical scavenging activity of the extract was measured according to Zou et al. (Zou, Liu, Zhang, & Xiang, 2017). Briefly, 10 µl of extracts in DMSO at different concentrations (5000–9.77 µg/ml) was added to a 190-µl methanol solution of DPPH (0.1 mM) in a well of 96-well plates. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 minutes. Absorbance readings were taken at 517 nm. The percentage of the radical scavenging activity of the extract and standard against DPPH was calculated according to the following formula:

$$\text{DPPH radical} - \text{Scavenging activity } (\%) = \left[\left(A_0 - A_1\right)/A_0\right] \times 100.$$

where A_0 is the absorbance of the control (containing all reagents except the test compounds), and A_1 is the absorbance of the extract/standard. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting the inhibition percentage against the extract concentration. Tests were carried out in triplicate. Ascorbic acid was used as the positive control.

The ABTS radical scavenging activity of the extract was measured according to Zou et al. (Zou et al., 2017). Briefly, ABTS radical cations were prepared by mixing an equal volume of ABTS (7 mM in H_2O) and potassium persulfate (4.9 mM in H_2O), allowing them to react for 12–16 hours at room temperature in the dark. Then, the ABTS radical solution was diluted with 96% ethanol to an absorbance of about 0.7 at 734 nm. Ten microlitres of the extract in DMSO at different concentrations (5000–9.77 µg/ml) was added to 190 µl of the ABTS radical solution in a well of 96-well plates. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 minutes. Absorbance readings were taken at 734 nm. The percentage of the radical scavenging activity of the extract and standard against ABTS was calculated according to the following formula:

$$\text{ABTS radical} - \text{Scavenging activity } (\%) = \left[\left(A_0 - A_1\right)/A_0\right] \times 100.$$

where A_0 is the absorbance of the control (containing all reagents except the test compounds), and A_1 is the absorbance of the extract/standard. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting the inhibition percentage against the extract concentration. Tests were carried out in triplicate. Trolox was used as the positive control.

The anti-inflammatory activity was evaluated as described by Phosrithong and Nuchtavorn with slight modifications described by Yıldırım et al. (Phosrithong & Nuchtavorn, 2016; Yıldırım, Şen, Doğan, & Bitiş, 2019). Ten microlitres at different concentrations of the extract (5000–9.77 µg/ml) or standard indomethacine (250–0.49 µg/ml) was added to a 20-µl ethanol, 20-µl pure water, and 25-µl sodium borate buffer solution (0.1 M, pH 9) followed by the addition of 25 µl of a type V soybean lipoxygenase solution in a buffer (pH 9, 20,000 U/ml). After the mixture was incubated at 25°C for 5 minutes, 100 µL of 0.6 mM linoleic acid solution was added and mixed well, and the change in absorbance at 234 nm was recorded for 6 minutes. Indomethacine was used as a reference standard. The percent inhibition was calculated using the following equation:

$$\%\,\text{inhibition} = \left[\left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}}\right] \times 100.$$

A dose-response curve was plotted to determine the IC_{50} values. IC_{50} is defined as the concentration sufficient to obtain 50% of the maximum anti-inflammatory activity. All tests and analyses were performed in triplicate.

2.4 | Determination of the total phenolic content of MC

The total phenolic content of the extract was measured as described by Gao et al. with slight modifications described by Yıldırım et al. (Gao, Ohlander, Jeppsson, Björk, & Trajkovski, 2000; Yıldırım et al., 2019). Ten microlitres of the extract in various concentrations (5000–9.77 µg/ml) was mixed with 20 µl of the Folin–Ciocalteu reagent, 200 µl of H_2O, and 100 µl of 15% Na_2CO_3, and the absorbance was measured at 765 nm after 2 hours of incubation at room temperature. Gallic acid was used as a standard, and the total phenolics were expressed as the mg gallic acid equivalent per g powder ethanol extract.

2.5 | Experimental design

The animals were assigned to four groups (randomly and each one: n = 8) as the saline-pretreated control (Control), MC-pretreated control (MC), saline-pretreated AP, and MC-pretreated AP groups (MC + AP). To induce AP, the rats received 50 µg/kg of cerulein intraperitoneally (ip) two times at a one-hour interval (n = 16), while the control group received saline (Dur et al., 2016; Ozturk, Gul, Esrefoglu, & Ates, 2008). MC dissolved in distilled water or saline was administered by oral gavage (100 mg kg^{-1} day^{-1}) before the first cerulein injection once per day for 14 days. The rats were fasted for 12 hours before the initial cerulein injection. The dose of MC was chosen based on our previous study, which indicated that the selected dose of MC has an effective anti-inflammatory activity

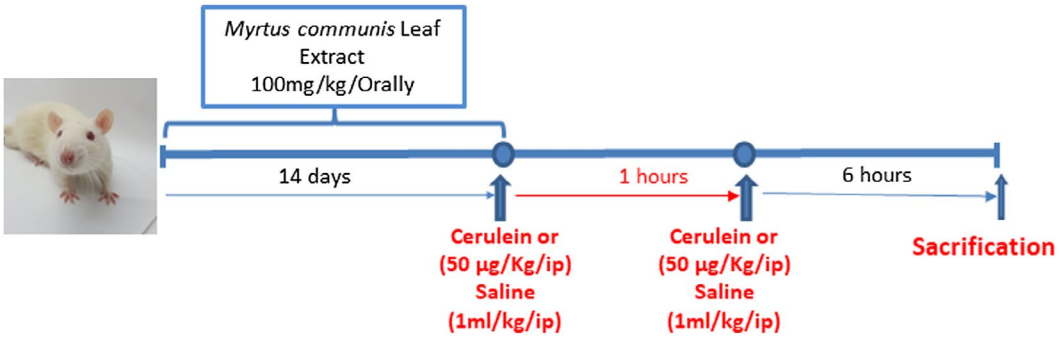

FIGURE 1 Experimental protocol of rats

(Sen et al., 2016, 2017). Six hours after the final injection of cerulein, blood samples were taken by cardiac puncture under sodium pentobarbital anesthesia (40 mg/kg/ip). Anesthetized animals were euthanized by cervical dislocation (Figure 1). A total of 1,000 μl of serum was obtained from 4 ml of blood. The serum samples were utilized for amylase and lipase analysis. The abdominal area was opened longitudinally, and the pancreas was carefully dissected and proportioned to body weight. Half of the pancreas tissue samples was stored (−80°C) for biochemical analysis, while the other half was immediately fixed with formaldehyde and hematoxylin-eosin staining for the histological evaluation. The parameters of the oxidative status in the pancreatic tissue, luminol and lucigenin chemiluminescence (CL), malondialdehyde (MDA), and reduced glutathione (GSH) levels were measured. Myeloperoxidase enzyme (MPO) activity, IL-1β, IL-6, and IL-10 levels in the pancreas were also tested.

2.6 | Serum amylase and lipase levels

The collected blood specimens were centrifuged (3,000 rpm, 10 min, 4°C). Amylase and lipase activity in the sera was measured using automated devices (Modular Systems, Abbott Diagnostics, CA, US) and enzyme assay kits (Amylase, REF: 7D58 and Lipase, REF: 7D80, Abbott Diagnostics, CA, US) in accordance with the manufacturer's prospectus.

2.7 | Malondialdehyde, glutathione levels, and myeloperoxidase enzyme activity

Freshly thawed from −80°C pancreatic tissue samples (0.2 to 0.5 g) were homogenized (Ultra Turrax homogenizer) with cold trichloroacetic acid (w/v, 10%) and centrifuged at 3,000 rpm for 15 minutes at 4°C. The supernatant was removed and recentrifuged at 10,000 rpm at 4°C for 5 minutes. A total of 0.75 ml of the supernatant and an equal volume of a TBA solution were boiled for 15 minutes. MDA levels were measured for products of lipid peroxidation by observing the formation of thiobarbituric acid reactive substances, which have a maximum absorbance of 532 nm as previously described. The results were calculated and stated as nmol MDA/g tissue (Beuge & Aust, 1978).

GSH levels were measured using a modified Ellman procedure (Beutler, 1975). After centrifugation at 10,000 rpm for 10 minutes at 4°C, 0.5 ml of the supernatant was added to 2 ml of the 0.3-mol/L $Na_2HPO_4.2H_2O$ solution. A 0.2-ml solution of DTNB, 0.4 mg/ml of 1% sodium citrate was added, and the absorbance was read at 412 nm.

MPO enzyme activity as a marker of the polymorphonuclear leukocyte gathering in inflamed tissues was tested spectrophotometrically. Freshly thawed −80°C pancreatic samples (0.2–0.5 g) were homogenized in a cold potassium phosphate buffer (PBS, 20 mM K_2HPO_4, pH 7.4) and centrifuged at 12,000 rpm for 10 minutes at 4°C. The obtained pellet after discarding the supernatant was re-homogenized with a cold K_2HPO_4 solution that contained HETAB (0.5%,w/v). MPO activity was assessed by the measurement of the H_2O_2-dependent oxidation of o-dianisidine 2HCl. A change in the enzyme activity determined at 460 nm and 37°C was described as a U/min change. The calculated MPO activity was referred to as (U/g tissue) (Hillegass, Griswold, Brickson, & Albrightson-Winslow, 1990).

2.8 | Chemiluminescence assay

Light-emitting chemical reactions of the reactive oxygen species (ROS) were measured with a Junior LB 9509 luminometer apparatus (EG&G, Germany). Before the measurements tissue specimens were cut for approximately 20–25 mg weight and put into test tubes containing PBS + HEPES [(500 mM phosphate buffered saline + 20 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)]. Luminol (chemical name is 5-amino-2,3-dihydrophthalazine-1,4-dione) or lucigenin (chemical name is bis-N-methylacridium nitrate) enhancers were diluted in DMSO and added for an ultimate final concentration of 0.2 mmol/L (4 μl) to the test tubes containing PBS + HEPES and tissue samples. The readings were done at 1 minute periods for 5 minutes. After the CL measurements the estimate results for a counting period of 5 minutes were calculated as the area under curve (AUC), compared to wet weight and given as relative light unit/mg tissue (rlu/mg tissue) (Haklar, Sayin-Ozveri, Yüksel, Aktan, & Yalcin, 2001; Simmonds et al., 1992).

2.9 | Tissue IL-1β, IL-6, and IL-10 levels

Freshly thawed −80°C pancreatic tissue samples were homogenized and rotated (6,000 rpm, 10 min, 4°C). An enzyme-linked immunosorbent assay (ELISA) was performed to determine the IL-1β, IL-6, and IL-10 levels according to the manufacturer's manual (EBIOSCIENCE, Thermo Fisher Scientific, MA, USA).

2.10 | Histological assessment

The collected tissue samples were evaluated with light microscopy. Samples were stained with hematoxylin-eosin dye. Each section was evaluated, and histological scoring was done using the technique from Warzecha et al. (2008) (Table 1). The sections were photographed using a digital camera attached light microscope (Olympus BX51 and Olympus DP72, Tokyo, Japan).

2.11 | Statistical analyses

The statistical analysis of the parameters was done by a one-way analysis of variance (ANOVA) supplemented with Tukey's multiple comparison tests. Histological scores were assessed by the

TABLE 1 Criteria for microscopic scoring of pancreatic damage

Score	Appearance
0: No edema	Interlobular edema
1: Interlobular edema	
2: Interlobular and moderate interlobular edema	
3: Interlobular and severe interlobular edema	
0: Absent	Leukocytic infiltration
1: Scarce perivascular infiltration	
2: Moderate perivascular and scarce diffuse infiltration	
3: Abundant diffuse infiltration	
0: Absent	Grading of vacuolization
1: Less than 25%	
2: 25%–50%	
3: More than 50%	
0: Absent	Hemorrhage
1: 1–2 hemorrhagic foci per slide	
2: 3–5 hemorrhagic foci per slide	
3: More than 5 hemorrhagic foci per slide	
0: No necrosis	Necrosis
1: Less than 15% of pancreatic cells involved	
2: 15%–35% of cells involved	
3: More than 35% of cells involved	

Mann–Whitney *U* test (Graph Pad Prism 6.0; San Diego, CA). The variances were postulated as significant if $p < .05$ and were stated as mean ± S.E.M.

3 | RESULTS

3.1 | Antioxidant/anti-inflammatory activity and total phenolic content of MC

MC with an IC_{50} value of 12.66 ± 0.18 μg/ml showed a stronger antioxidant activity when compared with the standard (IC_{50} for Ascorbic acid: 17.60 ± 0.37 μg/ml) against the DPPH radical. In addition, MC with an IC_{50} value of 17.29 ± 0.13 μg/ml exhibited an antioxidant activity as potent as the standard $_{50}$ for Trolox: 17.22 ± 0.00 μg/ml) against the ABTS radical. The MC exhibited remarkable anti-inflammatory activity with an IC_{50} value of 70.33 ± 2.75 μg/ml against a 5-lipoxygenase enzyme (IC_{50} for standard indomethacin: 22.39 ± 0.26 μg/ml). The total phenolic content of the MC was found to be 472.7 ± 2.36 mg/g extract as the gallic acid equivalent.

3.2 | Serum lipase and amylase levels

In comparison with the control group, the lipase and amylase levels in the sera were elevated in the AP group ($p < .0001$). This increase was remarkably diminished in the MC + AP group ($p < .0001$), while it still remained significantly higher than in the control group ($p < .01$–$.05$; Figure 2a,b).

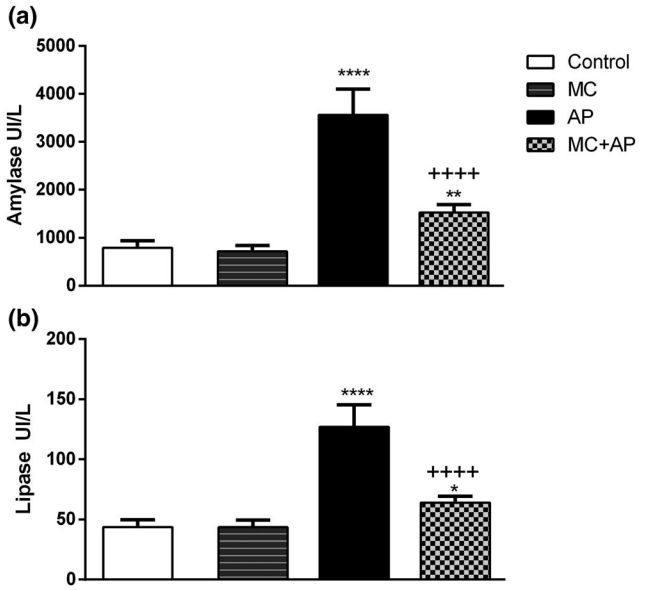

FIGURE 2 (a) Amylase and (b) lipase levels in sera of Control, MC, AP, and MC + AP groups. Each group consists of eight animals. The range bars show the *SEM*. $^{*}p < .05$, $^{**}p < .01$ and $^{****}p < .0001$ in comparison with saline treated control group; $^{++++}p < .0001$ in comparison with AP group. MC: *Myrtus communis* leaf ethanol extract treatment; AP: Cerulein-induced acute pancreatitis

3.3 | MDA, GSH levels, and MPO activity

In comparison with the control group, a significant elevation was observed in the MDA levels of the AP group ($p < .01$). This elevation significantly decreased in the MC + AP group ($p < .01$; Figure 3a).

Concomitant with increased MDA levels, GSH was significantly depressed in the AP group in comparison with the control group ($p < .01$); however, a marked elevation of GSH levels was observed with MC treatment in the MC + AP group ($p < .05$; Figure 3b).

MPO activity was found to be significantly increased in the AP group in comparison with the control group ($p < .0001$). In the MC + AP group, pancreatic MPO activity was diminished with MC treatments in comparison with the AP group ($p < .001$; Figure 3c).

In addition, in terms of all examined parameters, there were no significant differences between the control and MC groups.

3.4 | Pancreatic ROS levels

Pancreatic ROS release, which was determined by luminol or lucigenin-based CL, showed a marked elevation in the AP group in comparison with the control group ($p < .05$–$.001$, respectively). The increase in the CL release of the AP group was significantly decreased by MC applications ($p < .01$–$.05$, respectively; Figure 4a,b).

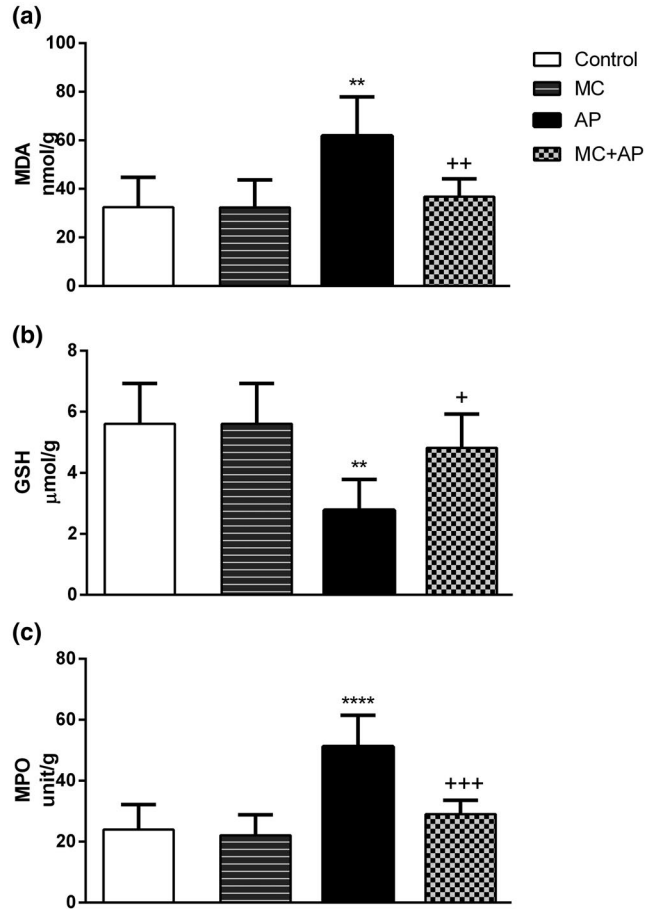

FIGURE 3 (a) MDA, (b) GSH and (c) MPO activity levels in the pancreatic tissues of Control, MC, AP, and MC + AP groups. Each group consists of eight animals. The range bars show the *SEM*. $^{**}p < .01$ and $^{****}p < .0001$ in comparison with control group; $^{+}p < .05$, $^{++}p < .01$ and $^{+++}p < .001$ in comparison with AP group. AP, cerulein-induced acute pancreatitis; MC, *Myrtus communis* leaf ethanol extract treatment

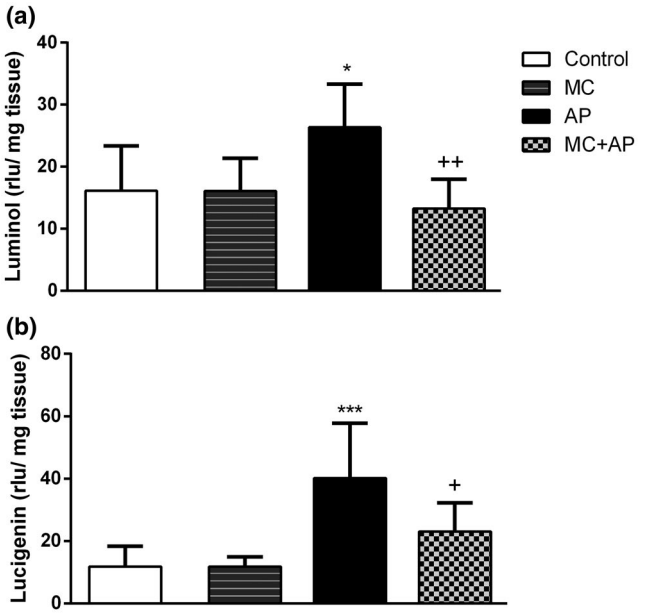

FIGURE 4 (a) Luminol and (b) lucigenin chemiluminescence levels in the pancreatic tissues of Control, MC, AP, and MC + AP groups. Each group consists of eight animals. The range bars show the *SEM*. $^{*}p < .5$ and $^{***}p < .001$ in comparison with control group; $^{+}p < .05$, $^{++}p < .01$ in comparison with AP group. AP, cerulein-induced acute pancreatitis; MC, *Myrtus communis* leaf ethanol extract treatment

3.5 | Pancreatic cytokines

The IL-1β and IL-6 levels of the AP group were found to be markedly higher than in the control group ($p < .0001$), and the treatment of rats with MC decreased these elevations ($p < .001$–$.01$, respectively; Figure 5a,b). The levels of anti-inflammatory IL-10 were found to be significantly lower in the AP group ($p < .0001$), while they were found to be increased in the MC group in comparison with the AP group ($p < .05$; Figure 5c).

3.6 | Pancreatic edema index

The pancreatic edema index is explained by the wet weight/body weight ratio of the pancreas (Carvalho et al., 2010). Edema caused by cerulein significantly increased pancreatic weights in comparison with the control group ($p < .01$). The increased pancreatic weights were found to be lower with MC treatment ($p < .05$) (Figure 6a).

3.7 | Histological assessments

The results have been expressed as a predominant histological grading of each sign of pancreatic damage in each experimental group of animals (Table 2). In both the control and the MC groups, regularly organized acinar structures and Langerhans islets in the parenchymal tissues were observed (Figure 7a,b). In contrast, in the AP group, acinar cells with moderate cytoplasmic vacuolization, edema, and inflammatory cell infiltration were observed, and these appearances significantly increased in the histological damage score ($p < .01$; Figures 6b and 7c; Table 2). In the AP + MC group, vacuolization in acinar cells, interstitial edema, and inflammatory cell infiltration were observed to be decreased when compared with the AP group (Figure 7d; Table 2). Accordingly, the histopathologic damage scores were alleviated with MC in AP in comparison with the AP group ($p < .001$; Figure 6b). In addition, there was no marked difference between the MC group and the control group.

4 | DISCUSSION

The pathophysiology of AP is not yet fully explained (Cavestro et al., 2015). AP is usually mild in patients, with approximately 20%–30% developing a severe form of single or multiple organ dysfunction (Leppäniemi et al., 2019). Gallstones, alcohol, drugs, and genetic factors are the risk factors for AP in humans (Lankisch, Apte, & Banks, 2015).

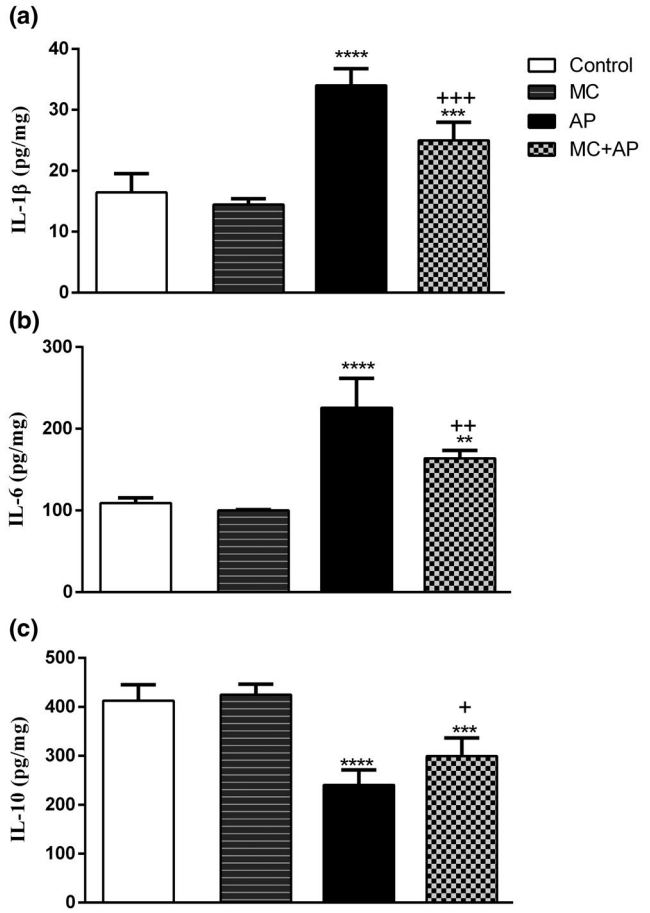

FIGURE 5 (a) IL-1β, (b) IL-6, and (c) IL-10 levels in the pancreatic tissues of Control, MC, AP, and MC + AP groups. Each group consists of eight animals. The range bars show the *SEM*. $^{**}p < .01$, $^{***}p < .001$ and $^{****}p < .0001$ in comparison with control group; $^{+}p < .05$, $^{++}p < .01$ and $^{+++}p < .001$ in comparison with AP group. AP, cerulein-induced acute pancreatitis; MC, *Myrtus communis* leaf ethanol extract treatment

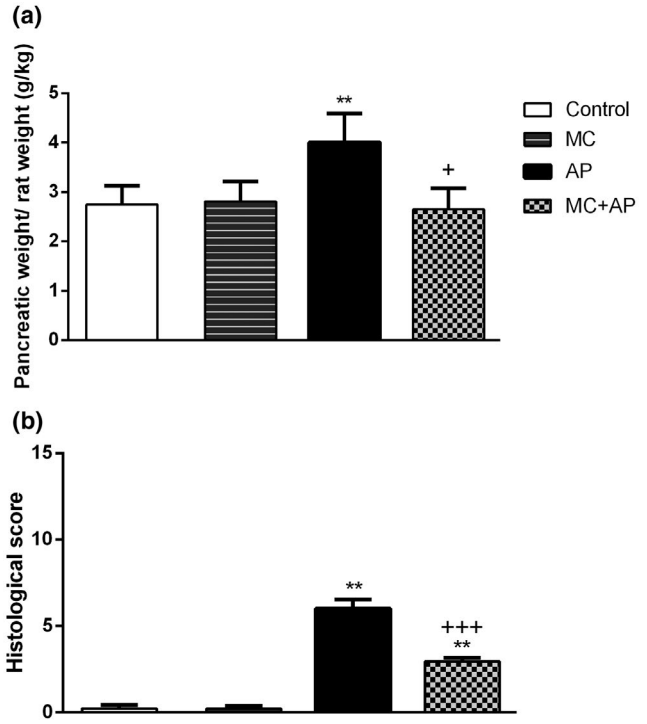

FIGURE 6 (a) The pancreatic wet weight/body weight ratio and (b) histological score of pancreatic tissue of Control, MC, AP, and MC + AP groups. Each group consists of eight animals. The range bars show the *SEM*. $^{**}p < .01$ in comparison to control group; $^{+}p < .05$ and $^{+++}p < .001$ in comparison to AP group. AP, cerulein-induced acute pancreatitis; MC, *Myrtus communis* leaf ethanol extract treatment

Although the circumstances that cause AP in humans and animals are dissimilar, the mechanisms of cellular injury, such as the premature intrapancreatic activation of trypsinogen observed are similar (Cruz-Santamaría, Taxonera, & Giner, 2012; Lankisch et al., 2015). Premature intrapancreatic activation of trypsin causes an increase in ROS and inflammatory mediators. This triggers cellular damage and clinical manifestations of the disease (Goodchild et al., 2019; Pérez et al., 2015). Previous studies have demonstrated that ROS formation is closely linked to AP (Kim, 2008; Thareja, Bhardwaj, Sateesh, & Saraya, 2009). In addition, one of the major antioxidants, GSH, decreases in the early stages. This decrease also determines the severity of the disease (Escobar et al., 2009). Recently, the published guidelines for AP treatment in China stated that Traditional Chinese Medicine (TCM), which involves the use of single plants or formulas, can be used as a promising complementary therapy for AP. It has also been demonstrated by experimental and clinical studies that plants and formulations used in TCM are effective in the treatment of AP (Li, Zhang, Zhou, Zhang, & Li, 2017; Miao et al., 2018). In this study, the beneficial effects of MC on AP have been explored. The results showed that MC treatment reduces oxidative damage and acute inflammation.

Cerulein, an analog of cholecystokinin, is widely used for the induction of the AP model in animals (Ceranowicz, Cieszkowski, Warzecha, & Dembiński, 2015; Lerch & Gorelick, 2013). Cerulein leads to the synthesis of excessive amounts of zymogen enzymes, such as amylase and lipase, and pancreatic liquid contents, thus causing pancreatitis.

TABLE 2 Predominant histological grading of each sign of pancreatic damage in each experimental group of animals

Control	Edema	Leukocytic infiltration	Vacuolization	Necrosis	Hemorrhage
Control	0.1	0	0.1	0	0
MC	0	0	0	0	0
AP	2	2	2	0	0
MC + AP	1	1	1	0	0

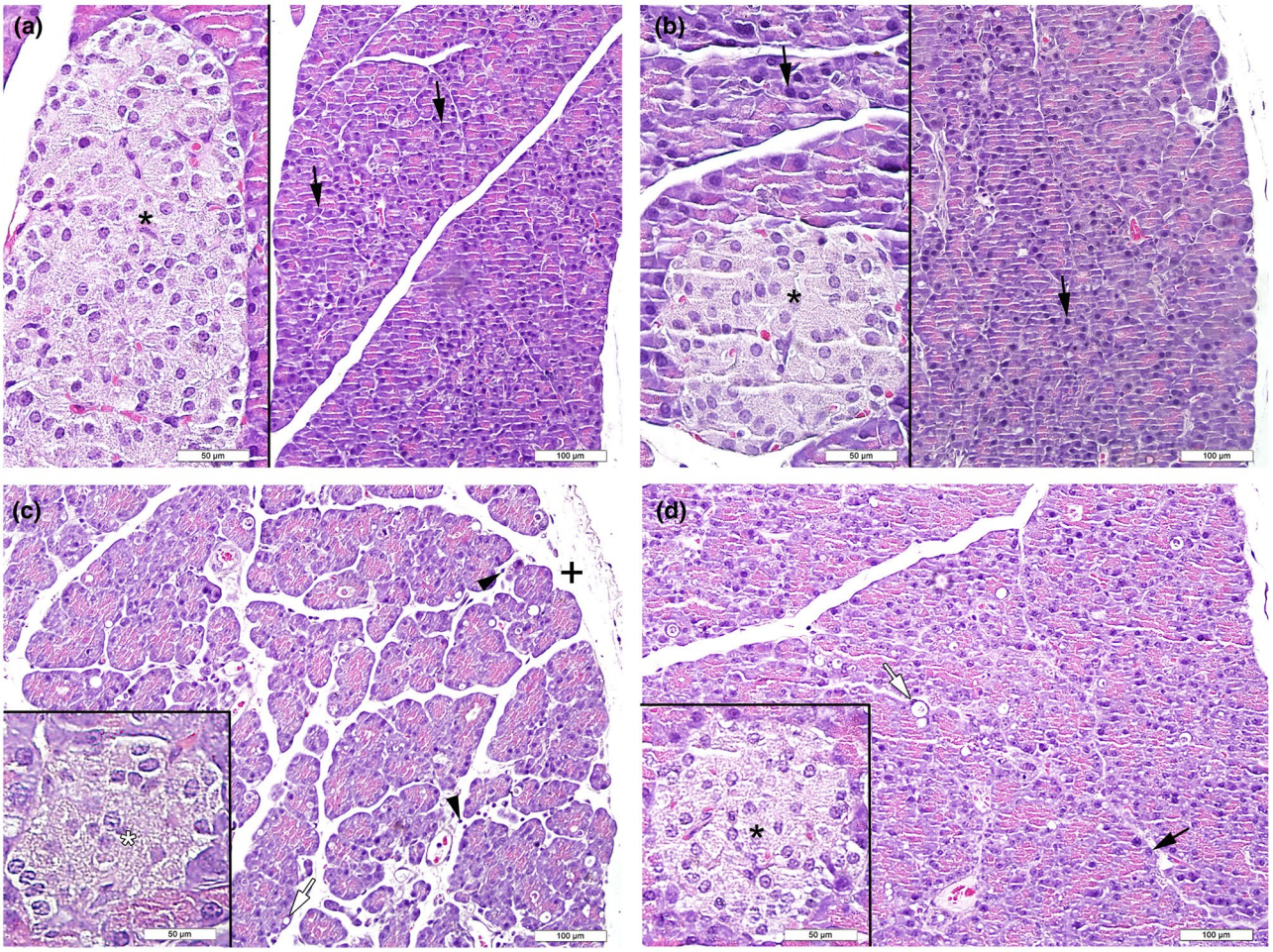

FIGURE 7 Demonstrative images of Control, MC, AP, and AP + MC groups. (a) Control group, (b) MC group, (c) AP group, (D) AP + MC group. Black arrow: Acinar structure with regular morphology. Asterisk (*): Regularly organized Langerhans islet. White arrow: Vacuolization in the acinar cell. Black arrowhead: Inflammatory cell infiltration. White asterisk: Abnormally organized Langerhans islet. Plus (+): Interstitial edema. Hematoxylin & Eosin stain, bars: 50 and 100 µm. AP, cerulein-induced acute pancreatitis; MC, *Myrtus communis* leaf ethanol extract treatment

Pancreatic enzymes are produced and stored in pancreatic acinar cells as zymogen granules. Proteases present in zymogen granules occur in an inactive form. Physiologically, zymogen granules are secreted by exocytosis at the apical part of pancreatic acinar cells to the lumen of pancreatic acini, and then they are transferred to the duodenum, where trypsinogen is activated by duodenal enterokinase to trypsin. Newly formed molecules of trypsin activate the next molecules of trypsinogen and other inactive pancreatic proteases. When AP occurs, this process is disturbed. The administration of supramaximal doses of cerulein zymogen vacuoles accumulate at the basolateral part of the acinar cells and then fuse with lysosomes to form large vacuoles. Lysosomal enzymes, especially catepsin D, lead to a premature intracellular activation of pancreatic digestive proteases. This process leads to the damage of cellular organelles and the fusion of large vacuoles containing active digestive enzymes with the basolateral cell membrane of acinar cells. Active digestive enzymes enter the interstitial space of the pancreas, leading to the development of AP (Adler, Rohr, & Kern, 1982; Kern, Adler, & Scheele, 1985; Saito, Hashimoto, Saluja, Steer, & Meldolesi, 1987). This mechanism was observed in cerulein-induced AP as well as in other models of AP (Saluja et al., 1989). Cerulein also leads to the accumulation of inflammatory cells in the pancreas and vacuolization in acinar cells (Kim, 2008). Moreover, cerulein has been shown to induce nuclear transcription factor NF-κB and cytokine expression by causing ROS formation in in vitro studies (Yu, Lim, Namkung, Kim, & Kim, 2002). The cerulein-induced AP model used in this study led to the development of acute edematous pancreatitis. In rats, the severity of this model of AP is minimal and corresponds to a clinical mild edematous AP (Kim, 2008).

Adequate tissue blood flow is necessary for the appropriate function and maintenance of organs' integrity. Previous clinical (Gullo et al., 1996; Warshaw & O'Hara, 1978) and experimental animal studies (Ceranowicz et al., 2015; Dembiński et al., 2001; Vollmar & Menger, 2003; Waldner, 1992) have shown that a reduction in the pancreatic blood flow may be a primary cause of AP. Moreover, a reduction in the pancreatic blood flow is observed in AP caused by primary nonvascular factors, and the development of AP is associated with microvascular disturbances with a subsequent formation of thrombi in the capillaries, the activation of leukocytes, and the release of digestive enzymes and

pro-inflammatory cytokines (Gress, Arnold, & Adler, 1990; Kusterer, Enghofer, Zendler, Blochle, & Usadel, 1991; Tomkötter et al., 2016; Vollmar & Menger, 2003). In light of this knowledge, the inflammatory status of pancreatic tissues through MPO activity and cytokines has been investigated. It is well-known that leukocyte recruitment within the inflamed pancreas begins in the early phase of AP (Escobar et al., 2009). The increase in MPO activity shows widespread inflammation in many inflammation models, including AP (Gurler et al., 2019; Ozgul et al., 2019; Sen et al., 2016). This increase in enzyme activity causes the formation of oxidant molecules by neutrophils and initiates oxidant damage. Therefore, the inhibition of this inflammation could prevent tissue damage. MC treatment may decrease MPO activity in colonic and liver tissues (Sen et al., 2016, 2017). Rossi et al. (2009) reported that *M. communis* has the capability to suppress adhesion molecules and to inhibit Leukotriene B4 generation and polymorphonuclear leukocyte infiltration. Concomitant with previous studies, the current results demonstrated a decrease in MPO activity with MC treatment, which indicates the anti-inflammatory effects of MC.

Acute pancreatitis causes an important increase in the tissue IL-1β and IL-6 levels. Maxia et al. (2011) have reported that *M. communis* oil inhibited inflammation in rats via mitigating IL-6 levels in the sera. In the carrageenan-induced pleurisy model, a reduction in the IL-1β level with *M. communis* treatment has also been reported (Rossi et al., 2009). In addition to these cytokines, IL-10 levels were also investigated in the AP model. The results showed a significant cerulein-induced decrease in IL-10, which was reversed by MC treatment. To the authors' knowledge, the effect of MC on the IL-10 level has not been studied previously in any inflammation model. For the first time, the current results showed that MC has an enhancing effect on IL-10.

There is a cross-talk between pro-inflammatory cytokines and ROS in AP (Escobar et al., 2009). It is well-known that the inflammation is moderated by a balance between pro-inflammatory and anti-inflammatory mediators. Thus, in light of the results, it is suggested that MC shows its anti-inflammatory effect by inhibiting this cross-talk. The histopathological findings confirmed these effects by showing a decrease in inflammation. Based on these results, the suppression of inflammation and the prevention of damage to pancreatic tissue can be considered to provide tissue integrity and to positively affect tissue blood flow.

Serum lipase or amylase levels are the major biomarkers in AP and the levels begin to rise within 3–6 hours in AP (Ozgul et al., 2019). It is reported that effective treatments in experimental AP models inhibited increased enzyme levels, even if the values did not decrease to control levels (Bulut et al., 2011; Ozgul et al., 2019). Similarly, although the amylase and lipase levels in this study did not decrease to the control levels, the results showed that because the enzyme levels were found to be significantly reduced in comparison with the AP group, MC protects tissue and re-regulates enzyme secretions.

The ROS levels in the pancreatic tissue were also examined using luminol and lucigenin CL probes. This is a simple technique for assessing oxidant formation in tissues. Increased CL values suggest that oxidant substances are produced in tissues due to increased inflammation, and these oxidant molecules play a role in damage. Both CL values significantly decreased with MC treatment, similar to recent reports (Hayder et al., 2004; Hennia et al., 2018).

Moreover, the oxidant-induced MDA level indicated that lipid peroxidation was also reversed. Increased lipid peroxidation in bile or pancreas tissue have been reported in humans with AP (Park et al., 2003). Increasing MDA levels and decreasing GSH levels reflect tissue damage in AP (Guleken et al., 2017; Huang & Cao, 2014; Mirmalek et al., 2016). The model verifies tissue damage, not only histologically but also biochemically, with GSH depletion and MDA elevation. On the contrary, similar to previous reports (Escobar et al., 2009; Sen et al., 2016, 2017), the study results showed that MC treatment exerted a notable reduction in lipid peroxidation and an increase in GSH levels. This can be explained by the fact that *M. communis* has a strong free radical scavenging capacity, as shown previously (Hayder et al., 2004; Hennia et al., 2018) and demonstrated in the present study.

In addition to the anti-inflammatory and antioxidant effects of *M. communis*, the present study revealed that a high phenolic compound content of *M. communis* was consonant with other studies (Amensour et al., 2009; Sen et al., 2016). Phenolic complexes are largely known to have antioxidant and anti-inflammatory activities (Lin et al., 2016). Therefore, the beneficial effects of MC on AP may be due to the existence of phenolic compounds, such as phenolic acids, flavonoids, and tannins found in MC.

This study has a limitation. The experiments were performed with the cerulein-induced AP model in rats. This model produces mild edematous AP; however it does not completely mimic human AP. Therefore, the effect of MC should be investigated in future studies using a more severe model with tissue necrosis to fully mimic the human disease.

5 | CONCLUSION

This study suggests that *M. communis* subsp. *communis* protects the antioxidant status of the pancreatic tissue in AP, ameliorates oxidative pancreatic injury via a neutrophil-dependent mechanism, and modulates anti-inflammatory and pro-inflammatory cytokines. *M. communis* subsp. *communis* may be a useful treatment strategy to protect pancreas from both AP and recurrent attacks.

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CONFLICT OF INTERESTS

The authors state that we have no potential conflict of interest for this study.

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