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Exploring the cytoprotective potential of oleuropein against indomethacin-induced gastric ulcer in rats

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Abstract: Indomethacin (INDO) can induce gastric ulcers via oxidative stress and apoptosis. This study aimed to investigate the protective effects of oleuropein (OLE), a natural antioxidant from olive plants, against INDO-induced gastric ulcers in rats, focusing on the modulation of growth factors and apoptotic pathways. Forty-eight male Wistar rats were divided into six groups: control, INDO, lansoprazole (LAN), and OLE doses (6, 12, and 18 mg/kg). INDO was administered to induce ulcers, followed by respective OLE treatments. Analyses included comet assays for DNA damage, assessment of oxidative/antioxidative enzyme activities, qRT-PCR for growth factor expression, Western blots for apoptotic protein levels, and histopathological examination. INDO significantly increased ulcer area, malondialdehyde (MDA) levels, and apoptosis while reducing antioxidant enzymes and growth factor expression. OLE (18 mg/kg), significantly reduced ulcer area ($P<0.001$), increased activities of superoxide dismutase (SOD) and glutathione (GSH), decreased MDA levels ($P<0.001$) and protected lymphocytes from DNA damage. OLE also attenuated the INDO-induced downregulation of EGF, EGFR, VEGF, VEGFR1, and VEGFR2 ($P<0.05$). OLE counteracted the increase in Bax and caspase-3/9 and the reduction in Bcl-2 expression ($P<0.05$). OLE showed strong protection against INDO-induced ulcers by reducing oxidative stress, regulating growth factors, and inhibiting apoptosis, supporting its potential for treating gastric ulcers.

Keywords: Oleuropein, gastric ulcer, apoptosis, oxidative stress, indomethacin

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INTRODUCTION

NSAIDs cause direct irritation of the gastric mucosa, leading to erosion of the stomach wall. This leads to gastric ulcers by damaging the epithelial cells in the stomach tissue (Tai and McAlindon, 2021). Since NSAIDs are acidic drugs, they penetrate through the stomach wall and ionize in the intracellular region. This causes apoptosis of epithelial cells within hours. Studies have shown that 25% of patients using NSAIDs develop ulcers, and 2-3% of these patients develop complications such as gastric bleeding or perforation (Bindu, Mazumder *et al*., 2020, Handa, Naito *et al*., 2014).

INDO, an NSAID, has a high potential to cause gastric ulcers. INDO has been shown to induce oxidative damage, characterized by an induction of lipid peroxidation and the formation of free radicals in the biological system (Maity, Bindu *et al*., 2009). The development of gastric ulcers in response to INDO administration is associated with elevated ulcer index values and impaired mucosal defense mechanisms. The ulcerogenic properties of INDO have been shown to be a result of disruption of EGF and VEGF and their corresponding receptors in the gastric mucosa (Musumba, Pritchard *et al*., 2009, Shaik and Eid, 2022).

In order to develop treatment strategies, it is important to understand the mechanisms that control gastric tissue reactions, particularly about gastric ulcers, which are initiated by VEGF and EGF interacting with their respective receptors. When VEGF, which regulates angiogenesis, the process by which new blood vessels form, reacts with its receptor (VEGFR1 and VEGFR2), it triggers multiple downstream signaling cascades known to suppress apoptosis (Long, Zhao *et al*., 2019, Magierowski, Magierowska *et al*., 2017). This suppression occurs through the reduction of pro-apoptotic proteins Bcl-2-associated X (Bax) and the increase of anti-apoptotic proteins B-cell lymphoma-2 (Bcl-2) (Antonisamy, Kannan *et al*., 2014, Chen, Huang *et al*., 2018). Likewise, EGF serves a crucial function in preserving and restoring the integrity of the gastric mucosa. Activation of EGF by binding to EGFR has been shown to increase Bcl-2 expression, thereby inhibiting apoptosis and promoting cell survival in gastric epithelial cells (Huang, Chen *et al*., 2013, Nur Azlina, Qodriyah *et al*., 2017).

During the process of ulcer healing, the production of apoptosis-related proteins, including Bax, Bcl-2, and caspases, plays a vital role in gastric epithelial cells. A rise in the Bax/Bcl-2 ratio generally signals a tendency towards cell death, whereas a decline suggests cellular survival (Zhou, Wong *et al*., 2001). In the process of apoptosis, caspases play a vital role, with caspase-3 and caspase-9

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being particularly significant. As the main effector caspase, caspase-3 is responsible for the proteolytic breakdown of cellular components. This degradation results in the distinctive morphological and biochemical alterations that are hallmarks of apoptotic cell death (Tian, Srinivasan *et al*., 2024). Caspase-9 acts as an initiator caspase in the mitochondrial/intrinsic apoptotic pathway, thereby triggering the activation of execution caspase-3 (Lee, Song *et al*., 2023). Activation of caspase-9 and caspase-3 is therefore a reliable indicator of apoptosis induction. It has been shown that the PI3K/Akt pathway activated by EGF and VEGF can promote cell survival by inhibiting the activation of caspases (Kan, Hood *et al*., 2017, Shahin, Abdelkader *et al*., 2018). In contrast, under conditions of cellular stress or when growth factor signaling is compromised, activation of these caspases can lead to increased apoptosis and thus contribute to gastric ulcer etiology (Hamerlik, Lathia *et al*., 2012, Lyros, Mueller *et al*., 2010). The balance between growth factors, anti-apoptotic proteins, and pro-apoptotic proteins is crucial for the maintenance of gastric mucosal integrity. Understanding the complex mechanisms involved in this balance could potentially lead to new therapeutic approaches for gastric ulcer and other related diseases. Recently, some natural biological substances have been extensively investigated for their potential use in ulcer treatment (Fu, Wu *et al*., 2018, Sisay Zewdu and Jemere Aragaw, 2020, Song, Kim *et al*., 2019). This study aims to investigate how OLE may protect the stomach from damage induced by INDO. This natural compound, extracted from olive plants, will be evaluated for its potential to prevent gastric injury, and we will explore the mechanisms through which it operates.

In the presence of elevated VEGF levels, Bcl-2 expression is frequently upregulated, thereby promoting cell survival and reducing apoptosis in gastric epithelial cells (Jiang, Fu *et al*., 2015, Sun, Jia *et al*., 2015). Caspases, in particular caspase-3 and caspase-9, are pivotal in the execution of the apoptotic process. Activation of these pathways can influence the activation of other proteins, including those involved in the apoptotic process. It has been demonstrated that the PI3K/Akt pathway, which is activated by both growth factors, can promote cell survival by inhibiting the activation of caspases (Kan, Hood *et al*., 2017, Shahin, Abdelkader *et al*., 2018).

Conversely, in circumstances of cellular stress or when growth factor signaling is compromised, the activation of these caspases may result in augmented apoptosis, thereby contributing to the etiology of gastric ulcers (Hamerlik, Lathia *et al*., 2012, Lyros, Mueller *et al*., 2010). The balance between growth factors, anti-apoptotic proteins, and pro-apoptotic proteins is crucial for the maintenance of gastric mucosal integrity. Understanding the complex mechanisms involved in this balance could potentially lead to new therapeutic approaches for gastric ulcer and other related diseases. Recently, some natural biological substances have been extensively investigated for their potential use in ulcer treatment (Fu, Wu *et al*., 2018, Sisay Zewdu and Jemere Aragaw, 2020, Song, Kim *et al*., 2019). This study aims to investigate how OLE may protect the stomach from damage induced by INDO. This natural compound, extracted from olive plants, will be evaluated for its potential to prevent gastric injury, and we will explore the mechanisms through which it operates.

Among natural compounds, OLE has recently attracted great interest (Ferrarini, Paes *et al*., 2022, Maio, Basile *et al*., 2022, Menezes, Peres *et al*., 2022, Motawea, Abd Elmaksoud *et al*., 2020, Rishmawi, Haddad *et al*., 2022, Sahin, Yilmaz *et al*., 2023).

OLE has shown a wide range of biological activities, including anti-inflammatory, antioxidant, and cardioprotective effects (fig. 1) (Silvestrini, Giordani *et al*., 2023). Research has demonstrated that OLE can suppress the generation of free radicals, neutralize reactive oxygen species, and regulate the functioning of enzymes with antioxidant properties (Alesci, Miller *et al*., 2022). In addition, OLE was found to show anti-apoptotic effects by regulating the expression of Bcl-2 family proteins and inhibiting the activation of caspases (Achour, Arel-Dubeau *et al*., 2016). Previous studies have shown that OLE may protect against oxidative stress-induced damage in various cell types, including gastric epithelial cells. Research by Alirezaei *et al*. demonstrated that OLE administration significantly mitigated gastric ulcers induced by ethanol (Alirezaei, Dezfoulian *et al*., 2012). Additionally, Koc *et al*. found that combining OLE with thymol provided substantial protection for gastric tissues (Koc, Cerig *et al*., 2020). Our hypothesis, given the potent antioxidant and anti-inflammatory activity of OLE, it may exert a protective effect on gastric tissue by modulating the growth factors VEGF and EGF. In this study, we aimed to investigate the effects of OLE as a potential therapeutic agent for ulcers on the complex interaction of growth factors and apoptotic regulators involved in gastric tissue repair.

MATERIALS AND METHODS

Experimental animals

Forty-eight male Wistar albino rats, weighing 300 and 350 grams, were utilized. The rats were sourced from the Experimental and Clinical Research Centre at Aydin Adnan Menderes University in Turkey and underwent a two-week acclimation period before the commencement of the study. Ethical approval was obtained from Aydın Adnan Menderes University Animal Ethics Committee for the conduct of animal experiments (ethical approval number: 2016 VI. 64583101/2016/124). The ethical rules determined by the ethics committee were strictly adhered to during experimental animals.

Table 1: Experimental animal groups.

Groups	Number of animals in each group (n)
Control group	n=8
INDO group (25 mg/kg INDO)	n=8
LAN group (30 mg/kg LAN + 25 mg/kg INDO)	n=8
OLE 6 mg/kg group (6 mg/kg OLE + 25 mg/kg INDO)	n=8
OLE 12 mg/kg group (12 mg/kg OLE + 25 mg/kg INDO)	n=8
OLE 18 mg/kg group (18 mg/kg OLE + 25 mg/kg INDO)	n=8

Table 2: Primers for qRT-PCR.

Primers	Sequence
VEGF	F: 5' ATCATGCGGATCAAACCTCACC 3' R: 5' GGTCTGCATTCACATCTGCTATGC 3'
VEGFR1 (Flt-1)	F: 5' CGACACTCTTTTGGCTCCTTCTAAC 3' R: 5' TGACAGGTAGTCCGTCTTTACTTCG 3'
VEGFR2 (Flk-1)	F: 5' GTACCAAACCATGCTGGATTGC 3' R: 5' CTTGCAGGAGATTTCCCAAGTG 3'
EGF	F: 5' CCACGGTTACATTCACTCC 3' R: 5' GCTATCCAAATCGCCTTC 3'
EGFR	F: 5' GCCATCTGGGCCAAAGATACC 3' R: 5' GTCTTCGCATGAATAGGCCAAT 3'
GAPDH	F: 5' AGGTCGGTGTGAACGGATTTG 3' R: 5' GGGGTCGTTGATGGCAACA 3'

Table 3: Ulcer area (mm^2), anti-ulcer effect (%), and macroscopic damage score of INDO, LAN, and different doses of OLE administration to gastric ulcer rats (n=8 in each group).

Groups	Ulcer area (mm^2)	Anti-ulcer effect (%)	Macroscopic Damage Score
Control	0.0 ± 0.0	-	-
INDO group	1181.63 ± 158.01^{a}	0.0	4.02^{a}
LAN group	170.47 ± 32.50^{b}	83.67	9.84^{b}
OLE 6 mg/kg group	64.72 ± 15.50^{c}	94.33	3.92^{c}
OLE 12 mg/kg group	$42.29 \pm 13.55^{c,d}$	96.09	$2.12^{c,d}$
OLE 18 mg/kg group	$31.54 \pm 5.66^{c,d,e}$	96.77	$1.77^{c,d,e}$
P <0.001	***		

The ulcer area(mm^2) indicates the mean gastric damage of the animals in the groups. The anti-ulcer effect% shows the inhibition in the ulcer area of the other groups against the damage caused by INDO. Treatment groups were statistically compared with the INDO-group, while the INDO-group was compared with the control group. All values are given as mean ± SE (n = 8). Statistical differences in the same column are indicated by different letters.

Table 4: The antioxidant-oxidant parameters of gastric tissue in rats with gastric ulcers (n=8 in each group).

Groups	Parameters				
	GSH (mg/g protein)	MDA (nmol/mg protein)	SOD (U/mg protein)	CAT (k/mg protein)	MPO (mmol/min/ mg protein)
Control	38.04 ± 4.42^{a}	11.32 ± 1.78^{b}	$10,55 \pm 1,39^{a,b}$	$0,63 \pm 0,22^{a}$	$5,81 \pm 2,01^{b}$
INDO (25 mg/kg)	14.82 ± 3.38^{c}	25.77 ± 3.24^{a}	$4,18 \pm 0,64^{d}$	$0,76 \pm 0,17^{a}$	$49,92 \pm 17,52^{a}$
LAN (30 mg/kg)	8.87 ± 1.29^{c}	26.12 ± 3.18^{a}	3.2 ± 0.57^{d}	0.45 ± 0.24^{a}	29.82 ± 7.11^{a}
OLE (6 mg/kg)	17.57 ± 3.40^{b}	$24.34 \pm 3.89^{a,c}$	$5.45 \pm 1.07^{b,c,d}$	0.60 ± 0.16^{a}	$7.07 \pm 2.34^{b,d}$
OLE (12 mg/kg)	25.35 ± 6.16^{b}	$13.68 \pm 2.98^{b,c}$	$10.68 \pm 1.93^{a,b,c}$	0.91 ± 0.19^{a}	$8.21 \pm 3.17^{b,d}$
OLE (18 mg/kg)	$34.41 \pm 8.36^{a,b}$	6.00 ± 1.05^{b}	$11.26 \pm 1.41^{a,b,c}$	0.64 ± 0.04^{a}	$15.00 \pm 6.28^{b,d}$
P<	0.001	0.001	0.001	≠	0.01

The data are expressed as the mean ± SEM (n = 8) for each group. ^{a,b,c,d} Different letters in the same column represent a statistically significant difference. "≠" no significant difference

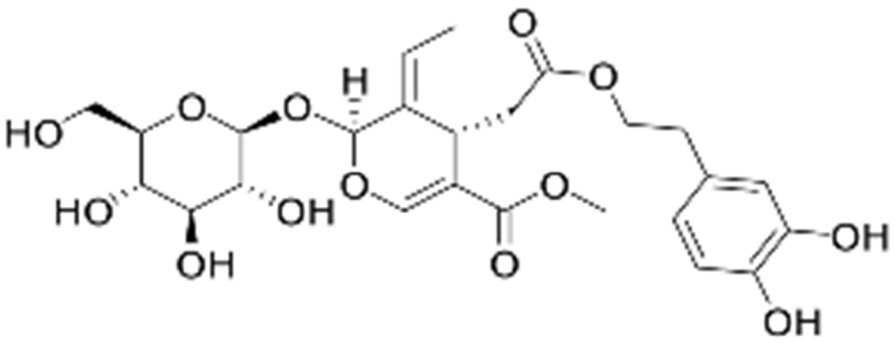

Fig. 1: Molecular structure of OLE

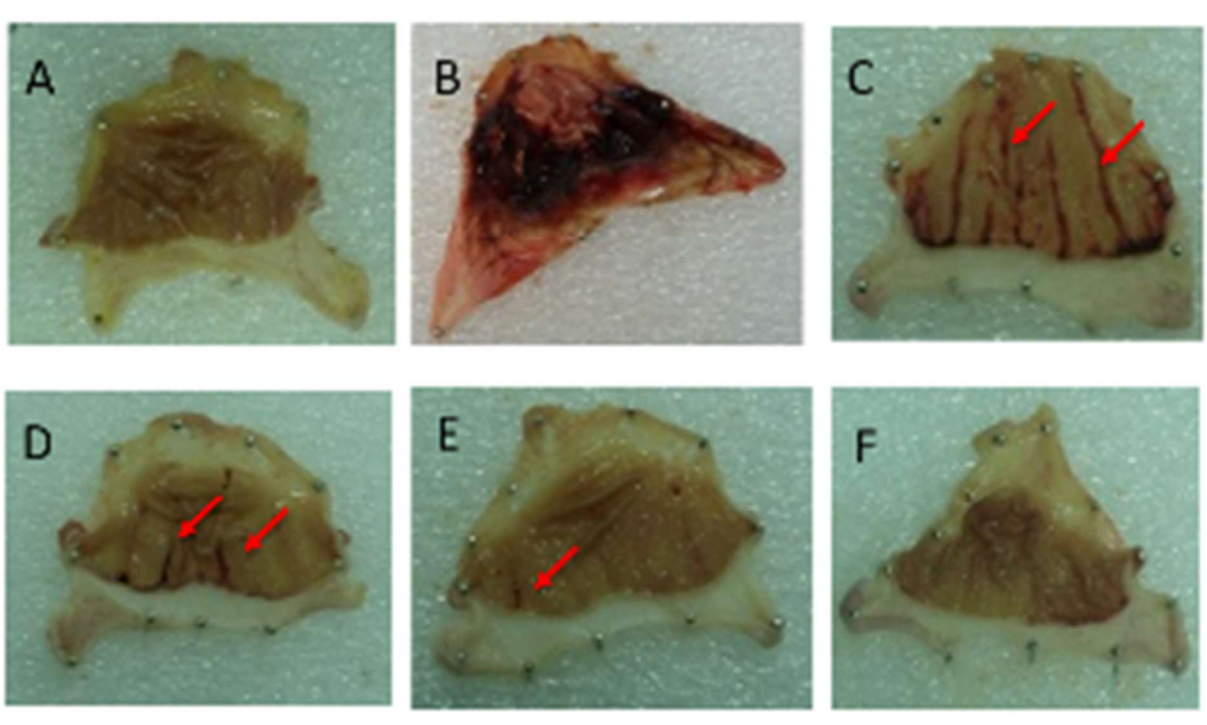

Fig. 2: Macroscopic view of gastric tissue. A) Control group. B) INDO group (25 mg/kg). C) LAN group (30mg/kg). D) OLE 6 mg/kg group. E) OLE 12 mg/kg group. F) OLE 18 mg/kg group.

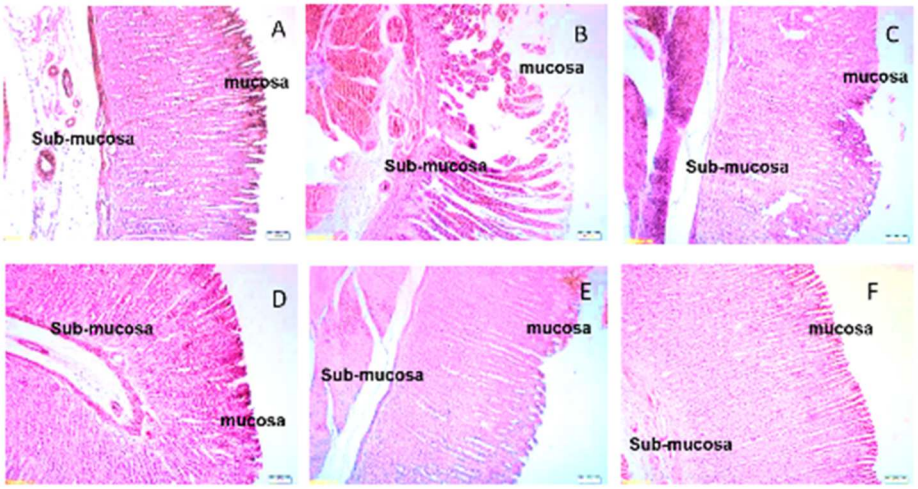

Fig. 3: Histological images of gastric tissue samples from rats. A) Control group. B) INDO group: ulcerative area, damaged epithelium, mucosa, and submucosa. C) LAN ulcerative area and epithelial necrosis. D) OLE 6 mg/kg group ulcerative area and epithelial necrosis. E) OLE 12 mg/kg group non-ulcerative area and non-epital necrosis. F). OLE 18 mg/kg group: non-ulcerative area and non-epital necrosis.

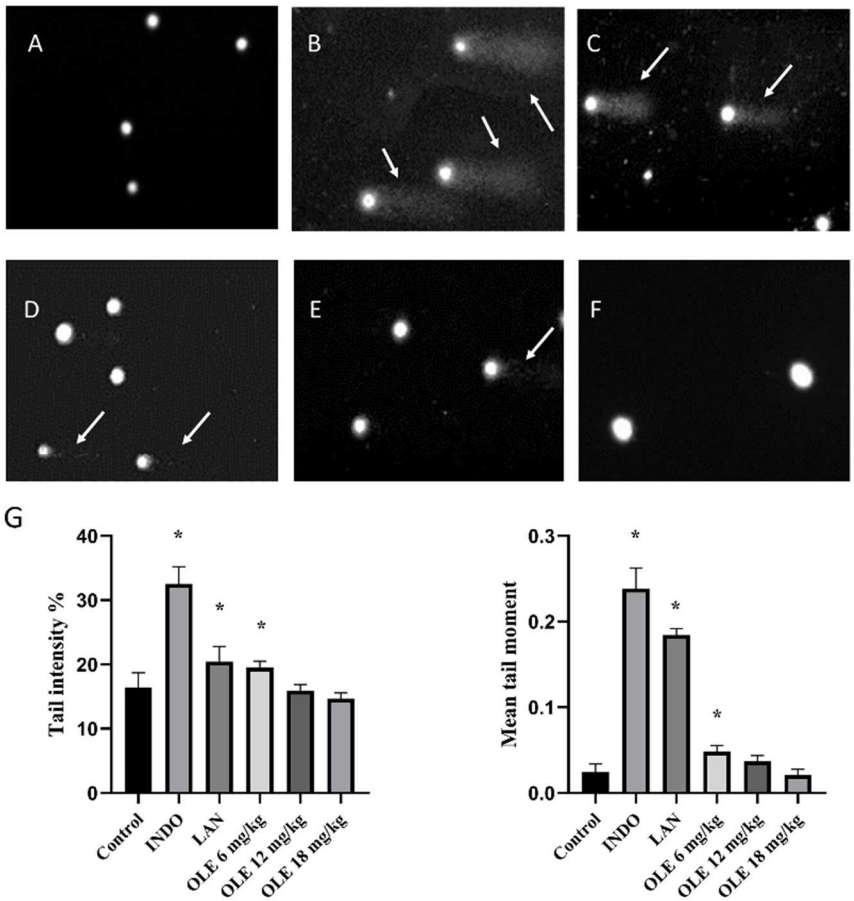

Fig. 4: Comet assay for cell DNA migration. A) Control group. B) INDO group (25 mg/kg). C) LAN group (30 mg/kg). D) OLE 6 mg/kg group. E) OLE 12 mg/kg group. F) OLE 18 mg/kg group. G) Effects of varying OLE dosages on the tailing intensity percentage and mean tailing moment measurements in the INDO-induced gastric ulcer experimental model. Each value represents the mean ± SE. * indicates $P<0.05$.

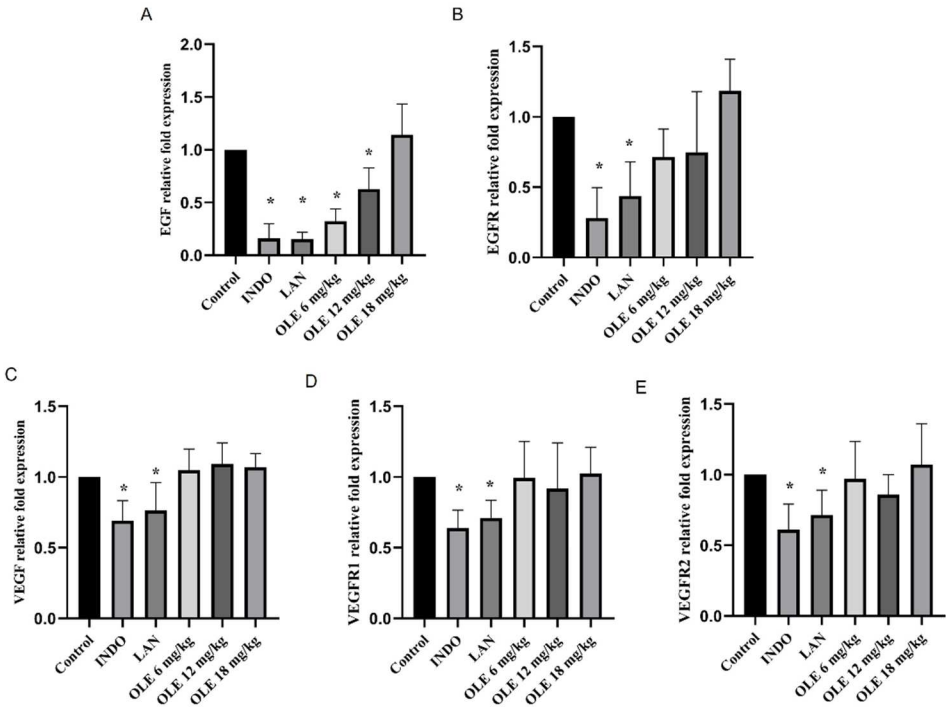

Fig. 5: Effect of OLE administration on EGF, EGFR, VEGF, VEGFR1, and VEGFR2 mRNA levels in all groups (n=8 per group). A) Expression mRNA for EGF, B) Expression mRNA expression, C) VEGF mRNA for VEGF, D) Expression mRNA for VEGFR1, E) Expression mRNA for VEGFR2. Each value represents the mean ± SE. * indicates $P<0.05$, compared to the control group.

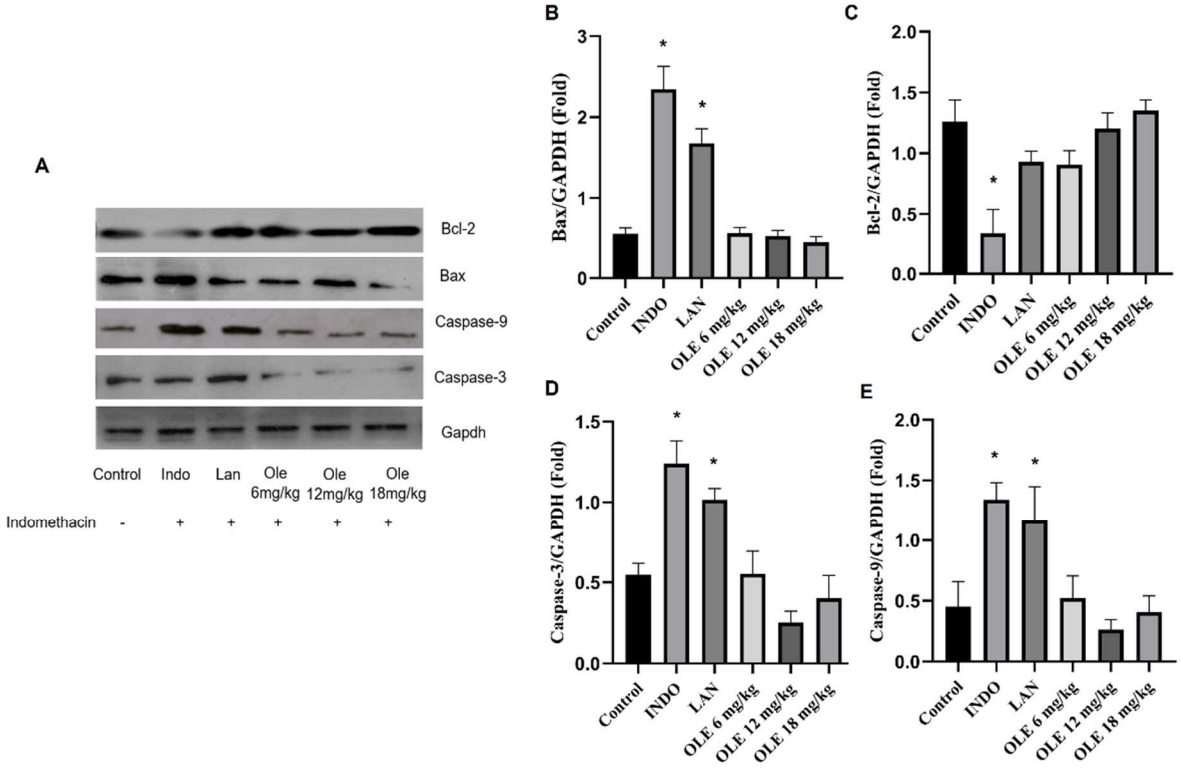

Fig. 6: Expression of apoptosis-related proteins in gastric tissues by western blotting. A) Representative western blot images of caspase-3, caspase-9, Bax, and Bcl-2. B) Quantitative western blot data for caspase-3, caspase-9, pro-apoptotic

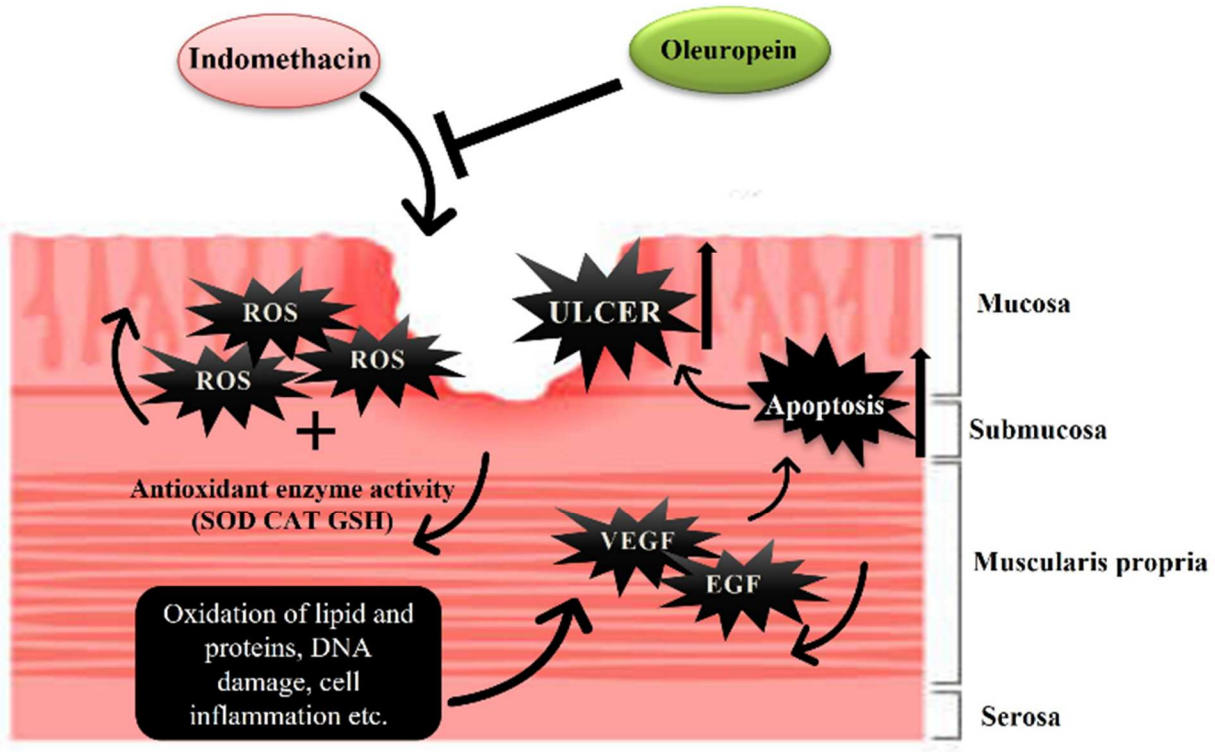

Fig. 7: Gastroprotective diagram of OLE in the gastric mucosa. Abbreviations: ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; ARE, antioxidant response element; SOD, superoxide dismutase; GSH, glutathione; CAT, catalase. When the stomach is exposed to indomethacin, large amounts of ROS are produced in the body and antioxidant enzyme activity decreases, resulting in excessive accumulation of ROS. Excessive accumulation of ROS can lead to lipid and protein peroxidation, causing DNA damage, stimulating the cell inflammatory response, increasing the expression of apoptotic proteins by suppressing the expression of VEGF and EGF in the acute period, and ultimately causing damage to the gastric mucosa.

Experimental protocol

Before initiating the experiment, the researchers measured the weight of the animals. The animals were then randomly divided into six groups with eight animals in each group (table 1). Before receiving the control, ulcer-inducing, or any other investigational drugs, the animals underwent a 24-hour fasting period. All chemicals used in the study were dissolved in deionized water. The study employed three treatment groups, each receiving a different dose of the drug. The treatment groups were orally administered an OLE solution at doses of 6, 12, and 18 mg/kg. The dosages used were determined based on previous research findings supported by our preliminary dose-based experiments, taking into account the typical consumption of olives and olive oil in the Mediterranean diet (Alirezaei, Dezfoulian *et al*., 2012 andreadou, Iliodromitis *et al*., 2006 andreadou, Sigala *et al*., 2007). Ten minutes after these treatments, all groups except the control were given an INDO solution at a dose of 25 mg/kg. Six hours after the administration of INDO, blood samples were obtained via cardiac puncture under ketamine-xylazine anesthesia, and all rats were subsequently euthanized. The stomachs of the rats were promptly removed for macroscopic, histological, and molecular analysis.

Measuring ulcer area

The abdominal cavities of the rats were incised along the greater curvature. Macroscopic lesions were examined in the mucosal area. The images of gastric tissues were photographed with a reference scale. Ulcer areas (UA) were measured by cellSens Imaging Software Olympus. To ascertain the inhibition percentage (I%) of areas of gastric hemorrhagic lesions, the following formula was employed: I% = [(UAcontrol–UAtreated) ÷ UAcontrol]x100%

Histopathological analysis

The gastric tissue specimens were fixed for 24 hours in a solution of 10% formalin. The dehydrated tissue samples were then embedded in paraffin wax and trimmed. Tissue samples were sectioned (5 µm thick) using a microtome (Leica RM2135, Germany). The tissue sections were transferred to clean glass slides. Following the staining of the tissues with hematoxylin and eosin (H&E) solution, the specimens were imaged with a camera-coupled light microscope (Olympus BX51, Tokyo, Japan) to observe histopathological lesions.

Biochemical analysis of gastric tissue

The stomach tissues were homogenized in chilled 10% PBS solution using a mechanical stirrer (IKA Overhead

Stirrer, Germany). Following centrifugation of the samples at 7,000 x g for 10 minutes at 4°C using a Hettich Centrifuge device (Germany), the resulting supernatant was extracted for the assessment of antioxidant and oxidant parameters.

Superoxide dismutase (SOD) level was measured spectrophotometrically at 560 (Sun, Oberley *et al*., 1988). The activity of the catalase (CAT) enzyme was measured by observing the breakdown of hydrogen peroxide (H_2O_2) at a wavelength of 240 nm (Boyacioglu, Kum *et al*., 2016). Spectrophotometry at 412 nm was used to measure Glutathione (GSH) activity, which was represented as milligrams per gram (mg/g) of tissue protein (Tietze, 1969). Levels of malondialdehyde (MDA) were assessed spectrophotometrically at 532 nm (Yoshioka, Kawada *et al*., 1979). Myeloperoxidase (MPO) activity was measured spectrophotometrically at 460 nm (Bradley, Priebat *et al*., 1982). Protein concentration in all supernatants was measured using QuantiPro™ BCA Assay Kit (Sigma-Aldrich, Germany).

Comet assay

To investigate the protective effect of OLE against DNA damage induced by INDO, researchers employed the comet assay technique (Collins, Dobson *et al*., 1997, Singh, McCoy *et al*., 1988). Given the DNA damage observed in lymphocyte cells following the administration of INDO in our previous study, the INDO group was identified as a positive control for this method, while the group that did not receive any treatment was designated as a negative control (Boyacioglu, Kum *et al*., 2016). The collected blood specimens were immediately diluted in phosphate-buffered saline (PBS). Lymphocyte cells were isolated using Histopaque solution, which allows the separation of mononuclear cells from other blood components. Subsequently, the cells were embedded in agarose 0.5% and streaked onto slides that had been pre-coated with agarose 1%. Cell lysates were then prepared with a radio-immunoprecipitation assay (RIPA, Thermo Scientific 89900) solution. The electrophoresis was conducted at 25 volts and 300 milliamps for one hour. This step results in the migration of negatively charged DNA fragments towards the anode, thereby forming a characteristic comet-like pattern. Subsequently, the slides were treated with 4',6-diamidino-2-phenylindole (DAPI) stain and examined using a Leica DM3000 fluorescence microscope (Wetzlar, Germany). Computer-based analysis software was utilized to evaluate the extent of DNA damage by quantifying the tail density and tail moment (Comet Assay IV, Perceptive Instruments, Bury St. Edmunds, UK).

Measuring growth factors by RT-qPCR

RNA isolation was conducted on gastric tissue using a total RNA isolation kit (No. RA101-02; Biomed, China). The purity of the genetic material was determined using a Nanodrop spectrophotometer (Thermo Nanodrop 2000). A commercial kit (MT403-01; Biomed, China) was employed to synthesize cDNA. Quantitative real-time PCR (qRT-PCR) was employed to assess the expression of target genes, utilizing Hieff UNICON® Universal Blue qPCR Master. Mix (SYBR Green No.11184ES08; Yeasn). Specific primers used were detailed in table 2. The $2^{-\Delta\Delta Ct}$ method was employed for data analysis, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) utilized as the internal control reference gene.

Western blot analysis

RIPA lysis buffer, a protein extraction reagent, was utilized to initially homogenize the tissue samples. The total protein content in the lysates was quantified using the BCA Protein Assay Kit (Sigma-Aldrich, Germany). Subsequently, proteins were separated via SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). These membranes were blocked with 3% bovine serum albumin (BSA) before overnight incubation at 4°C with primary antibodies specific to Bax, Bcl-2, caspase-3, and caspase-9. Following incubation with the secondary antibody for one hour, protein bands were visualized through the application of enhanced chemiluminescence (ECL) to the membranes. The intensity of these protein bands was quantified utilizing Image J software, with GAPDH serving as an internal control protein.

STATISTICAL ANALYSIS

Statistical analyses were conducted utilizing GraphPad Prism software (GraphPad Software, Inc., California, USA). The Shapiro-Wilk test was employed to assess data distribution, while Levene's test evaluated variance homogeneity. The selection of Kruskal-Wallis analysis of variance (ANOVA) or one-way ANOVA was contingent upon the distribution of data. For subsequent multiple comparisons, the researchers employed either the Mann-Whitney U test with Bonferroni correction or the Duncan test. A P value less than 0.05 was deemed statistically significant. The results are presented as mean and standard error.

RESULTS

The inhibitory effects of the treatments were assessed by comparing the ratio of the ulcerated area to the overall gastric surface. fig. 2 presents representative images of the stomach, while table 3 provides detailed information regarding the corresponding ulcer areas. The INDO group exhibited severe damage to the mucosal lining, as evidenced by an average ulcer area of 1181.63 ± 158.01 mm2. The LAN-treated group showed a significant reduction in ulcer area, averaging 170.47 ± 32.50 mm^2 ($P<0.001$). For the groups treated with OLE, the ulcer areas demonstrated a statistically significant, dose-dependent

decrease (P<0.001). Among these groups, the 18 mg/kg dose of OLE resulted in the smallest ulcer area (31,54 ± 5,66 mm^2) and the highest inhibition. The ulcer areas for the 12 mg/kg and 6 mg/kg OLE groups were measured at 42.29 ± 13.55 mm^2 and 64.72 ± 15.50 mm^2, respectively.

Histological analysis of gastric tissue samples revealed significant differences between the experimental groups. The INDO group exhibited severe histopathological changes characterized by loss of surface epithelial cells, extensive hemorrhage, and marked edema (fig 3). Conversely, the gastric tissue from the LAN group displayed focal mucosal necrosis. Importantly, the microscopic evaluation of the OLE-treated groups demonstrated a predominantly normal histological structure of the glandular mucosa across multiple examined sections, suggesting a protective effect of OLE against the INDO-induced gastric damage.

The results presented in table 4 show that the administration of different doses of OLE (18 mg/kg) resulted in a significant increase in the activities of SOD and GSH, with a significant decrease in MDA levels in gastric tissue (P<0.001). The results indicate that OLE administration effectively reduced oxidative stress in the gastric environment. It is worth noting that when OLE was administered orally, it did not lead to a substantial enhancement in CAT activity when compared to the control group. This observation suggests that the protective effects observed from OLE may not have been primarily mediated through this specific antioxidant enzyme. Furthermore, OLE (18 mg/kg) groups exhibited significantly reduced MDA activities compared to the INDO group, suggesting a reduction in both neutrophil infiltration and inflammatory response (P<0.001). Evaluation of the percentage of tail density and mean tail moment in isolated lymphocyte cells, as well as DNA damage levels, as shown by the comet assay images in fig. 4, revealed that the INDO group exhibited significantly higher levels of DNA fragmentation compared to the control group (P<0.05). The 12 and 18 mg/kg OLE groups did not show significant differences from the control group, suggesting that OLE treatment was effective in protecting against INDO-induced DNA damage in lymphocyte cells.

The evaluation of growth factor expression levels by qRT-PCR revealed that the administration of INDO led to significant reductions in the mRNA levels of several key factors, including EGF, EGFR, VEGF, VEGFR1, and VEGFR2 (fig. 5, P<0.05). Conversely, the treatment with OLE at 12 mg/kg and 18 mg/kg doses significantly attenuated the INDO-induced downregulation of these growth factors and their associated receptors.

Western blot analysis of the gastric tissue samples revealed that the INDO group exhibited an increased expression of the pro-apoptotic proteins Bax and caspase-3/9, along with a decreased expression of Bcl-2, indicating the induction of apoptosis (fig. 6A, P<0.05). However, the administration of OLE at 12 mg/kg and 18 mg/kg effectively counteracted the INDO-induced disturbance in Bax/Bcl-2 ratio and caspase activation, suggesting an apoptotic effect of OLE (fig. 6D-E, P<0,05).

DISCUSSION

Gastric ulcer is defined as endoscopic histological damage to the mucosal and submucosal lining of significant depth. Ulcer treatment is a dynamic and complex process that demands good coordination of the interaction between the different components of the cell and the connective tissue. Directly or indirectly, the negative effects of NSAIDs on the pathogenesis of gastric ulcers are a serious problem (Liu, Shang *et al*., 2017). Researchers have extensively utilized the INDO-induced gastric ulcer model to explore treatments for ulcers (Aboelella, Brandle *et al*., 2022, Tsutsumi, Gotoh *et al*., 2004). In this context, the present study aimed to investigate the protective effects of OLE, a natural compound found in olive leaves and fruits, against INDO-induced gastric ulceration. Previous research has indicated that OLE might have an impact on ulcers due to its antioxidative properties (Alirezaei, Dezfoulian *et al*., 2012, Koc, Cerig *et al*., 2020). However, the influence of OLE on the biological mechanisms involving growth factor signaling and apoptosis in INDO-induced gastric ulceration is not clear.

The findings suggest that OLE doses of 12 and 18 mg/kg significantly mitigated INDO-induced gastric ulceration, as evidenced by the observed reduction in ulcer severity. The gastroprotective effects of OLE were found to be similar in effectiveness to LAN, a widely used ulcer medication. This observation was supported by histopathological analysis of stomach tissue, which revealed that groups treated with OLE showed minimal mucosal layer injury compared to those given INDO. These findings are consistent with previous studies that have demonstrated the potential of NSAIDs to induce damage to the gastric mucosa and the protective effects of natural compounds such as OLE against this deleterious action (Siddique, Ahmad *et al*., 2023).

INDO administration has been demonstrated to elevate oxidative stress levels in gastric tissues. This phenomenon was evidenced by an increase in the level of MDA and a decrease in the levels of antioxidant enzymes, specifically SOD and GSH (Abdel-Tawab, Mostafa Tork *et al*., 2020). The study revealed that administering OLE at higher doses (12 and 18 mg/kg) effectively counteracted the INDO-induced increase in malondialdehyde levels and decrease in superoxide dismutase and glutathione activities. This finding suggests that antioxidant mechanisms play a role in the stomach-protective effects of OLE (Musa, Shady *et al*.,

2021). The observed effect may be attributed to OLE's capacity to neutralize free radicals, as it has demonstrated efficacy in mitigating oxidative stress.

MPO is an enzyme that is released by activated neutrophils and reflects the degree of neutrophil infiltration and oxidative damage (Cartwright, Zhou *et al*., 2024). In the present study, the administration of INDO resulted in a significant increase in MPO activity, which was markedly reduced by the treatment with OLE, particularly at higher doses. This finding indicates that the gastroprotective effects of OLE may involve the attenuation of neutrophil infiltration and the associated oxidative injury to the gastric mucosa.

The comet assay findings revealed that treatment with INDO significantly increased DNA damage in lymphocyte cells, as indicated by higher percentages of tail DNA and elevated mean tail moment values. Mir Hilal Ahmad *et al*., showed that INDO induced significant DNA damage (Hilal Ahmad, Fatima *et al*., 2018). OLE was shown to exhibit the capacity to protect against oxidative stress by effectively reducing the production of 8-hydroxydeoxyguanosine (8-OHdG), an indicator of oxidative DNA damage (Geyikoglu, Emir *et al*., 2017). This antioxidative action is attributed to the hydroxyl groups in OLE's structure, which can donate hydrogen atoms to reactive species, preventing oxidation (Koc, Cerig *et al*., 2019). In line with earlier research, this study showed that higher concentrations of OLE (12 and 18 mg/kg) successfully protected lymphocyte cells from DNA damage induced by INDO.

In the healing of gastric ulcers, VEGF and EGF play crucial roles as growth factors. VEGF is particularly important for its function in stimulating angiogenesis, the process of creating new blood vessels. This process is vital for supplying the healing tissue with necessary nutrients and oxygen. In the context of INDO-induced gastric ulcers, studies have shown that VEGF expression is significantly reduced, which may impair the healing process. The decrease in VEGF is usually associated with increased oxidative stress since ROS can negatively affect the expression and activity of angiogenic factors (Abdel-Raheem, 2010, Souza, Lemos *et al*., 2004). Conversely, EGF plays a critical role in promoting cellular proliferation and survival within the gastric mucosa. Upon binding to EGFR, it initiates a cascade of signaling events that are essential for cellular development and resistance to apoptosis. Inhibition of EGF signaling, such as through the use of EGFR inhibitors, has been shown to increase apoptosis rates in gastric epithelial cells, leading to further mucosal damage and ulceration (Crabtree, Jeremy *et al*., 2013, Tarnawski and Ahluwalia, 2021). Studies have demonstrated that EGF signaling enhances the production of anti-apoptotic proteins like Bcl-2, while simultaneously suppressing pro-apoptotic proteins such as Bax (Lam, Yu *et al*., 2007). In this study, INDO reduced the expression of EGF, VEGF, and their receptors. However, OLE (18mg/kg) did not significantly change the levels of growth factors and receptors compared to the control group. The reduction in the expression of EGF and VEGF supports the formation of ulcers and the tissue damage caused by INDO, and the fact that the application of OLE prevents the suppression of the growth factors shows its gastroprotective effect.

Apoptosis plays a crucial role in the development and progression of gastric ulcers. INDO-induced oxidative stress triggers apoptotic pathways, leading to the activation of caspases, which are crucial for apoptosis (Gebril, Ito *et al*., 2020). The balance between apoptotic proteins is essential for determining cell fate in the gastric epithelium (Soylemez, Ay *et al*., 2017). In particular, excessive production of ROS and MPO can induce the mitochondria-mediated apoptosis pathway (fig. 7). Some studies have shown that OLE can modulate the apoptosis-related proteins, thus inhibiting the apoptotic cascade and confirming its cytoprotective effects (Koc, Cerig *et al*., 2020).

This investigation revealed that INDO led to a notable increase in the expression of pro-apoptotic proteins, including Bax, caspase-9, and caspase-3, while simultaneously reducing the levels of the anti-apoptotic protein Bcl-2. Treatment with OLE, especially at the highest dose, was able to reverse these changes, suggesting that the gastroprotective effects of OLE may be mediated, at least in part, by its ability to modulate the apoptotic pathway.

While substantial progress has been achieved, it is imperative to acknowledge the extant limitations of our investigation. The research did not examine several critical factors, including the acidity levels within the stomach lining, the quantities of cyclooxygenase (COX) enzymes present, and the production of prostaglandin E2 (PGE2). It thus remains unclear whether its antiulcer mechanism is directly superior to that of proton pump inhibitor group drugs. Secondly, it is unclear whether OLE has significant adverse effects. Consequently, we must periodically treat rats with OLE. The objective of the present study was to investigate the protective effect of OLE against INDO-induced acute gastric ulceration. To ascertain the impact of OLE on ulcer healing and to determine whether rats exhibit adverse effects, we must administer OLE to ulcerated animals for an extended period.

CONCLUSION

OLE plays a multifaceted role in the context of oxidative stress, apoptosis, and the expression of VEGF and EGF in INDO-induced gastric ulcers. Its antioxidant properties help mitigate oxidative damage, while its modulation of

apoptotic pathways promotes cell survival. Additionally, OLE's ability to enhance the expression of growth factors such as VEGF and EGF supports the healing processes of the gastric mucosa. These findings underscore the potential therapeutic applications of OLE in managing gastric ulcers and related conditions.

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Conflict of interest statement

The authors declare that there is no conflict of interest related to this article.

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