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ANTIULCER POTENTIAL OF OLIVE LEAVES EXTRACT IN GASTRIC ULCER INDUCED BY INDOMETHACIN IN MALE RATS: ANTIOXIDANT AND ANTI-INFLAMMATORY EFFECTS

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ABSTRACT

Gastric ulcer (GU) is a severe gastrointestinal illness. The incidence and prevalence of GU are increasing globally. This work aimed to evaluate the potential gastro-protective effects of olive leaves extract (OLE), a natural antioxidant, in experimental rats of gastric mucosal damage induced by indomethacin (IND). The rats (n=40) were assigned into 4 groups including: control, ulcer (IND), and two protective OLE groups at two doses of low 300 and high 450 mg/kg b.wt (OLE 300 mg + IND and OLE 450 mg +IND). Each dose of OLE was orally given daily for 14 days. Then, the rats were sacrificed 4 h post IND given. The blood samples and gastric tissues were collected for biochemical analysis, calculation of ulcer index and histopathological examination. The results showed that OLE pretreatment and pre-induction of ulcer decline ulceration of gastric preserved the normal structure of gastric mucosa. There were significant decline in ulcer index, and total gastric acidity with significant increase of gastric pH level compared with IND group. The pretreatment with OLE significantly decreased the gastric tissue oxidative stress (thiobarbituric acid reacting substances (TBARS)) and serum pro-inflammatory cytokines, with a significant elevation in gastric antioxidant enzymes activities compared with IND ingested rats. The high dose of OLE (450 mg/kg) showed a better protective capacity compared with the low dose of OLE (300 mg/kg). The results showed that OLE had a potent gastroprotective activity on IND induced GU, which could be explained by its effect as an anti-inflammatory and antioxidant agent.

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Introduction

Gastrointestinal ulcers can be found from the esophagus to the colon anywhere in the gastrointestinal tract. Ulceration development of Peptic ulcer (PU) usually means mucosal injury with a diameter of more than 5 mm depending on mucosa and submucosa [1]. The main causes of PU are pepsin, gastric acid, non- steroidal anti-inflammatory drugs (NSAIDs), bile salts, helicobacter pylori, alcohol, and tobacco consumption [2]. The prevalence of PU is 5%-10%, and the incidence is about 1%-.3% in general population [3].

The NSAID are the most commonly used drugs globally [4]. They are used in a wide range for pain treatment, but there are some limitations on the long-term use of them such as serious gastrointestinal side-effects [5-6]. The IND as one of the NSAIDs family has been commonly used to treat arthritic diseases [7]. However, it has been recognized to induce the experimental stomach ulcers, and has been shown to be more likely to cause gastric damages [4].

Reactive oxygen species (ROS) have been well detailed as an administrator of stress within the gastric mucosa and the epithelial lining of the stomach [8]. Unfortunately, none of antiulcer drugs is without side effects or gives a 100% curative rate or a complete cure [9]. In addition, the high rate of recurrence has prompted a search for safe, easily available and inexpensive natural antiulcer agents [10]. It has been shown that the plant extracts have had encouraging results in the treatment of gastric injury [11]. The minimization of the chance of gastritis or stomach cancer decreases with the dietary supplementation of cancer prevention agents such as plant polyphenols, carotenoids, α-tocopherol and ascorbic acid that catch ROS within the gastric ulcer [12].

Olive leaves contain numerous and varied phenolic compounds that have important molecular characteristics such as flavonoids, secoiridoids, lignans ,simple phenols and acids [13]; The main compounds of olive leaves include oleuropein,

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flavonols(routine), flavones (diosmetin, apigenin, diosmetin, glucoside and luteolin-7-glucoside), phenols (tyrosol, hydroxytyrosol, flavan-3-Oleuropein, secoiridoids, and other derivatives) [14]. Oleuropein scavenges hydroxyl radicals and superoxide anions and inhibits neutrophils and radicals derived from hypochloric acids, which inhibits the oxidative stress. Phenolic compounds have shown anti-inflammatory and antioxidant properties in olives and leaves of olive trees, which improve the lipid profile and endothelial function, prevent lipoperoxidation, and reveal anti- thrombotic properties [15].
This study was done to examine the protective effects of olive leaves against peptic ulcers induced by IND in rats.

Material and Methods

I- Material

Experimental rats and Diet.

Male albino rats (n=40) (180 ± 15 g) were provided from King Fahd Research Center, KAU. The basal diet constituents were purchased from Baghafar Company for Pharmaceutical and Chemical, Jeddah, KSA.

Chemicals, kits and drugs

Indomethacin was obtained from local Pharmacy, Jeddah, Saudi Arabia. Other reagents, chemicals and kits were acquired from Sigma-Aldrich (St Louis, MO, USA).

II-Methods

The olive leaves extract preparation

Olive (*Olea europaea* L.) fresh leaves were collected from olive trees in Sakaka city, Aljouf region, Saudi Arabia. The leaves were washed and dried, then ground to the fine powder. The dried leaves (100 g) were mixed with ethanol 80 % under the agitation, after that they were filtered, and the extract was then concentrated under vacuum using a rotary evaporator [16].

Experimental induced gastric ulcer

A single oral dose of IND (30 mg/kg) was ingested to rats post fasted for 24 h to induce gastric ulcer [17].

Experimental protecol

The rats were divided randomly to 4 groups (10 rats/ each):

***Group I* (control).** The rats received distilled water inter-gastrically for 2 weeks.

***Group II* (ulcer group)**. The rats received distilled water intergastrically for 2 weeks and pre-oral given of IND at a dose level of (30 mg/kg) on empty stomachs after 24 h fasting.

***Groups III* and *IV* (protective groups).** The rats were given the OLE (300 and 450 mg/kg/day p.o), for 14 days prior to an oral dose of IND on 24 h empty stomachs. The dose was chosen according to Sarbishegi *et al*. [18]. All rats were sacrificed 4 h post ingested IND. Gastric was dissected out, then the greater curvature of gastric was cut. Gastric contents were emptied into a centrifuge tube. Cleaned gastric was washed with PSB (0.1M). Then treated either for the biochemical analysis or the histopathological examination.

Access of gastric ulceration

After the scarification, the rats' stomachs were removed, cut open along the greater curvature, washed with cold saline and examined for the ulcers. To quantify the ulcer index of gastric, a computer system was used with an image Pro Express analyzer. For ulcer area (mm^2) calculation, the sum of total GU areas of all lesions for each stomach was considered. Then, the total area of mucosa and the ulcers' area were calculated. The calculation of ulcer index and % of protection were done with the following equations according to Melese *et al*. [19]:

Ulcer index (UI) = Total area of mucosal ulcers / Total mucosal area

Percentage protection = UI (IND group) – UI (treated group) / UI (IND group) × 100

Gastric mucosal lesions biomarkers

The stomach juice was diluted with distilled water, then centrifuged at 300 rpm for 20 min. The pH value of gastric was assessed using pH meter [20]. Gastric total acidity was distinguished in the supernatant by titrating the contents with NaOH (0.01 N), using an indicator (phenolphthalein), then expressed as mEq/L and calculated as following [19]:

Acidity value =V Na OH × N × 100 N: Normality.

Determination of gastric oxidative stress biomarkers

Gastric tissue samples (100 mg) were homogenized in 10 v of 0.9% saline and centrifuged at 12000 g for 15 min using a Teflon pestle. The supernatant was collected, and the levels of lipid peroxides in terms of thiobarbituric acid reacting substances (TBARS), catalase (CAT) and superoxide dismutase (SOD) were measured by using ELISA kits according to the manufacturer's procedures.

Determination of serum pro-inflammatory cytokines

The blood samples were centrifuged for serum separation. The serum levels of tumor necrosis factor -α (TNF-α) and interleukin-6 (IL-6) were measured by ELISA kits. All the procedures were performed as described in the manufacturer's procedures.

Histopathological studies

The formaldehyde (10%) fixed gastric tissues from each group were stained with Hematoxylin and Eosin (H and E), then scanned under microscope.

Statistical

The percentage protection was illustrated as a percentage. The data was presented as mean ± SE. Statistics were performed by one-way (ANOVA) SPSS version 22, the analysis of variance, followed by LSD, and $p \leq 0.05$ was considered as significance difference.

Results

Gastric mucosal lesions biomarkers

There was a significant increase in ulcer index (mm^2) in IND group compared with the control rats ($p< 0.001$). The pretreatment with OLE to rats with the low dose (300 mg/kg) and the high dose OLE (450 mg/kg) in UI were recorded (19.45 ± 1.19 and 10.95±0.78, respectively) compared with (40.48 ± 3.07). There was a significant decrease compared with IND group ($p< 0.001$). At the same time, there was a significant difference in UI in the pretreated rats with the low dose compared with the control rats ($p< 0.05$). The high dose was more effective than the low dose, there was a significant difference in UI between the low and high doses ($p< 0.05$). The high dose of OLE recorded 72.95 % ulcer inhibition compared with 51.95 % in the pretreated group with the low dose. In IND group, there was a significant decrease of gastric pH with the significant increase in total gastric acidity compared with the control group. Pretreatment low and high doses of OLE (300 and 450 mg/kg, respectively) significantly elevated ($p<0.001$) the gastric pH with significantly decreasing the total gastric acidity compared to the IND group. However, the rats pretreated with 450 mg/kg OLE showed significantly ($p<0.05$) higher gastric pH with significantly lower total gastric acidity compared to 300 mg/kg OLE+ IND (Table 1).

Table 1: Effect of OLE on gastric mucosal lesions biomarkers in ulcer rats

Experimental groups	Ulcer index (mm^2)	Ulcer inhibition (%)	Gastric pH	Total acidity
Control	-	-	3.61± 0.12	55.82± 2.68
IND	40.48 ± 3.07 [a]	-	2.23± 0.16[a]	183.37 ± 4.29 [a]
OLE (300 mg/kg)**+IND**	19.45 ± 1.19 [a,b]	51.95	3.13± 0.17[a, b]	78.79±6.05 [a, b]
OLE (450 mg/kg)**+IND**	10.95±0.78 [b,c]	72.95	3.51±0.11 [b,c]	51.05±2.97[b,c]

The results are illustrated as mean ± SE (n = 10). [a] Significant versus control , [b] significant versus IND. [c] significant between low and high dose groups. $p \leq 0.05$

Oxidative stress biomarkers

The administration of rats with IND significantly elevated ($p< 0.001$) gastric tissue TBARs concentration, with significantly reducing ($p \leq 0.001$) both gastric tissue CAT and SOD concentration compared to the control rats. The pretreatment of rats with OLE at both 300 and 450 mg/kg significantly reduced ($p \leq 0.001$) the gastric tissue TBARs concentration with significantly elevating ($p< 0.001$) both gastric tissue CAT and SOD concentration compared to IND group. However, the rats pretreated with 450 mg/kg OLE showed significantly both higher gastric tissue CAT and SOD concentration with significantly lower ($p<0.05$) TBARs concentration compared to 300 mg/kg OLE+ IND (Figures 1,2 and 3).

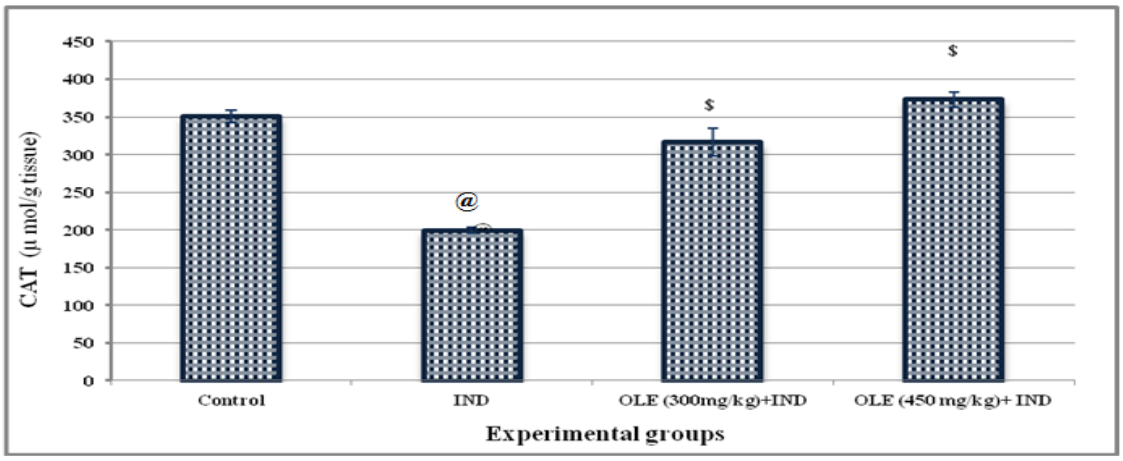

Figure 1: Effect of OLE on gastric catalase (CAT) (µmol/g tissue) activity in ulcer rats

The results are illustrated as mean ± SE (n = 10). [@]Significant versus control , [$] significant versus IND. [*] significant between low and high dose groups. $p \leq 0.05$.

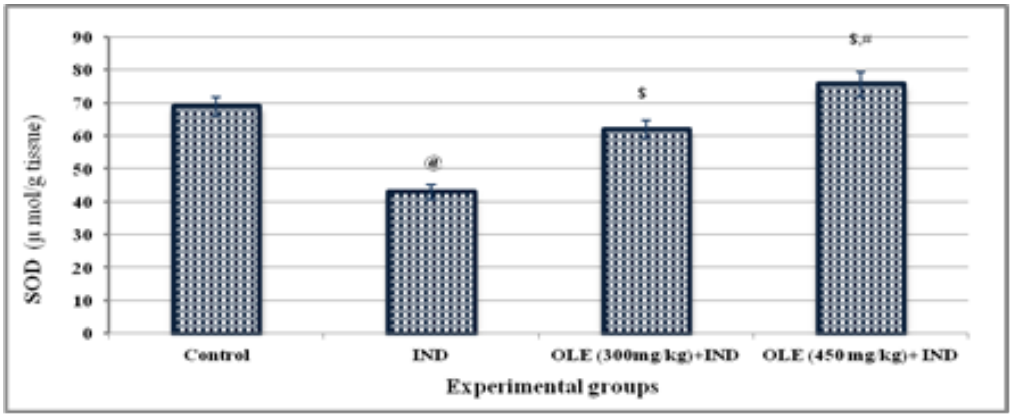

Figure 2: Effect of OLE on gastric superoxide dismutase (SOD) activity in ulcer rats
The results are illustrated as mean ± SE (n = 10). [@]Significant versus control , [$] significant versus IND. [*] significant between low and high dose groups. $p \leq 0.05$

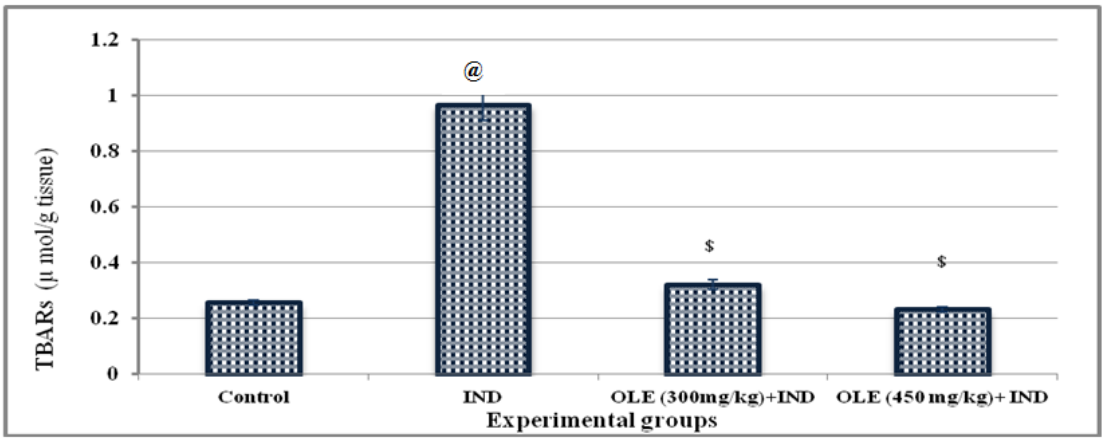

Figure 3: Effect of OLE on gastric thiobarbituric acid reacting substances (TBARS) levels in ulcer rats
The results are illustrated as mean ± SE (n = 10). [@]Significant versus control, [$] significant versus IND. [*] significant between low and high dose groups. $p \leq 0.05$

Pro-inflammatory cytokines

The administration of rats with IND significantly increased both serum IL-6 and TNF-α compared to the control rats. The pretreatment of rats with OLE at both 300 and 450 mg/kg significantly reduced serum IL-6 and TNF-α concentration compared to IND group. However, the rats pretreated with 450 mg/kg OLE showed significantly lower serum IL-6 and TNF-α concentration compared to 300 mg/kg OLE+ IND (Table 2).

Table 2: Effect of OLE on *serum* interleukin-6 (IL-6) and tumor necrosis factor -α (TNF-α) in ulcer rats

Experimental groups	**IL-6** (ng/ml)	**TNF-α** (ng/ml)
Control	14.16 ± 0.47	1.29 ± 0.06
IND	25.51± 0.73 [a]	3.02 ±0.15 [a]
OLE (300 mg/kg)**+IND**	18.51 ± 1.13[a, b]	1.80 ± 0.14 [a, b]
OLE (450 mg/kg)**+IND**	14.53±0.39 [b,c]	1.41 ± 0.10 [b,c]

The results are illustrated as mean ± SE (n = 10). [a] Significant versus control , [b] significant versus IND. [c] significant between low and high dose groups. $p \leq 0.05$

Gastric histopathological changes

The normal appearance of gastric mucosal in the control rats has been represented in (Fig 4. A). In IND group showing necrosis of gastric mucosa, the loss in some areas of architecture of superficial epithelium and gastric pits, the infiltration of the mononuclear cellular, dilated blood vessels, gastric mucosa fell away into a deep ulcer whose base contained necrotic and inflamed debris (Fig 4. B and Fig. 4.C). The pretreatment with OLE (300 mg/kg) + IND the stomach showed the atrophy of gastric mucosa, dilatation of the gastric pits and the lumen of the glands with the prevalence of mucous secreting cells (arrows) (Fig 4. D). Gastric of rats pretreated orally with OLE (450 mg/kg)+IND showed mild dilation of the lumen of the

gland (D), other sections apparently near normal glands showed minimal areas of the congested blood capillaries (Fig 4. E and Fig. 4.F).

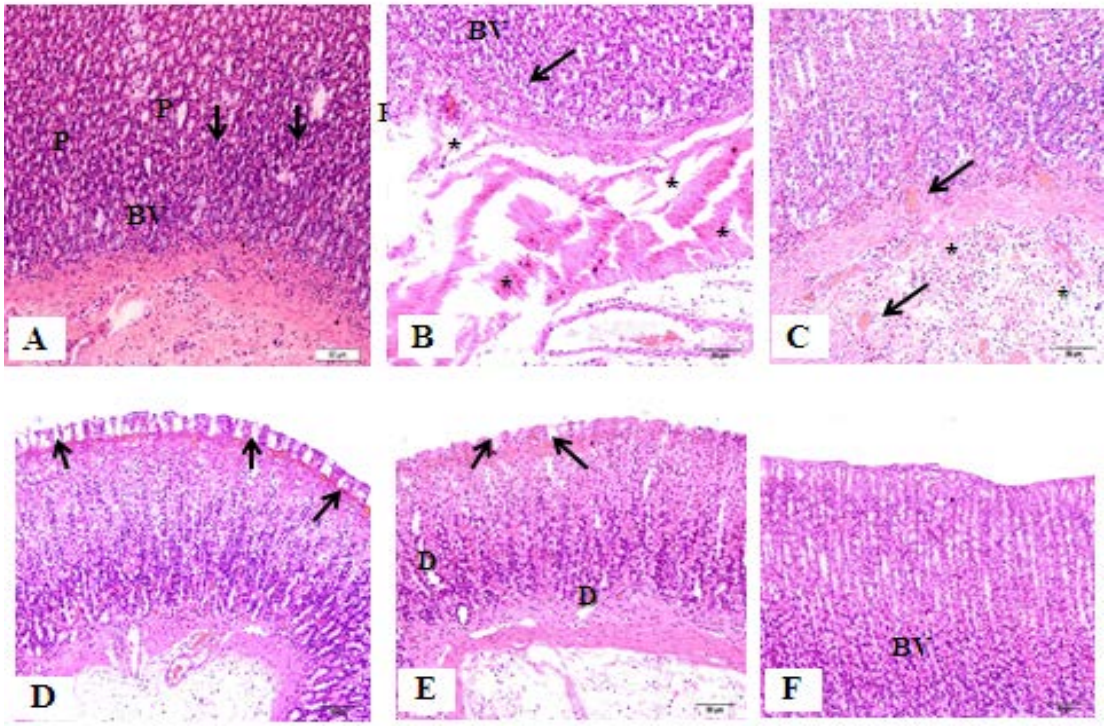

Figure 4: Effect of OLE on the stomach tissue histopathological changes detected by H & E staining in IND-induced peptic ulcer in rats.

The photomicrograph of control showed the typical appearance of gastric mucosal. Parietal cells (P) had central rounded nuclei, eosinophilic cytoplasm, mucous neck cells with flat basal nuclei and pale foamy cytoplasm (arrows) [A]. The stomach of the IND group showed necrosis of gastric mucosa, congestion of submucosal edema associated with hemorrhage (arrows). The area with the loss of architecture of superficial epithelium and gastric pits have also been noted (*). Notice the mononuclear cellular infiltration and dilated blood vessels (BV) [B]. As well, gastric mucosa fell away into a deep ulcer whose base contained inflamed necrotic debris [C]. In OLE pretreated rats (300 mg/kg) + IND, the stomach showed the atrophy of gastric mucosa, the dilatation of the gastric pits and the lumen of the glands with the prevalence of mucous secreting cells (arrows) [D]. Gastric of rats pretreated orally with OLE (450 mg/kg)+IND showed the mild dilation of the lumen of the gland (D), other sections apparently near normal glands showed minimal areas of the congested blood capillaries (BV) [F].

Discussion

Gastric ulcer is produced by the disproportion between the defensive factors (cellular mucus, cell shedding, cell proliferation and mucin secretion) and the aggressive factors (pepsin and acid)[21]. This study tested the gastroprotective effect of OLE on GU induced by IND. In the present study, considering the index of ulcers, the total acidity increased significantly by IND. The ulceration resulted from IND was caused by several reasons, including the initiation of malondialdehyde, the generation of ROS and leukocyte infiltration [22]. The low PH value was associated with the causation of the ulcers, and the destruction of stomach mucosa in the experimental animals [23].

The pretreatment with OLE significantly reduced Ulcer index and the total acidity of gastric, and elevated pH value in gastric compared to the group of IND. The high dose of OLE (450 mg/kg) had better effect than the low dose of OLE (300 mg/kg) on IND –induced peptic ulcer .The effect of OLE might be due to the decrease of both gastric juice volume and acid pepsin secretion in addition to the protection of mucosa by gastric mucin activity. A previous study reported that the extract of OLE had a complex effect on the stomach and duodenum luminal mucosa [24]. Some of these actions were important for the cure of ulcers, and others were important for the prevention of the ulcer relapses [25].

The OLE extract directly affected acid secretion and increased mucosal resistance to harmful agents [24]. These effects were due to the active compounds in OLE such as Oleuropein, and a phenolic compound that was found in a large amount in OLE [26]. Olive leaves also contain tyrosol, hydroxytyrosol and caffeic acids which have been identified as the main active ingredients. In addition, OLE contains vanilla acid, p- coumaric acid, luteolin, rutin, vanillin, diosmetin-7 glucoside ,luteolin-7 glucoside, diosmetin, and apigenin-7 glucoside. [15].

Oxidation marker was initiated by free radicals. Oxidative stress played a role in the toxicity mechanism done by IND [27]. The gastric ulcer resulted in increased free radicals accumulation which led to a severe mucosal damage [28]. The mucosal tissue was protected if there was a balance between free radical formation and scavenger's mechanism. The imbalance between them caused oxidative stress and tissue damage [29].

In the experimental animals, the IND was a precursor of reactive oxygen metabolites that led to the mucosal injury [27]. These free radicals destroyed SOD, CAT (antioxidant enzymes), which played a vital role in the cell protection in opposition to the oxidative damage [30]. Recent experiment results were consistent with these previous data. The IND- induced gastric ulceration was accompanied by severe oxidative stress in gastric tissue that damaged biomolecules such as lipids. This was evident in the stimulated lipid peroxidation, leading to the increased MDA accumulation and reduced gastric CAT [31].

In the current study, OLE induced a significant increase in CAT and SOD enzymes, while the concentration of TBARs significantly decreased compared to the IND group. This could be as a result of OLE bioactive compounds which played an important role as a powerful antioxidant scavenger such as Oleuropein that scavenged hydroxyl radicals and superoxide anions and inhibited neutrophils and radicals derived from hypochloric acids, which in turn inhibited the oxidative stress [15].

Cytokines are a heterogeneous group of polypeptides which have multifunctional acts as modulating, triggering and regulating of inflammatory and immune responses [32]. The IL-6 and TNF-α inhibition would ultimately reduce the destruction of tissue by reactive oxygen species [33].The current study demonstrated that there were significant increases in both cytokines IL-6 and TNF-α compared with the control rats. The study findings agreed with Appleyard. *et al* [34], who reported that the indomethacin improved the synthesis of pro-inflammatory molecules, such as IL-6 and TNF-α, which contributed to the injury of the mucosa.

Compared to the IND group, the pretreatment of rats with OLE at 300 and 450 mg / kg significantly reduced serum IL-6 and TNF-α concentration. The anti-inflammatory activity of OLE can be attributed to the suppression of TNF-α and IL-6 production, which affected the myeloperoxidase catalytic reactions [35].

The histopathological examination of the IND group showed the loss of the architecture of superficial epithelium and gastric pits, infiltration of the mononuclear cellular, dilated blood vessels, gastric mucosa falling away into a deep ulcer whose base contained inflamed necrotic debris. This could be as a result of the oxidative stress or a reduction in antioxidant enzymes [31]. However, the histopathological results of OLE group showed a significant gastro-protective effect against IND-induced peptic ulcer due to OLE’s active constituents [24].

Conclusion

The results of the current work proved that OLE's antioxidant and anti- inflammatory activities have inhibited physiological and histopathological changes caused by IND. Therefore, OLE exhibited a possible therapeutic option to prevent IND-induced GU.

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