Synergistic gastroprotective and antioxidative effects of natural olive oil and usnic acid isolated from *Usnea longissima*, a lichen species in Anatolia (Türkiye), in the indomethacin ulcer model created in rats

ABSTRACT

Usnea longissima, a medically important lichen growing up in forests in Anatolia (Türkiye). In this study, the gastroprotective effect of usnic acid (UA) was investigated using an indomethacin (IND)-induced gastric ulcer model in rats. While 25, 50, 100 and 200 mg/kg UA doses were dissolved in 2 ml of olive oil (OO) and administered to rats, only OO was given to one group. In addition, lansoprazole (LAN) and ranitidine (RAN) and IND were dissolved in water and administered to rat groups. IND administration caused very high levels of damage to rat stomachs. On the other hand, when four doses of UA, OO, RAN and LAN were administered, it was determined that hyperaemia's in the stomach of rats was significantly reduced. After macroscopic analysis of gastric tissues, the activities of superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO) and nitric oxide synthase (iNOS and cNOS) enzymes as well as glutathione (GSH) and lipid peroxidation (LPO) levels were determined in these tissues. After IND application, it was detected increases in MPO, CAT and iNOS activities in gastric tissues and decreases in SOD, cNOS and GSH amounts. Four doses of UA, OO, RAN and LAN applications reversed the trend, bringing them closer to healthy levels.

Keywords: Indomethacin, iNOS, gastroprotective effect, myeloperoxidase, usnic acid, olive oil

INTRODUCTION

The ulcer is a condition that results in damage to the gastric mucosa, caused by various factors such as anti-inflammatory drugs, stress, alcohol, *Helicobacter pylori*, etc. (Dejban et al., 2020). Factors such as inhibition of bicarbonate secretion, decrease in gastric blood flow, loss of protection of gastric mucosal cells and stress play an important role in the pathogenesis of ulcer, which consists of various factors (Atalay et al., 2015). Two cyclooxygenase enzymes (COX-1 and COX-2) converts arachidonic acid into prostanoids in definite ratios. Non steroid anti-inflammatory drugs (NSAIDs) used to reduce inflammation, pain and fever inhibit COX enzymes and ultimately prostaglandin synthesis is inhibited (Atalay et al., 2015; Atalay et al., 2016; Dejban et al., 2020; Odabasoglu et al., 2008). Generally, the imbalance between the defence mechanism of the gastric mucosal cells and the factors causing toxicity causes the formation of this condition. One of the main aggressive factors is the NSAIDs. The use of NSAIDs is increasing due to diseases that occur throughout the life period. The risk of peptic ulcer complications is four times higher in NSAID users (Lanas et al., 2015).

How to cite this article
Odabasoglu, F., Uyanık, S., Atalay Dumlu, F., Aydın Berktas, O., Aslan A. (2023). Synergistic gastroprotective and antioxidative effects of natural olive oil and usnic acid isolated from *Usnea longissima*, a lichen species in Anatolia (Türkiye), in the indomethacin ulcer model created in rats. *Journal of Advances in VetBio Science and Techniques*, *8*(3), 196-209. https://doi.org/10.31797/vetbio.1351161

Research Article

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Article info
Submission: 28-08-2023
Accepted: 24-10-2023
Online First: 24-11-2023
Publication: 15-12-2023

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
http://dergipark.org.tr/vetbio

The indomethacin-induced ulcer model in the rat is a widely used experimental model to investigate the gastric protective potential of various agents (Ahmed et al., 2021; Berktas et al., 2021; Halici et al., 2011; Karakus et al., 2009). The ulcer induced by oxidative stress, inflammation and especially NSAIDs; Today, it has become widely used in therapy thanks to newly discovered molecules. Therefore, these molecules can be an alternative to drugs used mainly in therapy (Bi et al., 2014). Many lichen species and molecules isolated from them have been shown to have a pronounced gastric protective effect. On the other hand, there are records that olive oil provides gastric protection (Odabasoglu et al., 2008). Lichens are symbiotic organisms that live in ecosystems ranging from mountains to deserts and consist of a fungus, an algae, and/or a cyanobacterial partner. Some lichen species have been traditionally used to treat various diseases. In some societies, it is also consumed as food. On the other hand, lichens are also widely used in the manufacture of perfumes and paints (Atalay et al., 2016; Halici et al., 2005; Huneck, 1999; Odabasoglu, 2001). The fungus forms a thallus or lichenified stroma, which may contain characteristic secondary metabolites in all lichens (Atalay et al., 2015; Ahmadjian, 1993; Bayir et al., 2006; Odabasoglu et al., 2006). It has been reported that secondary metabolites isolated from lichens exhibit a wide variety of biological activities, including gastroprotective, antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative, proapoptotic, anticarsinogenic and cytotoxic effects (Atalay et al., 2015; Halici et al., 2006; Huneck, 1999; Odabasoglu et al., 2006, 2012, 2019; Solárová et al., 2020; Suleyman et al., 2003; Yingshu et al., 2021).

Usnea longissima usually lives on trees (epiphytic) and has a hanging thallus. This species of lichen is very sensitive to air pollution. *Usnea longissima* is widely used in folk medicine of different countries. It has also been noted in some studies that this lichen species is used in ulcers, expectoration, wound healing, and skin disorders (Ahmadjian, 1993; Brij, 1995; Halici et al., 2005; Odabasoglu et al., 2019; Yazici and Aslan, 2003). In addition, it was reported that *Usnea longissima* extracts have gastric protective effects and antioxidant properties (Halici et al., 2005).

Usnic acid, one of the most common lichen metabolites; It is a dibenzofuran derivative molecule and has been identified in many lichen genera such as Usnea, Cladonia, Hypotrachyna, Lecanora and Evernia. The characteristics of this lichen species have been recorded in many literatures (such as antiviral, antibiotic, antiprotozoal, antitumoral, anti-inflammatory, antipyretic, analgesic…) (Odabasoglu et al., 2006, 2019; Solárová et al., 2020; Yingshu et al., 2021). In addition, Odabasoglu et al (2006) noted that UA has gastroprotective and antioxidative effects (Odabasoglu et al., 2006).

The OO is consumed more in Mediterranean countries. Its use, which has become widespread in our country in recent years, has been supported by some studies on cancer and heart diseases. It has also been found to be protective against various diseases such as skin diseases, autoimmune disorders, ulcers and cholelithiasis. (Miraj et al., 2020). In addition, Odabasoglu et al. (2012) demonstrated a pro-apoptotic effect of olive oil in peri-implant tissues of Ti-implanted rabbit (Odabasoglu et al., 2012), and its gastroprotective effect in IND-induced rat' stomach (Odabasoglu et al., 2008).

However, since there was no study evaluating usnic acid in combination with olive oil, its gastroprotective effect was also discussed in this study. The effect was expressed through biochemical enzymes.

MATERIALS AND METHODS

Plant material

Usnea longissima Ach. was collected from the Giresun region (northern Anatolia) of Turkey and identified by Dr. Ali Aslan. Voucher specimen (KKEF-374) has been deposited in the herbarium of Kazım Karabekir Education Faculty, Ataturk University, Erzurum-Türkiye.

General analytical procedures

The chemical products used in the experiments were obtained from Sigma. Olive oil is bought from any grocery store. Column chromatography and thin layer chromatography were performed. UV-visible spectra and biochemical analyses were recorded by spectrophotometer.

Extraction of Usnea longissima and isolation of usnic acid (UA)

The dried Usnea lichen species was extracted and subjected to column chromatography after filtration and washing with solvent system. The pure usnic acid obtained was tested. Its chemical structure was determined by UV, IR, 1HNMRand 13CNMRmethods and also confirmed by comparing previously reported spectral data (Huneck and Yoshimura, 1996). The spectral information of UA: m.p. 194 0C; IR (KBr) υ cm-1: 2920, 1690, 1630, 1530, 1450, 1370, 1350, 1280, 1190, 1140, 1110, 1060, 1030, 960, 830, 810; 1H and 13C NMR data (see Table 1).

Table 1. ^{1}H and ^{13}C-NMR spectral data of Usnic acid in $CDCl_3$[a]

Position	δ_H (ppm)	δ_C (ppm)
1	-	157.2
2	-	107.2
3	-	159.4
4	-	111.2
5	-	165.8
6	-	103.4
1'	-	193.7
2'	596	100.2
3'	-	181.3
4'	-	61.1
5'	-	200.0
6'	-	105.9
4-Me	2.07	9.5
5-OH	11	-
6-COMe	-	202.2
6-COMe	2.65	33.2
4'-Me	1.74	34.1
6'-COMe	-	203.7
6'-COMe	2.65	29.8

[a] Chemical shift (δ) in ppm relative to TMS

Animals

Experiments were carried out with a total of 54 male Albino-Wistar rats weighing 180-200 g. The rats were obtained from Atatürk University, Faculty of Medicine, Department of Pharmacology and Atatürk University-Experimental Animal Teaching and Researcher Center Laboratory (ATADEM). Experiments were performed in accordance with ethical norms approved by the Ethical Committee of the Center for Experimental Animal Education and Research Center (No: 36643897-47). Animals were grouped and kept under standard conditions prior to experiments.

Indomethacin-induced gastric damages

Experimental animals were divided into nine groups. There are six animals in each group. All groups were fasted for 24 hours before being included in the experiment, and the experimental animals were treated appropriately. Experimental groups were compared with FAM and RAN groups. (Table 2-Experimental groups).

Table 2. Experimental treatments, each consisting of 6 rats

Groups	Treatment	Dose/kg body weight
Grup I	IND + UA + OO	25 mg + 25 mg + 2 ml
Grup II	IND + UA + OO	25 mg + 50 mg + 2 ml
Grup III	IND + UA + OO	25 mg + 100 mg + 2 ml
Grup IV	IND + UA + OO	25 mg + 200 mg + 2 ml
Grup V	OO	2 ml
Grup VI (Positive control)	IND + RAN	25 mg + 50 mg
Grup VII (Positive control)	IND + LAN	25 mg + 50 mg
Grup VIII (Negative control)	IND	50 mg
Grup IX (Healthy)	Healthy	-

Olive oil and olive oil and usnic acid were given orally to the animals at the indicated doses. After 10 minutes, IND was given orally to all animals. The healthy group received no treatment. At the end of the treatments, the animals were sacrificed with high-dose anesthesia and their stomachs were removed. Ulcer areas were evaluated macroscopically with millimetric paper. The protective effects were determined biochemically (Berktas et al., 2021; Suleyman et al., 2003).

Biochemical inspection of stomach tissues

After macroscopic analysis of gastric tissues, the activities of SOD, CAT, MPO and NOS enzymes as well as GSH and LPO levels were determined in these tissues. Stomach tissues were ground in a mortar with liquid nitrogen to prepare tissue homogenates. Approximately 0.5 g was weighed for each group and 4.5 ml of the appropriate buffer was then added. After mixing at low speed with the aid of a vortex, this mixture was homogenized for 20 minutes using an ultra-turrax homogenizer. All procedures were performed on ice. The homogenates were filtered and centrifuged at 4°C using a refrigerator centrifuge. All measurements were performed at room temperature using these supernatants.

Biochemical estimations

Superoxide dismutase activity

Superoxide dismutase enzyme activity in gastric tissues was determined according to the appropriate method (Sun et al., 1988). The degree of inhibition of this reaction was measured at 560 nm, and SOD activity was expressed as millimole per minute per milligram tissue (mmol/min/mg tissue).

Catalase activity

Catalase enzyme activity was determined according to the appropriate method and the decrease in absorbance was expressed as millimoles / minute (mmol/min/mg tissue) / milligram tissue (Aebi, 1984).

Total glutathione determination

The amount of glutathione in the stomach tissues was measured according to the method determined in the literature. It was measured spectrophotometrically at 412 nm after treatment with appropriate homogenates. The level of GSH in each sample was expressed as nanomoles per

milligram of tissue (nmol/mg tissue) (Sedlak and Lindsay, 1968).

Determination of lipid peroxidation

Lipid peroxidation levels were determined by estimating malondialdehyde (MDA) using the thiobarbituric acid test. Tissues treated with appropriate solvents were measured spectrophotometrically at 532 nm. Results are expressed as nmol MDA per gram tissue (nmol/g tissue) (Ohkawa et al., 1979).

Myeloperoxidase activity

Myeloperoxidase enzyme activity was determined according to the Bradley method. Changes in absorbance were recorded spectrophotometrically at 450 nm. MPO activity of tissues was expressed as µmol/min/mg tissue (Bradley et al., 1982).

Nitric oxide synthase activity

Nitric oxide synthase activity in tissues was determined according to the appropriate method. The absorption difference between 401 and 421 nm wavelengths was monitored spectrophotometrically. iNOS activity was calculated by subtracting cNOS activity from total NOS activity (Knowles et al., 1990).

Statistical analyses

Statistical calculations were made using the SPSS 22.0 program. The results are expressed as the mean ± Standard Deviation (SD). Firstly, to see whether all data are normally distributed or not, they were analysed by Kolmogorov-Smirnov tests and then the differences in variance were analysed statistically using a one-way analysis of variance (ANOVA) test. Differences between groups were reached using the Duncan option, and significance was reported at $p<0.05$.

RESULTS

Usnic acid (UA) was isolated from the diethyl ether extract obtained from the lichen *Usnea longissima*. The structure of usnic acid (Figure 1) was characterized by IR, MS, 1H- and 13C-NMR (Table 1-spectral data) and 2D-NMR spectroscopic methods and also confirmed by comparison of previously reported spectral data.

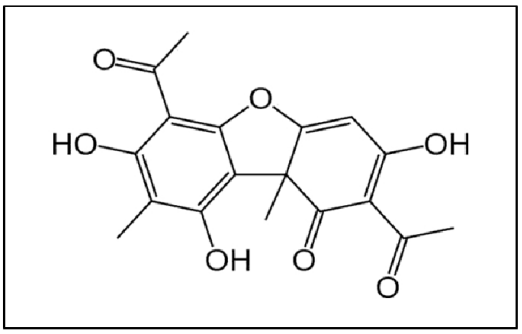

Figure 1. Structure of usnic acid.

The IND-induced gastric ulcer model is a useful and highly preferred experimental model to induce severe ulceration in rats. In this study, the IND-induced ulcer model was preferred to create an ulcer model in rats. 25, 50, 100 and 200 mg/kg UA doses dissolved in 2 ml olive oil and OO alone were administered to the experimental groups (Table 2- experimental groups). Positive controls (RAN and LAN) were dissolved in water and administered to rat groups. One group was given 25 mg/kg dose of IND dissolved in water (negative control). The resulting damages were measured in mm^2 with the help of millimetric paper. According to the results presented in Table 3 and Figure 2, administration of IND caused a very high level of damage in rat stomachs. On the other hand, it was determined that hyperaemia's were significantly reduced in stomachs of rats treated with four doses of UA, RAN, LAN and OO.

The doses of 25, 50, 100 and 200 mg/kg of UA prepared by dissolving in OO reduced ulcer areas by more than 95% compared to the IND group ($p<0.05$). UA at a dose of 100 mg/kg completely inhibited gastric lesions (Table 3 and Figure 2). On the other hand, OO, LAN and RAN reduced their mean damage areas by 70.3%, 92.9% and 72.7%, respectively. These results demonstrated that all doses of UA, LAN (50 mg/kg), RAN (50 mg/kg), and pure OO had significant ($p<0.05$) gastric protective effects against IND-induced gastric injury. While OO

prevented the formation of gastric ulcer by 70.3% when administered alone, this effect was found to be much higher when applied together with UA doses (25, 50, 100 and 200 mg/kg). These research results reveal that OO and UA have a synergistically effect when applied together. This synergistic effect is quite high compared to positive controls (LAN and RAN).

Table 3. Effects of usnic acid (UA), olive oil (OO), ranitidine (RAN) and lansoprazole (LAN) on damages in rat's indomethacin (IND)-induced gastric tissues

Treatment	N	Dose (mg/kg body weight)	Ulcer area (mm^2)[§]	% Inhibition[β]
IND + UA + OO	6	25 + 25 + 2 mL	0.63±0.01[d]	98.0
	6	25 + 50 + 2 mL	0.50±0.01[c]	98.4
	6	25 + 100 + 2 mL	0.00±0.00[a]	100.0
	6	25 + 200 + 2 mL	0.25±0.01[b]	99.2
IND + OO	6	25 + 2 mL	9.13±0.01[g]	70.3
IND + RAN	6	25 + 50	8.38±0.05[f]	72.7
IND + LAN	6	25 + 50	2.20±0.1[e]	92.9
IND	6	25	30.75±0.0 [h]	-
HEALTHY	6	-	0.00±0.00a	-

N: the number of rats. [§]Mean damage area±S.E.M. of six animals in each group. [B] % Inhibition in ulcer area in relation to indomethacin group. Indomethacin group was statistically compared with untreated groups. Other treated groups were statistically compared with indomethacin group. Means in the same column by the same letter are not significantly different to the Duncan test (=0.05). Results are means ± SE of three measurements [a-h]: statistical difference in the same column.

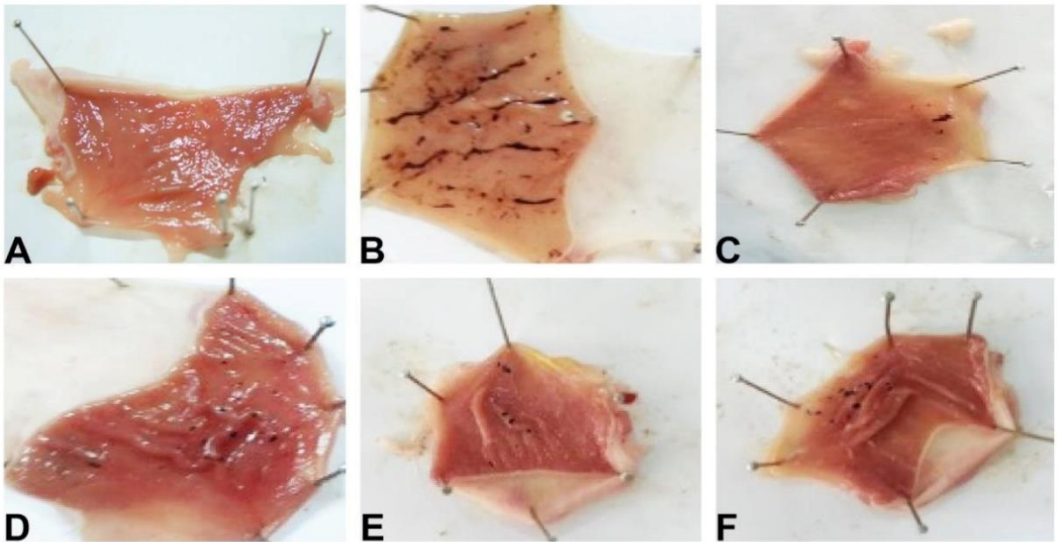

Figure 2. Ulcerous areas in indomethacin (IND)-induced rat's gastric tissues of orally administrated usnic acid (UA), olive oil (OO), ranitidine (RAN) and lansoprazole (LAN). Sections of the gastric tissues after administrations were obtained from some experimental groups. The **A**-**F** sections show some ulcerative areas: **A**, untreated group (healthy); **B**, the negative control group (IND, 25 mg/kg body wt.); **C**, IND-administrated plus OO (2 ml) + UA (200 mg/kg body wt.) group; **D**, IND-administrated plus OO (2 ml) group; **E**, IND-administrated plus LAN (50 mg/kg body wt.) group (positive control 1); **F**, IND-administrated plus RAN (50 mg/kg body wt.) group (positive control 2).

The enzyme activities in rat tissues were measured to determine their effects on antioxidant defence systems. The amounts of LPO and GSH in determining the synergistic effect of OO and UA are expressed in Table 4. Contrary to the applied IND, it appears to be almost as protective as positive controls. Likewise, MPO, SOD and CAT enzyme activities are also expressed in the Figures (Figure 3-4-5). Compared to healthy tissues, a significant protection was determined by the enzyme activities reduced by the application of IND ($p<0.05$).

Table 4. Effects of usnic acid (UA), olive oil (OO), ranitidine (RAN) and lansoprazole (LAN) on changes in levels of lipid peroxidation (LPO) and total glutathione (GSH) in rat's indomethacin (IND)-induced gastric tissues

Treatment	N	Dose (mg/kg body weight)	Amount of LPO (nmol/g tissue)	Amount of GSH (nmol/g tissue)
IND+UA+OO	6	25 + 25 + 2 mL	13.54±0.34^{d}	2.99±0.01^{b}
	6	25 + 50 + 2 mL	11.77±0.02^{b}	3.23±0.01^{e}
	6	25 + 100 + 2 mL	7.37±0.01^{a}	3.46±0.01f
	6	25 + 200 + 2 mL	7.31±0.02^{a}	3.48±0.01^{f,g}
IND+OO	6	25 + 2 ml	14.15±0.04^{e}	3.10±0.01^{c}
IND+RAN	6	25 + 50	12.00±0.01^{b,c}	3.10±0.01^{c}
IND+LAN	6	25 + 50	18.40±0.10^{f}	3.15±0.01^{d}
IND	6	25	35.09±0.40^{g}	2.10±0.01^{a}
HEALTHY	6	-	12.42±0.01^{c}	3.50±0.01^{g}

Means in the same column by the same letter are not significantly different to the Duncan test (P<0.05). Results are means ± SE of three measurements. N: The number of rats. $^{a-g}$: statistical difference in the same column.

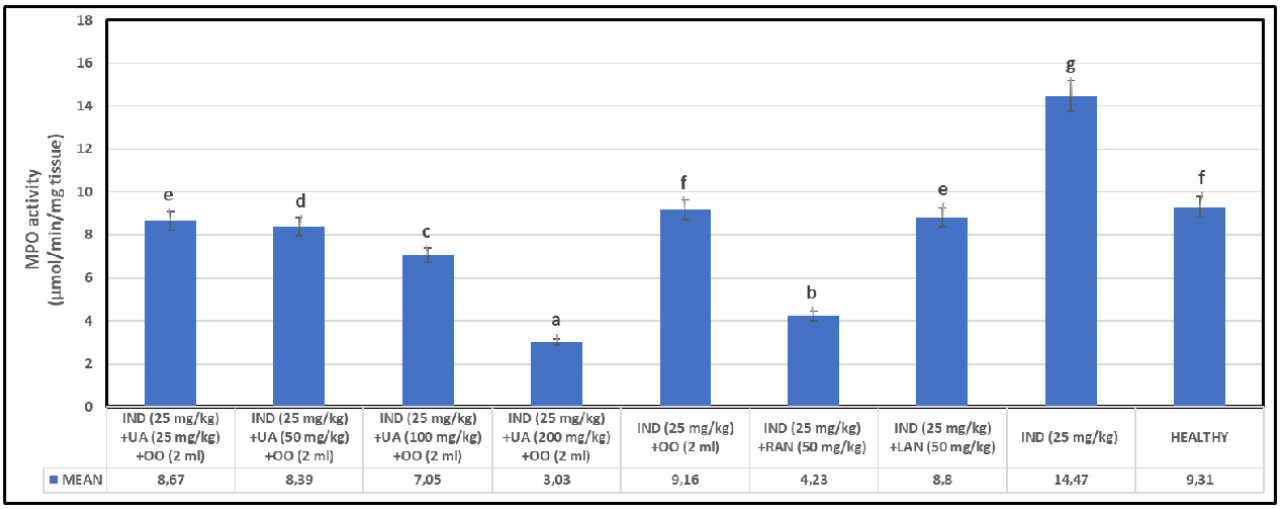

Figure 3. Effects of usnic acid (UA), olive oil (OO), ranitidine (RAN) and lansoprazole (LAN) on changes in enzymatic activity of catalase (CAT) in rat's indomethacin (IND)-induced gastric tissues. Means in the same column by the same letter are not significantly different to the Duncan test (P<0.05). Results are means ± SE of three measurements. N: The number of rats.

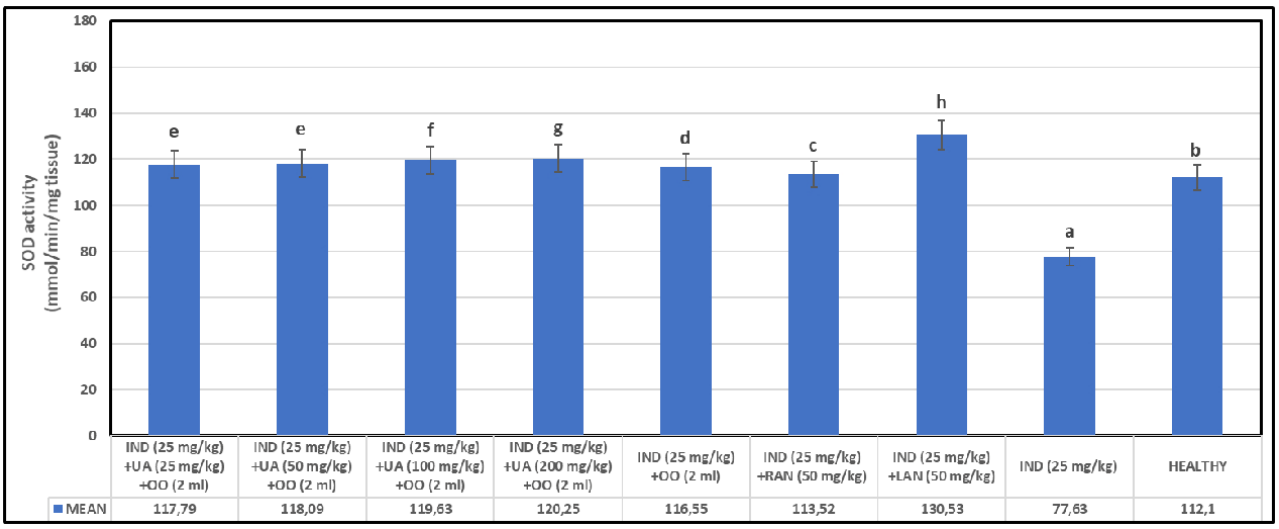

Figure 4. Effects of usnic acid (UA), olive oil (OO), ranitidine (RAN) and lansoprazole (LAN) on changes in enzymatic activity of superoxide dismutase (SOD) in rat's indomethacin (IND)-induced gastric tissues. Means in the same column by the same letter are not significantly different to the Duncan test (P<0.05). Results are means ± SE of three measurements. N: The number of rats.

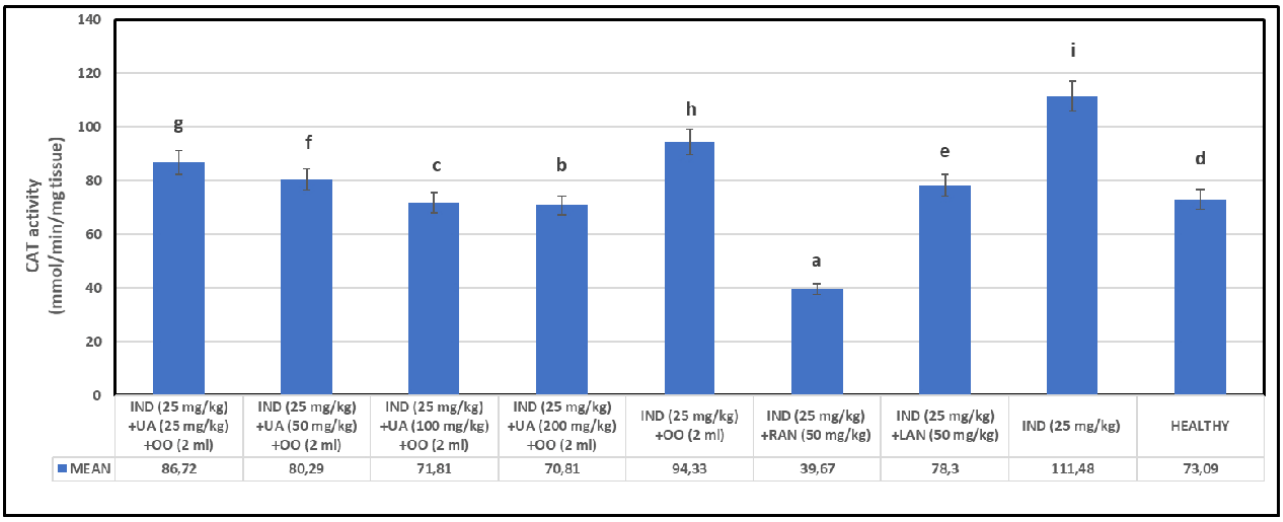

Figure 5. Effects of usnic acid (UA), olive oil (OO), ranitidine (RAN) and lansoprazole (LAN) on changes in enzymatic activity of myeloperoxidase (MPO) in rat's indomethacin (IND)-induced gastric tissues. Means in the same column by the same letter are not significantly different to the Duncan test (P<0.05). Results are means ± SE of three measurements. N: The number of rats.

In addition, levels of nitric oxide produced from the amino acid arginine, which is an important part of the antioxidant enzyme system, were also determined. cNOS, iNOS and tNOS activities produced in three different structures are shown in Table 5.

Table 5. Effects of usnic acid (UA), olive oil (OO), ranitidine (RAN) and lansoprazole (LAN) on changes in activities of inducible-nitric oxide synthase (iNOS), constitutive-nitric oxide synthase (cNOS) and total-nitric oxide synthase (tNOS) in rat's indomethacin (IND)-induced gastric tissue

Treatment	N	Dose (mg/kg body weight)	cNOS (nmol/min/mg tissue)	iNOS (nmol/min/mg tissue)	tNOS (nmol/min/mg tissue)
IND+UA+OO	6	25 + 100 + 2 mL	2.4±0.001^{e}	0.29±0.010^{b}	2.64±0.01^{d}
IND+OO	6	25 + 2 mL	1.96±0.003^{d}	0.41±0.003^{c}	2.36±0.01^{c}
IND+RAN	6	25 + 50	1.73±0.001^{b}	0.50±0.010^{d}	2.23±0.01^{b}
IND+LAN	6	25 + 50	1.84±0.003^{c}	0.50±0.003^{d}	2.32±0.01^{c}
IND	6	25	1.13±0.001^{a}	0.63±0.010^{e}	1.77±0.01^{a}
HEALTHY	6	-	2.41±0.001^{f}	0.26±0.010^{a}	2.68±0.01^{d}

Means in the same column by the same letter are not significantly different to the Duncan test (P<0.05). Results are means ± SE of three measurements. N: The number of rats. $^{a\text{-}f}$: statistical difference in the same column.

DISCUSSION

The protective effects of usnic acid in the formation of gastric ulcers have been noted in *H. pylori* ulcers (Safak et al., 2009) and IND-induced gastric lesions in rats (Odabasoglu et al., 2006). On the other hand, it has been reported in the literature that OO has protective effects against gastric damage (De la Lastra et al., 2001; Odabasoglu et al., 2008). All data obtained from the current study are consistent with the data in the literature. The results from the present study include data on the "synergistic gastro protective effect" between OO and UA, which will be presented for the first time in the literature.

The role of many factors (COX enzymes, TNF alpha, interleukins etc) in the pathogenesis of gastric mucosal damage caused by indomethacin, ethanol and other agents has been demonstrated. In recent years, besides the functions of these factors, attention has been drawn to the roles of reactive oxygen species (ROS) and antioxidants in the formation of IND

induced-stomach injury (Atalay et al., 2016; Dejban et al., 2020; Odabasoglu et al., 2006).

The present results (Figure 3-MPO enzyme activity) showed that administration of IND significantly increased the activities of myeloperoxidase enzyme (MPO) in gastric tissues ($p<0.05$). In addition, in the present experiments, it was determined that the activity of MPO in gastric tissues increased by the IND was reduced to the levels of the healthy control group after treatment with all UA doses, OO, LAN and RAN ($p<0.05$). The increase in activity of MPO as a result of IND administration may indicate that it is a marker of increased level of neutrophil infiltration into gastric damaged tissues. Similar results have been reported by various researchers (Atalay et al., 2016; Dejban et al., 2020; Nishida et al., 1998; Odabasoglu et al., 2006, 2008,).

The neutrophils and phagocytose cells are the most specialized cells of the immune system. Their activation and infiltration into tissues leads to inflammation and formation of lesions. MPO is a hem peroxidase enzyme stored in neutrophil granules. MPO activity increases, indicates the accumulation of neutrophils in tissues. Gastric ulcers and lesions that occur after treatment with IND and similar NSAIDs are a form of inflammation. MPO enzyme can be considered as control mechanisms for neutrophil infiltration into gastric mucosal tissues (Coskun et al., 1996; Dejban et al., 2020; Takeuchi et al., 1998; Wei et al., 2021). When there is an increase in the level of neutrophil infiltration into gastric damaged tissues, an increase in these enzyme activities is also observed. The peroxidases activated in damaged tissues increase the production of superoxides. Increasing superoxide trigger lipid peroxidation in membranes. As a result, the membranes are damaged and ulcers occur in the stomach tissues (Atalay et al., 2016; Kuzmanova et al., 2019; Odabasoglu et al., 2006; Ogaly et al., 2021; Tahir et al., 2022; Takeuchi et al., 1998, Valcheva et al 2019).

In the present study, lipid peroxidation levels (LPO) in gastric tissues were determined for all treatment groups on the ulcer formation process. Compared with the healthy rat group, it was determined that IND administration increased LPO levels ($p<0.05$). Contrary to IND, it was determined that all doses of UA, OO, LAN and RAN treatments decreased LPO levels ($p<0.05$). These results are in agreement with the literature (Atalay et al., 2016; Kuzmanova et al., 2019; Odabasoglu et al., 2006; Ogaly et al., 2021; Takeuchi et al., 1998; Valcheva et al., 2009). The results obtained from the present study indicate that the activated MPO enzyme in gastric damaged tissues increases the level of lipid peroxidation via superoxides. In the light of these data, it can be said that the excessive level of LPO causes damage to the gastric tissue membranes and ulcers occur in the stomach tissues.

After the ulcer formation process, enzymatic (SOD and CAT activities) and non-enzymatic (GSH amounts) antioxidant levels were determined in gastric tissues for all treatment groups. It was determined that IND application decreased SOD activities and GSH amounts, and increased CAT activities ($p<0.05$). Contrary to IND, it was determined that all doses of UA, OO, LAN and RAN treatments increased SOD and GSH levels while decreasing CAT amounts ($p<0.05$).

In previous literature, it has been reported that organisms have antioxidative defense systems against lipid peroxidation caused by ROS in cell membranes. These antioxidative systems can be enzymatic (such as SOD and CAT enzyme activities) or nonenzymatic (such as the amount of GSH) (Anvar et al., 2021; Atalay et al., 2016; Berktas et al., 2021; Bozkurt et al., 2017; Hanci et al., 2018; Odabasoglu et al., 2006; Ogaly et al., 2021; Takeuchi et al., 1998; Valcheva-Kuzmanova et al., 2019). In many previous studies, it has been shown that the administration of IND, an NSAID, reduces the amount of GSH and SOD activities in the stomach tissues (Anvar

et al., 2021; Atalay et al., 2016; Berktas et al., 2021; Bozkurt et al., 2017, Hanci et al., 2018, Ogaly et al., 2021; Valcheva-Kuzmanova et al., 2019). In contrast, administration of all doses of UA, OO, LAN and RAN resulted in a significant increase in SOD activities and GSH levels ($p<0.05$).

The amount of GSH is very important for the dissociation of thiol groups. Regular support of radicals and maintenance of this situation will be of vital importance for the cell. The current publication results reveal that all doses of UA, OO, LAN, and RAN increased the amount of GSH in the stomach of the rat. In previous studies, it has been reported that UA, OO, LAN and RAN have a protective effect by reducing oxidative stress and preventing intracellular GSH depletion against IND-induced gastric ulcer. Our findings are in agreement with previously recorded results. Previously reported studies have shown that both UA and OO stimulate both enzymatic and non-enzymatic antioxidant defence systems and have the potential to be used as a natural antioxidant. This also is true for our positive controls, LAN and RAN.

It has been reported that SOD activity in rat stomach tissues is inhibited when NSAIDs such as IND are used (Anvar et al., 2021; Berktas et al., 2021; Karakus et al., 2009, Ogaly et al., 2021; Valcheva-Kuzmanova et al., 2019). Our results are consistent with these reports and confirm previous results. The SOD enzyme plays an important role in preventing gastric damage by partially preventing oxidative damage caused by radicals. SOD enzyme converts the reactive superoxide radical ($O2^{-\bullet}$) to less reactive hydrogen peroxide (H2O2•). Formed H_2O_2 is used as substrate by CAT and MPO enzymes. In the present study, it was found that SOD activity was reduced by IND treatment. On the other hand, it was observed that inhibited SOD activity was activated to healthy tissue levels by UA, OO, LAN and RAN (Figure 4). These findings indicate that increased SOD activity is very important in gastric protection.

According to the results obtained in this study, CAT activity was found to be increased in IND-treated rat tissues compared to healthy rat tissues (Figure 5). On the other hand, all treatments reduced the trend to CAT levels in healthy tissue. Our findings are in agreement with the results reported in previous studies (Anvar et al., 2021; Atalay et al., 2016; Berktas et al., 2021; Bozkurt et al., 2017; Hanci et al., 2018; Ogaly et al., 2021; Valcheva-Kuzmanova et al., 2019).

The current research findings indicate that ROS production increases due to the elevated MPO activity in gastric tissues following IND application, and increased ROS initiate lipid peroxidation (LPO) and ultimately ulcers are formed. In other words, ROS formation and increased LPO level play a key role in the development of gastric mucosal lesions caused by IND. In parallel with the increase in ROS, the levels of H_2O_2 and OH• in the stomach tissues also increase. In response, the MPO and CAT enzymes increase and try to reduce the damage by consuming H_2O_2. After treatment with OO, LAN, RAN and all UA doses, H_2O_2 and all other ROS levels are reduced. In other words, the activities of MPO and CAT enzymes, which use H_2O_2 as a substrate, also decrease to healthy tissue levels.

It shows that indomethacin administration causes inhibition of cytoprotective NOS (cNOS) activities in gastric tissues, while also leading to significant activation of inducible NOS (iNOS) activities ($p<0.05$). In addition, in the current experiments, it was determined that after treatment with all UA doses, OO, LAN and RAN, increased iNOS and decreased cNOS activities with IND were brought to the levels of the healthy control group ($p<0.05$). In addition, in the current experiments, it was determined

that increased iNOS and decreased cNOS activities with IND after treatment with all UA doses, OO, LAN and RAN were brought to the levels of the healthy control group ($p<0.05$). Similar results have been reported by various researchers (Dejban et al., 2020; Nishida et al., 1998; Odabasoglu et al., 2006).

The nitric oxide (NO) is a biologically molecule with very important tasks. It is produced from the amino acid L-arginine by the nitric oxide synthase (NOS) enzyme. There are three isoforms of NOS: cytoprotective NOS (cNOS) produced in endothelial and wholebody tissues, inducible NOS (iNOS) produced by phagocytes and leukocytes and neuronal NOS (nNOS) produced in neuronal tissues. Many reports have been recorded that NO has a very important role in the whole organism, as well as a protective role against gastric erosions and ulcers. Rojas-Martínez et al (2013) reported that NO modulates the healing process of gastric ulcer. According to them, NO maintains the blood flow of the stomach as a vasodilator. On the other hand, when NO production is insufficient, it can lead to gastric mucosal damage, erosions and ulcers (Konturek et al., 1993). For these reasons, the amount of NO produced by cNOS in organisms must be stable. The organisms need extra NO in the inflammatory process and in case of tissue damage. Thus, it is tried to keep the NO amount stable by increasing the iNOS activity. In this case, it tells us that there has been a trauma or damage (Dejban et al., 2020).

It has also been widely accepted that NO produced by cNOS in digestive systems is cytoprotective and NO produced by iNOS is cytotoxic (Nishida et al., 1998; Odabasoglu et al., 2006). As one of the remarkable results of the present study, it was determined that cytotoxic-inducible iNOS activity was activated while cytoprotective-cNOS activity was inhibited by the IND ($p<0.05$). On the other hand, it was measured that all UA doses, OO, LAN and RAN treatments brought cNOS and iNOS activities closer to the healthy group level (Table 5-iNOS, cNOS, tNOS levels). According to the available data, the increase in the amount of LPO due to the increase in the activity of the MPO enzyme following the application of IND indicates membrane damage. Increases in MPO, CAT and iNOS activities and decreases in SOD, GSH and cNOS amounts seem to be effective in further increasing the level of damage in gastric tissues.

According to the results of the present experimental study, it can be said that UA and OO can restore and strengthen non-enzymatic and enzymatic antioxidative defence systems due to their ability to reduce lipid peroxidation. This ability may be the source of the gastroprotective effects of both UA and OO. Moreover, when UA and OO are applied together, these abilities increase synergistically. Taking these properties into account, drug preparations containing UA and OO together can be developed.

CONCLUSION

In conclusion, biochemical damages caused by IND in gastric tissues were approximated or eliminated to healthy groups with all UA doses, OO, LAN and RAN treatments. Among all these positive effects, the most striking thing is that UA showed a synergistic effect when applied together with OO.

ACKNOWLEDGMENT

Financial support: This research was not funded financially by any institution or company.

Conflict of interest: The authors have no commercial interest, financial interest and/or other relationship with manufacturers of drugs, laboratory supplies and/or medical devices, or commercial providers of medically related services.

Ethical statement: The rats were obteined from Atatürk University, Faculty of Medicine, Department of Pharmacology and Atatürk University-Experimental Animal Teaching and Researcher Center Laboratory (ATADEM). Experiments were performed in accordance with ethical norms

approved by the Ethical Committee of the Center for Experimental Animal Education and Research Center (No: 36643897-47).

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