Phytochemical analysis and gastroprotective activity of an olive leaf extract

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Abstract: Some medicinal features of olive leaf have been known for centuries. It has been traditionally used as an antimicrobial and to prevent and treat diabetes mellitus and heart disease. Whether olive leaf, a natural antioxidant, influences the gastric defense mechanism and exhibits gastroprotection against experimentally-induced gastric lesions remains unknown. In this study, the content of total phenols, total flavonoids and tannins in olive leaf extract (OLE) were determined. Seven phenolic compounds were identified and quantified (oleuropein, caffeic acid, luteolin, luteolin-7-*O*-glucoside, apigenine-7-*O*-glucoside, quercetin, and chryseriol). Furthermore, the protective activity of the OLE in gastric mucosal injury induced by a corrosive concentration of ethanol was investigated. In relation to the control group, pretreatment with OLE (40, 80 and 120 mg kg^{-1}) significantly ($p < 0.001$) attenuated the gastric lesions induced by absolute ethanol. The protective effect of the OLE was similar to that obtained with a reference drug, ranitidine. The results obtained indicate that OLE possesses significant gastroprotective activity, and that the presence of compounds with antioxidative properties would probably explain this effect.

Keywords: olive leaf extract; phenols; flavonoids; tannins; gastroprotection.

INTRODUCTION

Research on flavonoids and other polyphenols, their antioxidant properties, biological activities and their effects in disease prevention truly began in the last decade. There is an increasing interest in medicinal plant extracts, the greatest value of which may be due to constituents that contribute to the modulation of the oxidative balance *in vivo*. Benefits of the olive (*Olea europaea* L.) leaf have been

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known for centuries and it has been traditionally used to prevent and treat different diseases.

Olive leaf is used to enhance the immune system, as an antimicrobial and in heart disease. Folk medicine uses also include hypertonia, arteriosclerosis, rheumatism, gout, diabetes mellitus, and fever.[1] Recently, experimental animal studies have demonstrated hypoglycemic,[2,3] hypotensive,[4] anti-arrhythmic,[5] anti-atherosclerotic,[6] and vasodilator effects,[7] as well as a stimulatory effect on the activity of the thyroid.[8] Antimicrobial,[9–12] antiviral,[13,14] anti-tumor,[15,16] and anti-inflammatory activity[17] were also reported.

Despite the number of papers published on olive leaf and the effects of its constituents, none has focused on its influence on the gastric defense mechanism and gastroprotective activity.

It is well known that oleuropein, one of the iridoide monoterpenes, is the main phenolic constituent of olive leaves, which is thought to be responsible for their pharmacological effects. Furthermore, olive leaves contain triterpenes, flavonoids, and chalcones.[1,18] Its chemical content makes olive leaf one of the most potent natural antioxidant.

The gastric mucosa plays the role of a barrier that limits exposure of the gastric mucosal cells to numerous injurious luminal agents and irritants of exogenous and endogenous origin. Pretreatment with different substances could effectively prevent the gastric mucosa from the development of erosions and ulceration. This action, called gastro- or cyto-protection is not related to the inhibition of gastric acid secretion and is known to account for gastroprotection by various irritants. The role of oxygen-derived free radicals in the generation of gastric injury is also well-known. Effects of reactive oxygen species (ROS) on the gastric mucosa in various experimental models of stress-induced mucosal injury were proven. Previous studies demonstrated that the damaging action of absolute ethanol could be attributed to the enhancement of the ROS and the ROS-dependent increase in lipid peroxidation and inhibition of antioxidative enzyme activity.[19]

In light of the above considerations, the chemical composition and the protective effect of an olive leaf extract (OLE) on ethanol-induced gastric mucosal damage in rats were investigated, since in this experimental model the pathogenesis of the lesions has been related with production of reactive oxygen species. This study represents the first step in the recognition of the gastroprotective properties of the olive leaf.

EXPERIMENTAL

Materials

Standardized dry olive leaf extract, EFLA® 943, was purchased from Frutarom Industry Ltd. (Wädenswil, Switzerland). Ranitidine tablets were obtained from Galenika a.d. (Belgrade, Serbia). Sodium bicarbonate (analytical grade) was purchased from Sigma-Aldrich (Schnelldorf, Germany). Analytical grade reagents ethyl acetate, acetone, hydrochloric acid (HCl) and

absolute ethanol were purchased from Merck (Darmstadt, Germany). HPLC grade acetonitrile (MeCN) and methanol were also purchased from Merck (Darmstadt, Germany). Reference HPLC standards were purchased from Carl Roth (Karlsruhe, Germany).

Determination of total phenols content

The total content of phenols was determined by the Folin–Ciocalteu method.[20] A total of 100 μl of a methanolic solution of dry extract (17.5, 13.1, and 8.8 μg ml^{-1} final quantity) was mixed with 0.75 ml of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at 22 °C for 5 min; 0.75 ml of sodium bicarbonate (60 g l^{-1}) solution was added to the mixture. After 90 min at 22 °C, the absorbance was measured using a Hewlett Packard 8453 UV–Vis spectrophotometer (Agilent Technologies, Santa Clara, CA) at λ_{max} 725 nm. Results are expressed as gallic acid equivalents (GAE), and presented as mean ± ± standard deviation (*SD*) of three determinations.

The percentage content of tannins was calculated using the method described in the European Pharmacopoeia, Ph. Eur., 6.0.[21] The content of tannins, expressed as pyrogallol percentage, is presented as the mean ± *SD* of three determinations.

Determination of total flavonoids content

The percentage content of flavonoids expressed as hyperoside was calculated using the method described in the Deutsches Arzneibuch, DAB (German Pharmacopoeia) 10.[22] Briefly, the sample was extracted with acetone/HCl under a reflux condenser; the AlCl$_3$ complex of the flavonoid fraction was extracted with ethyl acetate and measured by a UV–Vis spectrophotometer at λ_{max} 425 nm. The content of flavonoid, expressed as the hyperoside percentage, is presented as the mean ± *SD* of three determinations.

High pressure liquid chromatography (HPLC) procedure

A HPLC fingerprint of the extract and quantification of the identified compounds was achieved by HPLC (Agilent Technologies 1200). Detection was performed using a diode array detector (DAD) and the chromatographs were recorded at λ = 260 nm (for flavonoids and oleuropein) and at 325 nm (for caffeic acid). The spectra recorded at 360 nm were used to identify luteolin and chryseriol. HPLC separation of components was achieved using a LiChrospher 100 RP 18e (5 μm), 250 mm×4 mm i.d. column with a mobile phase flow rate of 1.0 ml min^{-1}. The mobile phase A consisted of 500 ml of H$_2$O plus 9.8 ml of 85 % H$_3$PO$_4$ (w/w), while B was MeCN. A combination of gradient modes: 92–75 % A, 0–8 min; 75–60 % A, 35–55 min and 60–50 % A, 55–60 min. The sample was prepared by dissolving 66.4 mg of the extract in 10 ml of methanol, filtered through 0.20 μm PTFE membrane filters. The identification was realized according to retention time and spectra matching. Once spectra matching succeeded, the results were confirmed by spiking with the respective standards to achieve a complete identification by means of the so-called peak purity test. Peaks not fulfilling these requirements were not quantified. Quantification was performed by external calibration with standards.

Gastric lesions induction and evaluation

This study was approved by the Ethical Committee, School of Medicine, University of Belgrade, and run in accordance to the statements of the European Union regarding the handling of experimental animals. Wistar male rats (*n* = 30), weighing between 200 and 220 g were randomly divided into 5 groups. The animals were placed in individual metabolic cages. Before the experiment, they were fasted overnight, but had free access to water.

The first, control group, received distilled water intragastrically (*i.g.*) 30 min prior to administration of 1.0 ml absolute ethanol. Three different doses of OLE were applied on the next three groups, and finally, the last group (positive control) received 50 mg kg^{-1} of raniti-dine, an H$_2$ receptor antagonist, as a reference drug. For extrapolation of the dosage from humans to rats, the metabolic body size or food intake rather than body weight was used as the criterion.[23,24] Hence, 40 mg kg^{-1} of OLE was administered as the minimum dose but higher doses of 80 and 120 mg kg^{-1} were also given to test for a dose response. Both OLE and rani-tidine were suspended in distilled water before administration. One hour after *i.g.* applied ethanol, the animals were sacrificed under the light ether anesthesia, the abdomen was opened by a midline incision, the stomach was removed, opened along the greater curvature, rinsed gently with water and pinned open for macroscopic examination and for photodocumentation by a digital camera (Hewlett Packard PhotoSmart R507). The areas of gastric lesions were measured by planimetry using the NIH ImageJ computer program[25] and the ulcer index (*UI*) were estimated from the formula:

$$UI = (\text{Ulcerated area/Total stomach area}) \times 100$$

Results are expressed as means $\pm$ *SD*. Statistical analysis was achieved using the *t*-test. Differences with $p < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

Many different commercial preparations of olive leaf and extracts are available and vary in strength. Various extraction techniques, as well as different origin of the olive leaves, results in some differences in the chemical composition of the extracts. Standardization of commercially available extracts is strictly based on their oleuropein content, although other constituents of olive leaf are not less important in explaining its medicinal features but data are unavailable.

In this study, an olive leaf extract standardized to 18–26 % of oleuropein, with confirmed stability and chemical and microbiological purity, was employed.

Group of active constituents

Quantitative analysis of the contents of total phenols, flavonoids and tannins were performed.

The total phenols content of the OLE, determined by the Folin–Ciocalteu method, was 197.8$\pm$11.3 µg GAE *per* g of dry extract. This indicates the expectable high total phenols content (19.8 %) of the OLE. Further phytochemical investigation yielded flavonoids and tannins, 0.29 and 0.52 %, expressed on total dry OLE, respectively.

Flavonoids are a widely distributed group of polyphenolic compounds, identified in recent years as antioxidants in various biological systems. It is well known that one of the important effects of flavonoids is the scavenging of oxygen-derived free radicals, and that flavonoids can prevent injury caused by free radicals, including experimentally induced gastric mucosal injury.[26,27]

Low concentration of tannins are known to "tan" the outermost layer of the gastric mucosa and to render it less permeable and more resistant to chemical and

mechanical injury or irritation.[28,29] The gastroprotective effect of tannins was experimentally confirmed when the administration of tannins was found to significantly lower stomach free radical concentrations in rats.[30]

In order to further elucidate the chemical composition of the OLE, the phenolic compounds were identified and quantified.

Analysis of the OLE by HPLC revealed a complex mixture of phenolic compounds (Fig. 1). It was intended to identify ten components of the OLE and seven of them were found.
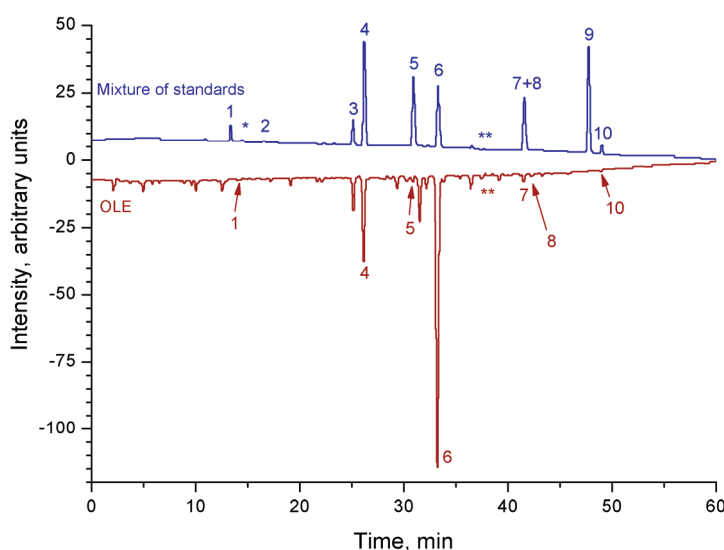

Fig. 1. HPLC chromatogram of the olive leaf extract recorded at 260 nm and compared to the standard mix of identified compounds. The numbers refer to the following: **1**, caffeic acid; **2**, vanillin; **3**, rutin; **4**, luteolin-7-*O*-glucoside; **5**, apigenine-7-*O*-glucoside; **6**, oleuropein; **7**, quercetin; **8**, luteolin; **9**, apigenine; and **10**, chryseriol. *Impurity from caffeic acid; **impurity from the oleuropein standard.

The major constituent of the OLE was oleuropein (Fig. 2), composing 19.8 % of the extract (Table I). The antioxidant properties of oleuropein are well known. It exhibited high antioxidant activity *in vitro*, comparable to a hydrosoluble analog of tocopherol[31] and exhibited strong antioxidant protection in oxidative stress during ischemia-reperfusion in an *in vivo* experimental model.[32]

The other identified components were caffeic acid, luteolin-7-*O*-glucoside, apigenine-7-*O*-glucoside and quercetin (Fig. 2). These constituents of the olive leaf also exert antioxidative properties, which was experimentally confirmed in several studies.[33,34] Moreover, it was shown that a total olive leaf extract had an antioxidant activity higher than that of vitamin C and vitamin E, due to the synergy between the flavonoids, oleuropeosides and substituted phenols.[33]
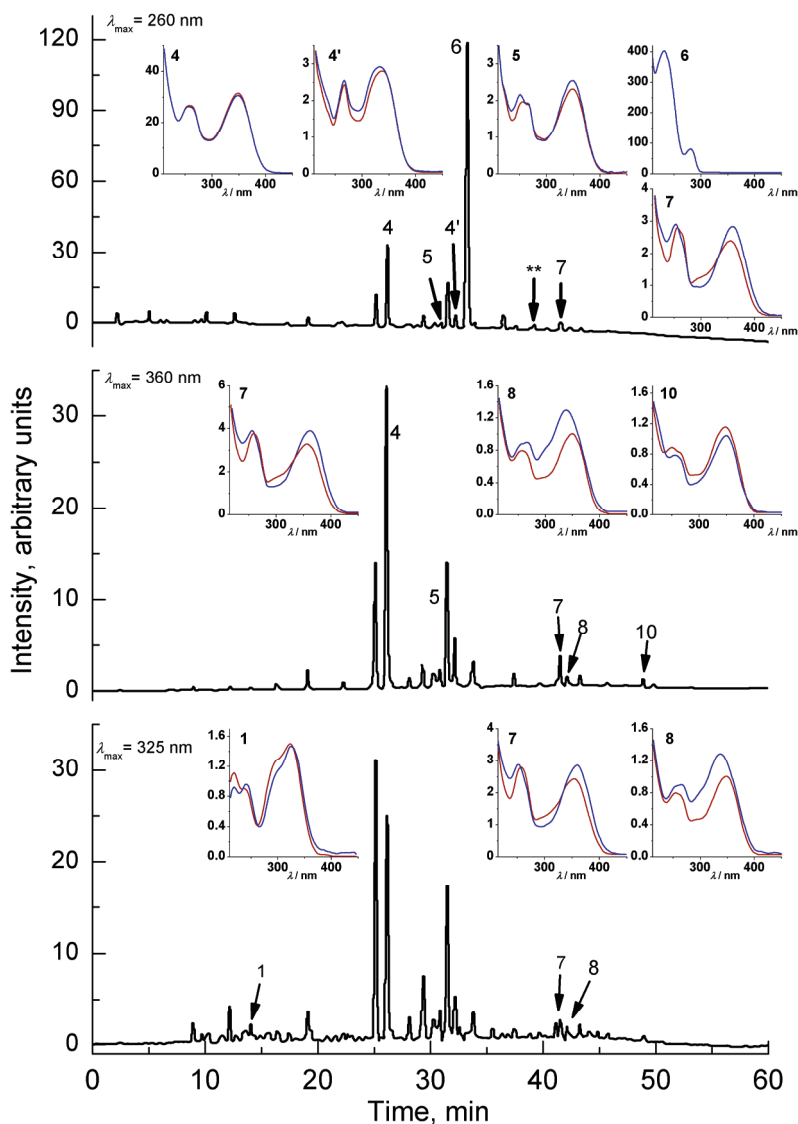
Fig. 2. HPLC chromatogram of the olive leaf extract recorded at 260, 360 and 325 nm with the spectrum of identified compounds and compared to the UV spectra of reference standards. The numbers refer to the following: **1**, caffeic acid; **4**, luteolin-7-*O*-glucoside; **5**, apigenine-7--*O*-glucoside; **6**, oleuropein; **7**, quercetin; **8**, luteolin and **10**, chryseriol (**4'** refers to a derivate of luteolin, taking into account the spectrum of the corresponding peak).
**Impurity from the oleuropein standard.

Luteolin and chryseriol were also isolated from the extract of olive leaves.[17] Since in this study they were present only in the traces (Table I), they did not significantly contribute to the total flavonoid content.

TABLE I. Quantitative determination of flavonoids, phenolcarbonic acids and oleuropein in the studied olive leaf extract

Compound name[a]	Amount	
	mg	%
Caffeic acid (**1**)	0.013	0.02
Vanillin (**2**)	Not found	–
Rutin (**3**)	Not found	–
Luteolin-7-*O*-glucoside (**4**)	0.027	0.04
Apigenine-7-*O*-glucoside (**5**)	0.046	0.07
Oleuropein (**6**)	13.147	19.8
Quercetin (**7**)	0.027	0.04
Luteolin (**8**)	Trace[b]	–
Apigenine (**9**)	Not found	–
Chryseriol (**10**)	Trace[b]	–

[a]The numbers refer to the compounds marked on the HPLC chromatogram (Figs. 1 and 2); [b]determination was not possible – present in the extract under the limit of quantitative analysis

Luteolin-7-*O*-glucoside is widespread in plant species and its anti-radical activity is well-known. Its presence in olive leaf was previously confirmed,[12,35] as well as its anti-ulcer activity.[29] This flavonoid was also identified and quantified in this study, composing 0.04 % of the extract (Table I).

Apigenine, vanillin and rutin were identified in olive leaf in some analytical studies.[12,33,35] Their presence was not confirmed in this investigation, but 0.04 % of quercetin, 0.07 % of apigenine-7-*O*-glucoside and 0.02 % caffeic acid (Table I) were found.

Quercetin is the most abundant of the flavonoid molecules and it is found in many medicinal botanicals. It has been reported to prevent gastric mucosal lesions induced by ethanol.[36] Quercetin increases the amount of neutral glycoproteins in the gastric mucosa[37] and thus participates in the recovery of the mucosal defensive capacity against aggression from absolute ethanol. Other possible mechanisms include inhibition of lipid peroxidation,[36] inhibition of the gastric proton pump,[37] and scavenging of free radicals associated with a significant enhancement in the glutathione peroxidase activity.[38]

Radical scavenging abilities for apigenine-7-*O*-glucoside and for caffeic acid were also reported.[33]

Effect of intragastrically applied OLE on gastric lesions induced by absolute ethanol

There are various plant-originating gastroprotectors with different compositions that have been used in clinical and folk medicine due to their beneficial effects on the gastric mucosa. The documented literature has centered primarily on their pharmacological action in experimental animals. Many studies have demonstrated that substances with antioxidant properties (especially polyphenolic compounds) may protect against the gastric-damaging effects of absolute ethanol.[39–42] The beneficial properties of the polyphenols of olive leaf, the same as in

olive oil, are further enhanced by their good bioavailability. The results obtained for oleuropein and its metabolites, tyrosol and hydroxytyrosol, indicated that they are readily absorbed through the gastrointestinal tract, resulting in significant levels in the circulation.[43,44]

In this study, the protective effect of OLE, a natural antioxidant, on the gastric mucosal damage induced by absolute ethanol in rats was studied.

The administration of absolute ethanol to fasted rats resulted in severe gastric damage, visible from the outside of the stomach as thick reddish-black lines. After opening, gastric lesions were found in the mucosa and consisted of elongated bands, 1–10 mm long, usually parallel to the long axis of the stomach. They were located mostly in the corpus, the portion of the stomach secreting acid and pepsin. No visible lesions developed in the non-secretry part of the stomach.

The effect of absolute ethanol and pretreatment with OLE applied *i.g.* in graded concentrations, as well as ranitidine, on the ulcer index, *UI*, is shown in Fig. 3. Ethanol caused typical widespread gastric lesions on 14.7 ± 5.5 % of total stomach area. Pretreatment with all three doses of OLE significantly ($p < 0.001$) reduced gastric lesions induced by absolute ethanol. The best result was obtained in group pretreated with 80 mg kg^{-1} of OLE, when the ethanol caused gastric lesions on only 2.6 ± 1.4 % of the total stomach area. The gastroprotective effect of OLE was similar to that achieved by pretreatment with the known anti-ulcer drug, ranitidine, when the *UI* was 3.6 ± 0.8 %. Hence, the useful role of gastric anti-sec-
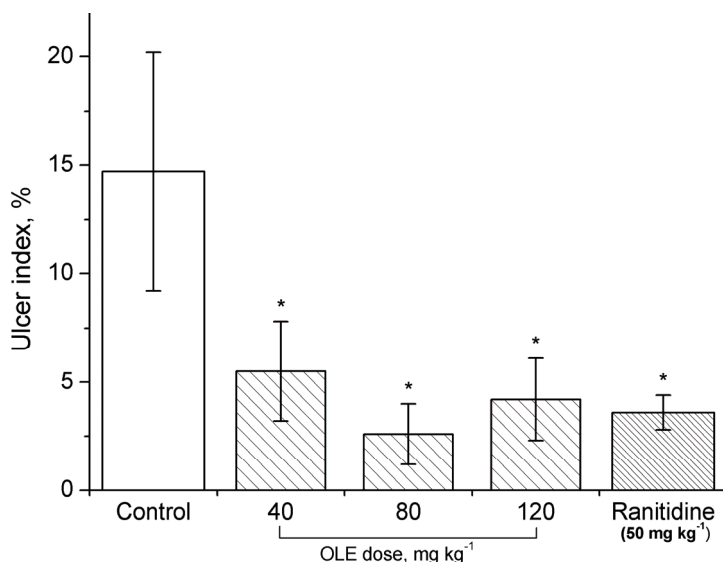

Fig. 3. Effect of intragastric pretreatment with olive leaf extract (OLE) applied in graded doses ranging from 40 up to 120 mg kg^{-1} and ranitidine (50 mg kg^{-1}) on the ulcer index induced by absolute ethanol. The asterisk indicates statistical significance of inhibition ($p < 0.001$), as compared to the control value.

retry medication in the prevention of gastric mucosal damage was also confirmed.

The obtained results indicate that the gastroprotective potential of OLE most probably results from the ability of its constituents to scavenge reactive oxygen species, produced in ethanol-induced gastric injury, which initiate lipid peroxidation. The actual potential is probably related to its ability to maintain the integrity of the cell membrane, by its anti-lipid peroxidative activity and to protect in this way the gastric mucosa against oxidative damage, and by its ability to strengthen the mucosal barrier, the first line of defense against exogenous and endogenous ulcerogenic agents.

CONCLUSIONS

The investigated olive leaf extract caused a significant attenuation of the gastric damage induced by a corrosive concentration of ethanol, suggesting a respectable gastroprotective activity. This activity could be related to its antioxidative properties, since phytochemical analysis of OLE showed a high content of phenolic compounds, well-known antioxidants. However, in order to elucidate the mechanism of OLE gastroprotective effect, and to understand better the actual potential, further investigation will be focused on the determination of lipid peroxidation and antioxidative enzyme activity in the gastric mucosa.

И З В О Д

ФИТОХЕМИЈСКА АНАЛИЗА И ГАСТРОПРОТЕКТИВНО ДЕЈСТВО ЕКСТРАКТА ЛИСТА МАСЛИНЕ

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Нека лековита својства листа маслине су позната вековима. Он се традиционално користи као антимикробни агенс и за превенцију и лечење шећерне болести и срчаних обољења. Остало је непознато да ли примена листа маслине утиче на одбрамбене механизме слузнице желуца и да ли показује гастропротективно дејство код експериментално индукованих лезија желуца. У екстракту листа маслине прво је одређен садржај укупних фенола, флавоноида и танина, а потом идентификовано и квантификовано 7 фенолних једињења (олеуропеин, кафена киселина, лутеолин, лутеолин-7-*О*-глукозид, апигенин-7-*О*-глукозид, кверцетин и хризериол). Испитано је и протективно дејство екстракта листа маслине код оштећења желудачне мукозе корозивном концентрацијом етанола. Претретман екстрактом листа маслине у дозама од 40, 80 и 120 mg kg^{-1} значајно је смањио оштећење желуца у односу на контролну групу ($p < 0,001$). Ово дејство је било слично дејству референтног лека, ранитидина. Добијени резултати указују да екстракт листа маслине поседује значајно гастро-

протективно дејство, које би се донекле могло објаснити присуством антиоксиданаса у његовом хемијском саставу.

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