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Hydroxytyrosol and oleuropein from olive leaves: Potent anti-inflammatory and analgesic activities

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Abstract

The goal of this study was to evaluate the analgesic and the anti-inflammatory activities of two polyphenols rich olive leaves extracts. At the tested doses (100, 200 and 300 mg/kg), Oleuropein - Rich Olive Extract (ORE) and Hydroxytyrosol - Rich Olive Extract (HRE) inhibited, significantly, writhing and reduced carrageenan-induced hind paw edema in a dose dependent manner. HRE was more effective than ORE to relieve inflammation and nociception. In addition, the acute toxicity test showed that ORE and HRE did not present acute toxicity up to a maximum dose of 1000 mg/kg.

Key words: *Olea europaea* L. cv. Chemlali, anti-inflammatory activity, analgesic activity, oleuropein, hydroxytyrosol, paw oedema, writhing test.

Introduction

The olive tree (*Olea europaea* L.) has been cultivated in the Mediterranean for more than a thousand years. The positive effects on health of its fruits, oil and leaves are well known. Olive leaves contain different groups of constituents such as iridoids, polyphenols, flavones and carbohydrates [1-5]. Fresh olive leaves contain up to 10% of polyphenols [6]. Oleuropein and hydroxytyrosol are the main active ones [7, 8]. Oleuropein is used as the typical marker compound of extracts (as recently in Pharmacopoea PhEur 5) and its concentration is significantly high in leaves [9, 10]. Hydroxytyrosol is an interesting metabolite obtained from oleuropein hydrolysis. It is incorporated in the aglycon of oleuropein and is thought to be released from this glycoside owing to the action of cellular esterase or acidic catalysis [11] (Fig. 1).

Until now, various bioactivities of oleuropein and hydroxytyrosol have been identified in several studies. Oleuropein has been reported to possess numerous properties for its hypoglycaemic [12], vasodilatory [13, 14], antimicrobial [15-17], hypotensive [18, 19], anti-inflammatory [20, 21], anti-rheumatic [21], antiatherogenic [22, 23] and antipyretic [24] effects. Many of these pharmacologic features of oleuropein are due to its potent antioxidant action [25-28]. Moreover, hydroxytyrosol has been shown to have several beneficial effects. Previous studies showed its antioxidant [29-32], hypoglycaemic [33], antithrombotic [34], hypocholesterolemic [35], anti-inflammatory [36-38] and antimicrobial activities [15, 39-41]. Furthermore, hydroxytyrosol has been proved to prevent oxidative damage in human erythrocytes [42], to contribute to eye health [43, 44], to down-regulation of the immunological response [45] and to reduce the risk of coronary heart disease and atherosclerosis [46, 47]. It is also considered an important anticancer [48, 49].

Studies on the efficacy of oleuropein and hydroxytyrosol purified from olive leaves, in moderating pain and inflammation, are still scarce. Therefore, the aim of the current study was to evaluate the anti-inflammatory and the antinoceptive effects of olive leaf extracts rich in oleuropein and hydroxytyrosol, by using *in vivo* experimental models in mice and rats.

Materials and Methods

Plant material and chemicals: Fresh leaves of *Olea europaea* L. cv. Chemlali were collected in June 2008 from Hammamet (Tunisia). The plant was identified in the biological laboratory of the Faculty of Pharmacy of Monastir according to the flora of Tunisia [50]. A voucher specimen (O.E-01.72) was deposited in this laboratory. Standards of oleuropein (Cas No.: 32619-42-4) and hydroxytyrosol (Cas No.: 10597-60-1) were purchased from Extrasynthese (Genay, France). The organic solvents used, purchased from Sigma, were 99% pure carrageenan (BDH Chemicals Ltd., Poole, England) and acetyl salicylate of lysine (ASL) from Adwya Laboratory, Tunisia.

Preparation of oleuropein-rich olive leaf extract: The extraction of oleuropein from olive leaves was conducted as previously reported by Savournin *et al.* [6] with little modifications. Briefly, olive leaf powder (500 g) was extracted with 50% aqueous methanol. The extract was filtered through Whatman filter paper (No. 2) and concentrated with a vacuum evaporator. After evaporation of methanol, the aqueous phase was extracted with chloroform, saturated with NaCl and finally filtered. This filtrate was dissolved in methanol and extracted three times with ethyl acetate. The ethyl acetate phase was totally evaporated to obtain the oleuropein-

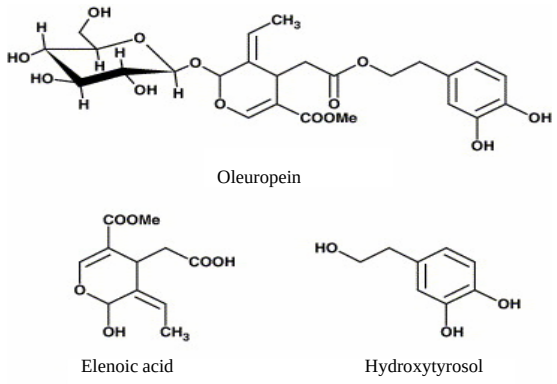

Figure 1. Structure of oleuropein and its metabolite hydroxytyrosol.

rich extract ORE (26 g). Identification was established using TLC and UV and found to be comparable to that of authentic standard.

Preparation of hydroxytyrosol-rich olive leaf extract: Dried and powdered leaves (200 g) were mixed with methanol and water (800 ml, 4:1 v/v) under agitation for 24 h. The mixture was filtered and concentrated by evaporation to dryness at 40°C. After that, 4 g of this extract was dissolved in 40 ml of a MeOH/H_2O (4:1). The solution was hydrolyzed at 100°C for 1 h using 20 ml of HCl (2 M), cooled and diluted with water (40 ml). The hydrophobic fraction was extracted by a separatory funnel three times with 100 ml of ethyl acetate, which was later removed by evaporation, to obtain hydroxytyrosol-rich extract HRE (5.4 g) [51]. Identification was established using TLC and UV and found to be comparable to that of authentic standard.

Animals: For studying the acute toxicity and the *in vivo* activities, male adult 'Wistar' rats (150-180 g) and Swiss albino mice (18-25 g) of both sexes were obtained from the Pasteur Institute (Tunis, Tunisia). They were housed in polypropylene cages with free access to standard pellet diet and water *ad libitum*. The animals were maintained under controlled conditions of humidity (40-45%), and temperature (22±2°C) with a 12 h light-dark cycle.

Housing conditions and *in vivo* experiments were approved according to the guidelines established by the European Union on Animal Care (CFE Council, 86/609). The rats were used for the anti-inflammatory evaluation, while the mice were used for the analgesic investigation and for the acute toxicity testing. Animals were divided into 'drug-treated test' and 'Tween 80-treated control' groups of six or eight animals per group.

Acute toxicity study: In this experiment, we used male Swiss mice weighing 20-25 g (eight per group). LD_{50} was assumed using 50% deaths within 48 h following intraperitoneal (i.p.) administration of the extracts at different doses (100, 200, 300, 400, 500 and 1000 mg/kg). The control group received only the vehicle (10% of Tween 80, 10 ml/kg) given by the same route. The mortality rate was determined and the LD_{50} was estimated according to the method described by Miller and Tainter [52].

Determination of analgesic activity: Analgesic activity of the extracts was tested in male and female Swiss albino mice (18-25 g) using the method of Koster *et al.* [53] and assessed by the acetic acid abdominal constriction test (writhing test). Mice (N = 6) of control group were treated cutaneously with vehicle (10% Tween solution), while those of test groups with HRE and ORE (100, 200 and 300 mg/kg). Acetyl salicylic acid (200 mg/kg i.p.) was administrated to mice as a positive control. Writhing was induced in mice by intraperitoneal injection (10 ml/kg) of 1% acetic acid after 30 min of the administration of tested extracts. The number of writhings was counted over a 30 min period. Antinoceptive activity was expressed as inhibition percent of the usual number of writhes observed in control animals. The percentages of inhibition were calculated according to the following equation:

$$\% \text{ inhibition} = \frac{((\text{Number of writhes})_{\text{control}} - (\text{Number of writhes})_{\text{treated group}}) \times 100}{(\text{Number of writhes})_{\text{control}}}$$

Determination of anti-inflammatory activity: Anti-inflammatory activity of extracts was evaluated by carrageenan-induced paw oedema in rats [54]. Male 'Wistar' rats were divided into eight groups of eight animals each. Experimental groups: (i) 10% Tween 80 solution (2.5 ml/kg), i. p.; (ii) reference drug (acetyl salicylate of lysine (ASL), 300 mg/kg), i.p.; (iii, iv and v) HRE at 100, 200 and 300 mg/kg, respectively; (vi, vii and viii) ORE at 100, 200 and 300 mg/kg, respectively.

Animals were pre-treated with drugs 60 min before injection of carrageenan. Inflammation of the hind paw was induced by injecting 0.05 ml of 1% carrageenan suspension into the sub-plantar surface of the right hind paw. Measurements of foot volumes were accomplished by using a plethysmometer (model 7150, Ugo Basile, Italy). The measures were determined at 0 h (V0 before edematogenic agent injection) and 1, 2, 3, 4, 5 and 24 h intervals later (VT). The difference between VT (1, 2, 3, 4, 5 and 24 h) and V0 was taken as the oedema value. Inhibition percentage of the inflammatory reaction was determined for each animal by comparison with controls and calculated by the following equation:

$$\% \text{ inhibition} = \frac{((VT - V0)_{\text{control}} - (VT - V0)_{\text{treated group}}) \times 100}{(VT - V0)_{\text{control}}}$$

Statistical analysis: The results obtained in the assays were expressed as mean±S.E.M. Mean comparisons between groups were analyzed through ANOVA and parametric Student's *t*-test for independent samples. P≤0.05 was considered significant.

Results

Toxicity studies: In mice, no death occurred within 48 h after a dose of ORE and HRE at 100, 200, 300, 400, 500 and 1000 mg/kg. Symptoms associated with toxicity such as locomotor ataxia, convulsion and diarrhoea were not observed. The mice weights had a normal variation. LD_{50} was estimated to more than 1000 mg/kg. Hence, we supposed that ORE and HRE at the doses of 100, 200 and 300 mg/kg i.p. injected to animals, were safe.

Analgesic activity: As can be seen from Table 1, oleuropein-rich extract significantly reduced the number of writhing induced by a 1% acetic acid solution from the dose of 100 mg/kg, the percentage of protection being 29.06%. This dose-dependent protective effect reached 54.91% and 61.09% at the doses of 200 and 300 mg/kg, respectively. The HRE, also induced a potent dose-dependent antinociceptive activity at all doses. Inhibitory ratios at the dose of 100, 200 and 300 mg/kg were 39.35, 65.44 and 73.45%,

respectively. The inhibition ratio produced by the positive control ASL was 77.11%.

Anti-inflammatory activity: The intraperitoneal administration of the ORE and HRE reduced significantly the paw oedema induced by the carrageenan. ORE (100, 200 and 300 mg/kg, i.p.) exhibited anti-inflammatory activity in a dose-dependent manner with a maximum percent inhibition of paw oedema of 69.84, 83.53 and 88.77, respectively, as compared with the control group. However, the standard drug, ASA (300 mg/kg) showed significant ($P<0.001$) anti-inflammatory activity with 81.32% inhibition after 3 h. HRE (100, 200 and 300 mg/kg, i.p.) exhibited 83.93, 84.93 and 92.57% oedema inhibition, respectively, after 2 h of carrageen injection. The activity possessed by the HRE was higher to that shown by ORE and the reference drug ASL (300 mg/kg, i.p) (Table 2).

Discussion

The present investigation showed anti-inflammatory and antinoceptive activities of oleuropein and hydroxytyrosol from olive leaves. At the consumed doses, ORE and HRE may be considered as relatively safe, as they did not cause either any lethality or changes in the general behaviour of the acute toxicity study.

Although some studies had previously confirmed the antinoceptive effect of hydroxytyrosol [55], at the best of our knowledge, this is the first research dealing with the analgesic activity of oleuropein. Esmaeili-Mahani *et al.* [56] quantified recently some identified compounds of an ethanolic olive leaf extract using high performance liquid chromatography (HPLC). Results showed that oleuropein (356 mg/g), hydroxytyrosol (4.89 mg/g), tyrosol (3.73 mg/g) and caffeic acid (49.41 mg/g) were the main compounds. It was demonstrated that this extract has analgesic effect, but the mechanisms of extract-induced analgesia and which compounds are responsible of this activity, were not obvious. Therefore, in agreement with our results, it is clear that hydroxytyrosol, as well as oleuropein, contribute in a large part to the analgesic effect of the olive leaf extract. In acetic acid-induced writhing in mice, oleuropein- and hydroxytyrosol-rich extracts reduced significantly the number of writhing; which is associated with the release of endogenous substances including serotonin, histamine, prostaglandin and bradykinin [57]. It is well-known that abdominal constriction response is very sensitive and able to detect analgesic activity of compounds that may appear inactive in other methods. Local peritoneal receptors are postulated to be partly implicated in abdominal writhing response [58, 59].

The mechanism of the reaction to this nociceptive stimulus seems to be related with prostanoid system. Experimental results obtained by several researchers indicated increased levels of lipoxygenase product [60, 61] as well as increase of peritoneal fluid level of PGE_2 and $PGF_{2\alpha}$ [62, 63]. Hence, it is possible that oleuropein

Table 1. Effects of oleuropein- and hydroxytyrosol-rich olive leaf extracts on acetic acid-induced writhing in mice (N = 6).

Extract	Concentration (mg/kg)	Number of writhes	Inhibition of writhing (%)
Control	-	72.83 ± 3.53 ***	-
ORE	100	51.66 ± 3.38 **	29.06
	200	32.83 ± 2.13 **	54.91
	300	28.33 ± 2.06 ***	61.09
HRE	100	44.16 ± 3.25 ***	39.35
	200	25.16 ± 2.92 ***	65.44
	300	19.33 ± 1.86 ***	73.45
Reference drug (ASL 200 mg/kg)		16.66 ± 1.36 ***	77.11

Values are expressed as mean ±S.E.M. (N = 6).
**P≤0.01 significant from control.
***P≤0.001 significant from control.
ASL: Acetyl salicylate of lysine.

Table 2. Effects of oleuropein- and hydroxytyrosol-rich olive leaf extracts on carrageenan-induced paw oedema.

		Mean swelling thickness (10^{-2} mm) ± S.E.M. (% inhibition)					
		1 h	2 h	3 h	4 h	5 h	24 h
Control 1	-	32.83±2.92	83.00±1.67	116.00±2.28	118.83±5.70	94.66 ±2.87	67.00±1.89
ORE	100	22.50±2.25 ** (31.47)	31.33±1.50 *** (62.24)	39.16±1.72 *** (66.23)	35.83±2.63 *** (69.84)	35.16±5.30 *** (62.85)	41.83±2.31 *** (37.56)
ORE	200	13.00±3.16 *** (60.40)	13.66±6.28 ** (83.53)	21.66±5.16 *** (81.32)	36.83±10.68 *** (69.00)	39.83±9.19 *** (57.92)	38.16±12.15 *** (43.03)
ORE	300	6.83±0.75 ** (79.18)	11.00±2.28 *** (86.74)	15.00±4.47 *** (87.06)	13.33±2.65 *** (88.77)	18.00±1.26 *** (80.98)	27.83±3.06 *** (58.45)
HRE	100	12.16±2.56 *** (62.94)	13.33±1.86 *** (83.93)	19.83±5.11 *** (82.90)	29.33±11.99 ** (75.31)	38.16±14.44 ** (59.68)	37.00±2.44 *** (44.77)
HRE	200	11.33±2.58 *** (65.48)	12.50±1.87 *** (84.93)	18.50±1.51 *** (84.05)	26.33±1.21 *** (77.84)	30.33±1.63 *** (67.95)	31.16±1.16 *** (53.48)
HRE	300	5.50±1.87 *** (83.24)	6.16±1.94 *** (92.57)	18.16±2.04 *** (84.33)	15.33±1.21 *** (87.09)	28.33±1.21 *** (70.07)	25.50±3.01 *** (61.94)
ASL	300	16.50±1.22 *** (49.74)	19.66±1.03 ** (76.30)	21.66±0.51 *** (81.32)	25.50±1.37 *** (78.54)	26.50±1.04 *** (72.00)	34.83±1.72 *** (48.00)

Values are expressed as mean ± S.E.M. (N = 8).
*P≤0.05 significant from control.
**P≤0.01 significant from control.
***P≤0.001 significant from control.
ASL: Acetyl salicylate of lysine.

and hydroxytyrosol participate in the inhibition of prostaglandin synthesis or action and so, their peripheral analgesic property is probably linked to their anti- inflammatory effects.

In the current study, the anti-inflammatory activity was investigated by carrageenan-induced rat paw oedema, the commonly used experimental model for the anti-inflammatory evaluation of natural products [54]. Carrageenan-induced oedema is a biphasic effect. The first phase (90-180 min) of the inflammation is due to the release of histamine, serotonin and similar substances. The second phase (270-360 min) is associated with the activation of kinin-like substances and the release of prostaglandins, proteases and lysosome [64].

It is well known that polyphenols have *in vivo* anti-inflammatory properties [65, 66] by suppressing the tumour necrosis factor-α (TNF-α), inhibiting arachidonic acid-induced ear oedema, inhibiting the 12-*O*-tetradecanoylphorbol-13-acetate-induced ear oedema and oxazolone-induced allergic oedema. Furthermore, hydroxytyrosol-20 was demonstrated to inhibit inflammatory swelling by suppressing proinflammatory cytokine IL-1β and TNF-α in a rat inflammation model [55]. Oleuropein has anti-inflammatory effect[67, 21] by inhibiting the lypoxygenase activity, inhibiting the production of leucotrien B4 [27] and decreasing the concentration of interleukin-1β [68]. Experimental results showed that ORE and HRE seem to inhibit both phases: blocking histamine and serotonin release within the first phase, and preventing the release of some of the inflammatory mediators via blocking the prostaglandin and kinin-like substances action within the second phase.

Conclusions

Results of pharmacological tests performed in the present study suggest that the oleuropein- and hydroxytyrosol-rich olive leaf extracts are safe and present potent analgesic and anti-inflammatory effects.

Considering high consumer demand due to the beneficial health effects, olive leaf by-products generated can be used beneficially as food supplement in the battle of inflammation and pain and the development of new drugs.

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