Anti-inflammatory and analgesic activities of olive tree extract

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ABSTRACT

Objective: The aim of this study was the *in vivo* evaluation of analgesic and anti-inflammatory effect of an olive tree extract with high polyphenols content.

Methods: This olive tree extract was obtained from Moroccan olive fruits and leaves using an eco-extraction free of chemical solvents and toxic additives. Anti-inflammatory activity was evaluated using carrageenan and histamine-induced paw edema methods. Analgesic activity of the olive tree extract was estimated against a hot plate, acetic acid induced writhing and formalin tests.

Results: The extracts showed significant anti-inflammatory and analgesic activities with a dose-dependent manner. Anti-inflammatory activity of olive tree extract at 250 and 500 mg/kg doses was more important compared to the used standard drugs ($p<0.05$), in both carrageenan and histamine-induced paw edema

tests. In analgesic assays, results showed that 500 mg/kg dose of olive tree extract has a significant analgesic effect through both peripheral and central mechanisms.

Conclusion: Our findings suggest that olive tree extract is safe and has potential anti-inflammatory and analgesic activities, which promote this use as a food supplement against pain and inflammation related to inflammatory diseases.

INTRODUCTION

The correlation between inflammatory response and many chronic diseases like rheumatoid arthritis, cardiovascular diseases and some types of cancer has been reported [1, 2]. Excessive production and accumulation of oxygen and reactive nitrogen species (ROS and RONS), and arachidonic acid metabolites play a key role in the pathogenesis of cardiovascular diseases and cancer [3, 4]. In the process of inflammation, arachidonic acid is converted by cyclooxygenase (COX) and lipooxygenase (LOX) to prostaglandins, thromboxanes, prostacyclin, and leukotrienes [5]. These inflammatory mediators are responsible for pain and other inflammatory symptoms. C-reactive protein concentration rises during inflammation [6]. Plasma thromboxane B2 (TXB2) increases platelet aggregation and leukotrienes B4 (LTB4) promotes migration of neutrophils to inflamed tissue [7]. Oxidative stress enhances inflammation by activating nuclear factor kappa B (NF-$\kappa\beta$) and activator protein-1 (AP-1) transcription factors and affecting cellular signaling processes [8]. NF-kB activation is associated with cancer progression. Thereby, ROS, COX-1/2 and NF-$\kappa\beta$ inhibitors,

may have a potential therapeutic effect on inflammation-depending diseases [9, 10].

In this sense, olive phenolic compounds are well known for their potential health benefits including the reduction of coronary heart disease risk, the prevention of some cancers and for their anti- inflammatory properties [11-13]. In fact, olive phenolic compounds are considered to possess anti-inflammatory properties, and therefore, were proposed as a alternative natural approach to prevent and/or treat chronic inflammatory diseases [14, 15]. Anti- inflammatory mechanisms of olive polyphenols are suggested to include: inhibition of pro-inflammatory enzymes, such as COX-2, LOX [16]. Its also inducible nitric oxide synthase (iNOS); inhibition of phosphoinositide 3-kinase (PI 3-kinase), tyrosine kinases, NF-kB, and AP-1; and down-regulation of various pro-inflammatory cytokines such as chemokines, tumor necrosis factor alpha (TNF-a), interleukins including (IL-1 b, IL-6, IL-8), and monocyte chemotactic protein-1 (MCP-1) [14, 17-21]. It is reported that olive polyphenols relieved pain and have an analgesic activity in the case of some inflammatory diseases [22, 23].

In the present study, we have examined the possible antinociceptive and anti-inflammatory effects of a phenolic olive tree extract using *in vivo* experimental models. Namely, the carrageenan and histamine- induced hind paw oedema model tests for the anti-inflammatory activity and hot plate, acetic acid-induced abdominal writhing and formalin tests to assess the analgesic activity. Indomethacin and diclofenac sodium (with known anti-inflammatory effects), and tramadol hydrochloride and acetylsalicylic (with known analgesic properties) were used as positive controls.

MATERIALS AND METHODS

Plant materials

The olive tree extract (OTE) was ecologically obtained from Moroccan olive fruits and olive leaves/young sprouts, according to the previously described protocol [24]. They have been taken from stressed olive trees planted in a rocky desert harsh environment of Morocco. These suffering olive trees belong to a farm of the renowned Company Atlas Olive Oils. Actually, OTE is marketed in the world (France, Switzerland, Belgium, etc.) under the brand name OLIVIE FORCE/OLIVIE RICHE (see more at www.olivie.ma).

Chemicals

Carrageenan; histamine; indomethacin; diclofenac sodium (DFS); acetylsalicylic acid (ASA); tramadol hydrochloride (Tramadol Hcl); formalin; acetic acid were purchased from Sigma-Aldrich (Paris, France).

Animals

For studying the acute toxicity and the *in vivo* activities, male adult Wistar rats (120-180 g) and Swiss albino mice (20-25 g) of both sexes were obtained from the animal breeding unit of the Faculty of Science Dhar El Mahraz-Fez-Morocco. They were housed in polypropylene cages with free access to food and water. The animals were maintained under controlled conditions of temperature (22±2°C) with a 12 h light-dark cycle. The animals were used after an acclimatization period of 7 d in the laboratory environment. Housing conditions and *in vivo* experiments were approved according to the guidelines established by the European Union on Animal care (CEE Council 86/609). Animals fasted overnight before any experiments.

Acute toxicity study

Swiss albino mice (male) weighing 20-25 g were divided into two groups (five per group). Mice in the experimental group were received increasing OTE's dose (100, 250, 500 and 1000 mg/kg) while those in the control group received only 0.9% NaCl solution (10 ml/kg). The mortality rate was determined and the LD50 was estimated within the 24h following orally administration, according to the method described by Creton *et al.* [25].

In vivo anti-inflammatory activity

Carrageenan-induced rat paw oedema

OTE's anti-inflammatory activity was evaluated using carrageenan- induced paw oedema in rats. Male Wistar rats were divided into 8 groups of five animals each. (1) Control group (10 ml/kg of 0.9% NaCl solution); (2) and (3) groups received reference drugs (10 mg/kg of indomethacin and diclofenac sodium); (4), (5), (6), (7) and groups (8) were orally administered OTE in 50, 100, 250, 500 and 1000 mg/kg doses, respectively.

Animals were pre-treated with drug and OTE 60 min before injection of carrageenan. Inflammation of the hind paw was induced by injecting 0.1 ml of 0.5% carrageenan suspension into the sub- plantar surface of the right hind paw. Measures of the paw circumference were determined at 3, 4, 5 and 6h (after edematogenic agent injection) intervals later (St) using the method of Bamgbose and Noamesi [26]. The difference between St (3, 4, 5 and 6h) and S0 was taken as the oedema size. The inhibition percentage of the inflammatory reaction was determined for each animal by comparison with controls and calculated by the following equation:

$$\% \text{ inhibition} = \frac{(St - S0)control - (ST - S0) \text{ treated}}{(St - S0)control} \times 100$$

Histamine-induced rat paw oedema

Eight groups of rats (five rats each) were used for this test. Group (1) served as a control group (10 ml/kg of 0.9% NaCl solution), animals in groups (2) and (3) were orally treated with indomethacin and diclofenac sodium (10 mg/kg), while rats in groups (4), (5), (6), (7) and (8) received the OTE at doses of 50, 100, 250, 500 and 1000 mg/kg, respectively.

Animals were treated by drug controls and OTE 1h before histamine injection (0.1 ml of a 1% solution in 0.9% NaCl solution) into the plantar region of the right-hand paw. Paw size was measured before injection of histamine and at 3, 4, 5 and 6h, after injection. The paw oedema was measured using the cotton thread method according to Farshid *et al.* [27]. The average increase in paw size of each group was determined. The percentage inhibition was obtained using this formula:

$$\% \text{ inhibition} = \frac{(St - S0)\text{control} - (St - S0)\text{treated}}{(St - S0)\text{control}} \times 100$$

With St = the paw size for each group after histamine treatment. And S0 = paw size for each group before histamine injection.

In vivo analgesic activity

Hot plate test

Twenty-five Swiss albino mice (20-25 g) were divided into 5 groups of five mice per group. Group (1) received control solution (0.9% NaCl solution), group (2) received tramadol hydrochloride (10 mg/kg), while groups (3), (4) and (5) received 100, 250 and 500 mg/kg of OTE, respectively. 1h after the orally administration, mice were placed onto a hot plate (55±2 °C), and the reaction time for licking of paw or jumping for the control and treated

mice was recorded (in seconds) [28]. A cutoff time of 15 s was used to avoid damage to the paw [29]. The percentage increase in reaction time was determined thus:

$$\% \text{ increase in reaction time} = \frac{Tt - T0}{T0} \times 100$$

Aceta writhing test

Overnight fasted mice were divided into five groups of five each. Groups (1) and (2) received control solution (0.9% NaCl solution) and acetylsalicylic acid (10 mg/kg), while groups (3), (4) and (5) received 100, 250 and 500 mg/kg of OTE, respectively. 1h after, the animals were intraperitoneally injected with acetic acid (0.6%, v/v in 0.9% NaCl solution) [30], the analgesic activity was quantified by counting the total number of writhes over a period of 25 min after a latency period of 5 min [31]. The percentage of analgesic activity was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{number of writhes (control)} - \text{number of writhes (test)}}{\text{number of writhes (control)}} \times 100$$

Formalin licking test

Formalin licking test was carried out using male mice under same experimental conditions of acetate writhing test. 1h after orally OTE administration, 20 µl of 1 % formalin solution (in 0.9% NaCl solution) was injected subcutaneously into the plantar surface of the right hind paw of each mouse. Licking the injected paw time was measured over 30 min divided into two phases. The early phase was observed during the first 5 min and the late phase was recorded in 15-30 min [32]. These phases represented neurogenic and inflammatory pain responses, respectively [33]. The percentage of inhibition was obtained by the following formula:

$$\% \text{ inhibition} = \frac{\text{reaction time (control)} - \text{reaction time (treated)}}{\text{reaction time (control)}} \times 100$$

Statistical analysis

Statistical analyzes were performed using GraphPad Prism software version 6.00 (GraphPad Inc., San Diego, California). Data were analyzed by analysis of variance (ANOVA Analysis of Variance) followed by posthoc Dunnet test if the sample distribution follows a normal distribution or by the Kruskal-Wallis if the sample distribution does not follow the normal law. Values between groups were considered statistically significant for at $P<0.05$.

RESULTS

Toxicity test

Results (data not shown) showed no signs of toxicity which could be attributed to the administrated material (OTE), even at high doses. On the other hand, OTE, containing more than 15% of polyphenols (w/w), had no evidence of toxicity in mice and rats (mortality, tremor, convulsions, loss of reflex, sedation, and diarrhea). This, in fact, was in concordance with Soni *et al.* (2006) findings, that reported NOAEL (No Observed Adverse Effect Level) in rats after aqueous olive fruit extract administration (with high polyphenols content), even though at high doses as 2 g/kg/day. As a result, we suppose that OTE's orally administration (to mice) at the doses of 100, 250 and 500 mg/kg will be safe.

Anti-inflammatory activity

Results plotted in tables 1 and 2 illustrate the dose-dependent OTE's effect on paw oedema formation after induction by carrageenan (table 1) and histamine (table 2).

Table 1: Effect of olive tree extract on carrageenan-induced rat paw oedema in rats.

Dose (mg/kg)	Inhibition (%)			
	3h	4h	5h	6h
50	[a]0.27±0.13	[a]10.00±2.20	[a]20.21±2.21	[a]0.45±0.05
100	[b]44.45±3.45	[b]60.00±2.33	[b]64.24±2.22	[b]16.67±1.00
250	[b]50.27±3.61	[b]75.00±2.76	[c]73.40±1.56	[c]37.50±1.11
500	[c]66.76±3.69	[c]80.00±2.11	[c]78.73±1.72	[d]50.00±4.27
1000	[c]67.81±5.06	[c]81.97±2.63	[c]78.79±1.18	[d]50.00±3.00
Indomethacin*	[d]19.05±2.20	[b]53.24±2.54	[d]60.99±2.33	[e]8.34±1.22
Diclofenac sodium*	[b]47.11±2.22	[b]69.34±3.25	[d]63.12±1.65	[e]3.34±0.18

a–eValues in the same column with different superscripts are significantly different ($p<0.05$) [mean±SD, n= 6].
*Reference drugs (indomethacin 10 mg/kg and diclofenac sodium 10 mg/kg).

Table 2: Effect of olive tree extract on histamine-induced rat paw oedema in rats.

Dose (mg/kg)	Inhibition (%)			
	3h	4h	5h	6h
50	[a]0.13±0.03	[a]8.90±0.55	[a]18.96±0.59	[a]0.26±0.04
100	[b]25.00±2.43	[b]52.00±1.54	[b]60.50±1.35	[b]12.00±1.67
250	[c]51.00±2.39	[c]70.90±1.67	[c]69.00±2.22	[c]34.00±1.50
500	[d]62.70±0.84	[d]76.00±2.03	[d]76.30±0.70	[d]45.50±1.34
1000	[d]63.98±0.57	[d]78.22±1.18	[d]77.10±0.86	[d]47.00±2.37
Indomethacin	[e]15.50±0.50	[b]51.00±0.59	[b]56.80±1.04	[a]3.21±0.79
Diclofenac sodium	[f]43.90±1.01	[e]64.96±1.66	[b]58.60±1.09	[a]2.60±0.10

a–fValues in the same column with different superscripts are significantly different ($p<0.05$) [mean±SD, n= 6].
*Reference drugs (indomethacin 10 mg/kg and diclofenac sodium 10 mg/kg).

The subplantar injection of carrageenan-induced a progressive local oedema reaching its peak at the 3rd hour (table 1). The orally administration of the OTE showed a dose-dependent reduction in carrageenan-induced paw oedema from the 3rd to the 6th hour. The highest OTE's inhibition activity (80%) was recorded after 4h at 500 mg/kg dose, compared to reference drugs indomethacin (53.24%) and diclofenac (69.34%) ($p < 0.05$). Besides, no significant difference (*p*<0.05) was observed when the treatment dose, rise to 1 g/kg (compared to 500 mg/kg dose).

Similarly, the paw oedema induced by histamine was reduced after OTE administration (table 2). High inhibition activity was observed for the 500 and 1000 mg/kg doses (76.00 and 78.22%), but with no significant difference ($p < 0.05$). Thus, OTE's administration has a significant anti-inflammatory effect compared to the used reference drugs, even at low doses like 250 mg/kg.

Analgesic activity

Graphs of fig. 1, 2 and 3 show results related to the OTE's analgesic activity assessed by means of the hot plate, acetic acid-induced abdominal writhing and formalin *in vivo* tests. Considering the results of anti-inflammatory tests, analgesic tests were performed at three OTE's doses (100, 250 and 500 mg/kg).

OTE administration has increased the insensibility to pain without loss of consciousness (analgesic activity) in mice placed onto the hot plate (55±2 °C). The observed OTE's analgesic effect was dose - dependent, reaching its maximum (36.77%) at 500 mg/kg dose (fig. 1). This effect was, however, compared to that observed (35.84%) for tramadol hydrochloride (no significant difference at *p*<0.05).
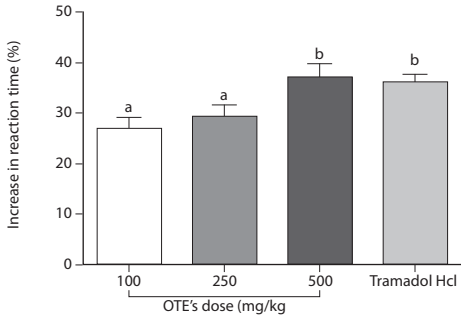
Fig. 1: Effect of olive tree extracted on hot plate-induced pain in mice. Data is expressed as means±SD [n= 5]. Different letters indicate significant differences ($p<0.05$)

On the writhing response in mice, OTE induced a potent dose-dependent antinociceptive activity at all used doses (fig. 2). This activity was up to 68% for 500 mg/kg dose, which was similar to that shown by the reference drug significantly (no differences at $p<0.05$).
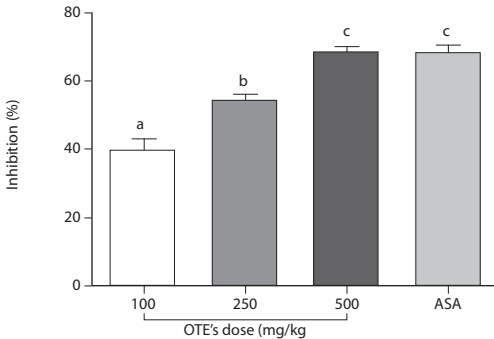

Fig. 2: Olive tree extract's analgesic activity in mice treated with acetic acid. Data are expressed as means±SD [n= 5]. Different letters (a-c) indicate significant differences ($p<0.05$)
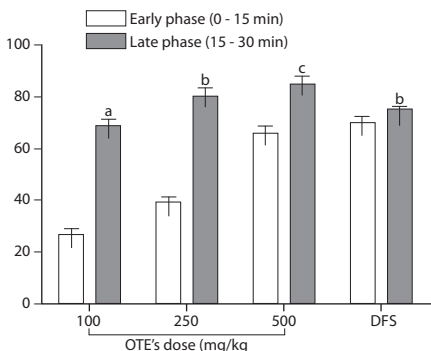
Fig. 3: Effects of olive tree extract on the formalin-induced licking response in mice. Data is expressed as means±SD [n= 5]. Different letters (a-c) indicate significant differences ($p<0.05$)

Two phases showed in fig. 3 represented neurogenic and inflammatory pain responses, respectively [31]. The subcutaneous injection of formalin solution into the plantar surface of the right hind paw of mice produced an analgesic response of licking of the treated paw. OTE showed dose-dependent effect in both early and late phases. The OTE's analgesic activity was incomparable to that of reference drug (diclofenac sodium) during the early phase (corresponding to the neurologic pain) (fig. 3). However, this effect was significantly higher in mice treated with 500 mg/kg dose (84.70

%) compared to diclofenac sodium (75.20 %) ($p<0.05$) in the late phase (corresponding to the inflammatory pain).

DISCUSSION

The carrageenan-induced paw oedema is frequently used as an experimental model for acute inflammation studying [34]. The inflammatory reaction carrageenan-induced (in rats) is a biphasic response, (i) oedema

formation involving the production of inflammatory mediators such as histamine, serotonin, and kinins; (ii) the biosynthesis of prostaglandin and other autacoids release and attributed to the induction of cyclooxygenase (COX)-2 in the tissue [35-37]. Actually, the results of this study suggest that the OTE could antagonize the action and/or inhibit the production of the circulating inflammation mediators. On injection, histamine acts as an inflammatory mediator, which increases vascular permeability [38-40]. Results herein presented show the anti-oedomatogenic effect of orally administered olive tree extract in rats injected by Histamine. This could be, in fact, attributed to the anti-inflammatory activity of studying extract through an antihistamine mechanism. The rich phenolic olive tree extracted may act by inhibiting the release and/or histamine action, which can explain its inhibitory activity on oedema development.

Thus, observed OTE's Anti-inflammatory effects could be related to its phenolic composition. Actually, OTE is a rich phenolic extract (15%, w/w), particularly hydroxytyrosol (2%, w/w). In general, olive phenolic compounds have been known to inhibit both COX-1/2 inflammatory enzymes (in a dose-dependent manner) more efficiently than ibuprofen drug [16]. In a clinical trial, Ghanam *et al.* [14] recently reported that olive tree extract relieved pain in rheumatoid arthritis patients and decreased the circulating inflammatory biomarkers such as hs-CRP, IL-6, TNF-α, and PGE2 significantly.

Moreover, hydroxytyrosol impedes PGE2 synthesis by indirectly blocking of inducible nitric oxide synthase and COX-(2) enzymes. This effect was raised by the prevention of transcription factors activation as NF-$\kappa\beta$, interferon regulatory factor-1 and transducer and activator of transcription 1a, which prevents the

activation of mouse macrophages J774 [41]. It is also known that hydroxytyrosol is capable of bringing about arylating/alquilant adducts in the cysteine residues of NF-$\kappa\beta$. The action of hydroxytyrosol on this factor blocks COX-(2) and 5-lipooxygenase transcription, reducing the PGE2 synthesis and, thus, the chronic inflammation associated with inflammatory diseases such as cancer [42]. Beside this, hydroxytyrosol possesses significant anti-inflammatory actions in inflammation animal models through the inhibition of pro- inflammatory cytokines expression (TNF-α and IL-1β) [20, 23].

Results from the current study reveal the effectiveness of natural OTE as oedema inhibitor compared to reference drugs (indomethacin and diclofenac sodium). These classical non-steroidal anti-inflammatory drugs (NSAIDs) mainly inhibit COXs. However, they have side effects such as irritation of the gastric mucosa, caused by the inhibition of prostaglandin biosynthesis, which has a protective role in the gastrointestinal tract. Many NSAIDs are also acids that may cause additional harm in the gastrointestinal tract [43]. Thus, the use of natural anti-inflammatory agents is one of the proposed solutions to overcome problems caused by side effects of NSAIDs.

Hot plate test was selected to investigate the central analgesic effect of OTE, which is known to elevate the pain threshold of mice towards heat [44]. It also indicates narcotic involvement with opioid receptor and measures the complex response to a non-inflammatory acute nociceptive input [45, 46]. The high percentage inhibition (36.77%) shown by OTE in this test suggests that OTE is a centrally acting analgesic. Acetic acid-induced writhing response is useful for the evaluation of peripherally acting analgesics. Acetic acid stimulates the tissue to produce several inflammatory mediators such as histamine,

serotonin, cytokines, and eicosanoids with an increase in peritoneal fluid levels of these mediators [47]. In this sense, OTE inhibited mice abdominal writhes, suggesting that OTE's antinociceptive activity could be related to the reduction of inflammatory mediator's liberation and/or to direct blockage of receptors resulting in peripheral analgesic response.

The formalin test is a valid model of pain and analgesic research for clinical pain compared to tests using phasic thermal or mechanical stimulation. It has two distinctive phases, reflecting different types of pain [45]. The early phase (0–5 min) reflects centrally mediated pain, which was a result of direct stimulation of nociceptors and believed to be a non-inflammatory pain.

The late phase (15–30 min) persistent period caused by local tissue inflammation. Experimental results demonstrated that substance P and bradykinin participate in the early phase, while histamine, serotonin, prostaglandins, nitric oxide, and bradykinin are believed to be involved in the late phase of the formalin test response. Our results show that OTE has an inhibitory effect on the analgesic response of both early and late phases of the formalin test. Moreover, significant pain relief activity observed in the late phase (compared to the early phase) indicates the peripherally acting protective effect of OTE, which was correlated with anti- inflammatory tests results. In that way, OTE attenuate pain response better than diclofenac sodium (NSAID), commonly used as a reference due to its anti-inflammatory and analgesic effects. This drug has the ability to reduce inflammation, swelling and pain by inhibiting either the release of arachidonic acid or the prostaglandin synthesis [48, 49]. This fact corroborates with published data about, *in vivo*, phenolic compounds antinociceptive effects, mainly attributed to flavonoids

[50, 51] and hydroxytyrosol [52]. It has also reported in a rodent model of opiate tolerance, that antiradical activity of olive phenolic compounds (hydroxytyrosol and oleuropein, amongst others) reinstates the analgesic action of morphine [22].

CONCLUSION

Results of pharmacological tests performed in the present study suggest that olive tree extract with high polyphenols content is safe and presented potential anti-inflammatory and analgesic activities, which are comparable with the reference drugs. This might be correlated with the phenolic compound composition of this extract, particularly hydroxytyrosol. Considering high consumer demand due to the beneficial health effects, olive tree extract can be beneficially used as a natural food supplement to contend inflammation and pain in the case of inflammatory diseases.

CONFLICTS OF INTERESTS

All authors have none to declare.

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