

Effect of Minor Components of Virgin Olive Oil on Topical Antiinflammatory Assays

Rocío de la Puerta^{a,*}, Eugenia Martínez-Domínguez^a, and Valentina Ruíz-Gutiérrez^b

^a Department of Pharmacology, Faculty of Pharmacy, University of Seville, 41012 Seville, Spain. Fax: 34-(9)-5-4233765. E-mail: puerta@fajar.us.es

^b Instituto de la Grasa, (C. S. I.C.), Padre García Tejero 4, 41012 Seville, Spain

* Author for correspondence and reprint requests

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Unsaponifiable Compounds, Olive Oil Phenolics, Topical Ear Edema

Interest in the health-promoting effects of virgin olive oil, an important part of the “Mediterranean diet”, prompted us to determine the antiinflammatory effects of erythrodiol, β -sitosterol and squalene, identified as major components of the so-called “unsaponifiable fraction” of virgin olive oil, as well as of the phenolic compounds from the “polar fraction”: oleuropein, tyrosol, hydroxytyrosol and caffeic acid. Their activities were compared to those of both, total unsaponifiable and polar fractions. This study was designed to analyse the antiinflammatory effect of these specific compounds from virgin olive oil on edema in mice induced by either arachidonic acid (AA) or 12-O-tetradecanoylphorbol acetate (TPA). The inhibition of the myeloperoxidase (MPO), marker enzyme of the accumulation of neutrophils in the inflamed tissue, was also investigated by the TPA model. The topical application of the olive oil compounds (0.5 mg/ear) produced a variable degree of antiinflammatory effect with both assays. In the auricular edema induced by TPA, β -sitosterol and erythrodiol from the unsaponifiable fraction of the oil showed a potent antiedematous effect with a 61.4% and 82.1% of inhibition respectively, values not very different to that of the reference indomethacin (85.6%) at 0.5 mg/ear. The four phenolics exerted a similar range of inhibition (33–45%). All compounds strongly inhibited the enzyme myeloperoxidase, indicating a reduction of the neutrophil influx in the inflamed tissues.

The strongest inhibitor of AA edema was the total unsaponifiable fraction which inhibition was 34%, similar to that obtained by the reference drug dexamethasone at 0.05 mg/ear. Among the phenolics, oleuropein also produced an inhibition of about 30% with the same dose, but all the other components were found less active in this assay. The anti-inflammatory effects exerted by both unsaponifiable and polar compounds might contribute to the potential biological properties reported for virgin olive oil against different pathological processes.

Introduction

In olive oil, the main type of lipid consumed in the Mediterranean area, the major nutritionally-relevant part constitutes triglycerides with a high content of monounsaturated fatty acids, but there are also many other minor compounds belonging to the so-called ‘unsaponifiable fraction’ that can be extracted by solvents after the saponification process. The composition and concentration of the components of this fraction are complex and specific for each type of vegetable oil. For example,

they vary between ‘extra virgin’ olive oil (made from first pressings of ripe olives) and other natural or refined olive oils. In general, hydrocarbons account for about 50% of the unsaponifiable fraction of olive oil (Lanzon *et al.*, 1994), with squalene as the major component (Bondioli *et al.*, 1993), together with antioxidant tocopherols (Kiritakis and Markakis, 1987), sterols and smaller amounts of triterpenic dialcohols (Table I).

Moreover, virgin olive oil contains various polyphenols (Gutfinger, 1981) which are usually removed from it, as well as from other edible oils, during the refining processes used in large scale commercial oil production (Brenes *et al.*, 1995). These substances constitute the ‘polar fraction’ in virgin olive oil, prevent its autooxidation and underly its exceptional stability (Vazquez Roncero,

Abbreviations: AA, arachidonic acid; TPA, 12-O-tetradecanoylphorbol acetate; MPO, myeloperoxidase; HTBA, hexadecyltrimethyl-ammonium bromide; COX, cyclo-oxygenase; 5-LO, 5-lipoxygenase.

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Table I. Components and concentrations in the oil of the unsaponifiable and polar fractions of virgin olive oil. (Based on references: Gutfinger and Letan, 1974; Vázquez Roncero *et al.*, 1976; Kiritsakis and Markakis, 1987; Visioli and Galli, 1995).

Sub-fraction	Components	Concentration (mg/kg oil)
Hydrocarbons	Squalene β -Carotene polycyclic aromatic hydrocarbons	1200–7500 0.3–0.7 traces
Sterols	β -Sitosterol campesterol, Δ^7 -stigmasterol brassicasterol	1800–2650 $\leq 4.0\%$ of total sterols $\leq 0.5\%$ of total sterols $\leq 0.1\%$ of total sterols
Terpene dialcohols Tocopherols	Erythrodiol + Uvaol α -tocopherol $\beta + \gamma$ -tocopherol δ -tocopherol	6–10 60–200 3% of total tocopherols $\leq 2\%$ of total of tocopherols
> 70 Flavour components	Hydrocarbons, aliphatic hydroxy compounds, terpenic hydroxy compounds, aldehydes, ketones, ethers, furans, thiophenes, esters	traces of each (proportions variable)
Polyphenols	Tyrosol Hydroxytyrosol Caffeic acid Oleuropein	50–800 (in total)

1978), as well as contributing to its characteristic flavour and taste. They include simple phenols based on cinnamic acid (e.g. caffeic acid), the secoiridoid glycoside oleuropein and its hydrolysis product hydroxytyrosol, also known as 2-(3,4-dihydroxyphenyl)-ethanol, and tyrosol (4-hydroxyphenylethanol). The concentration of these phenols in olive oil depends on many factors, including species, location, climate and maturation of the olive (Van Buren, 1970), being highest in the first expressed extra virgin olive oil.

These minor but important compounds found in the phenolic and unsaponifiable fractions indicate that virgin olive oil is a special product with valuable potential biological activities worth a detailed study. In earlier publications we reported inhibition of TPA-induced ear edema produced by a triterpenic and steroidal fraction from the unsaponifiable matter of virgin olive oil (De la Puerta *et al.*, 1997). Moreover, we found the phenolics from this oil selectively to inhibit the leukocyte 5-lipoxygenase enzyme, and exhibiting potent scavenger activity against reactive oxygen species (De la Puerta *et al.*, 1999). These interesting “*in vitro*” effects demonstrated by others authors (Chimi *et al.*, 1991) and ourselves, prompted us to evaluate the “*in vivo*” antiinflammatory action of these spe-

cific components of virgin olive oil, testing them by two well-established antiinflammatory assays.

Materials and Methods

Animals

Male Swiss albino mice weighing 20–25 g ($n=6$ per group) were used. The animals were supplied by “Centro de Producción y Experimentación Animal” from the University of Seville (Spain). All animals were maintained in suitable nutritional and environmental conditions throughout the experiments.

TPA-induced mouse ear edema

An edema was induced at the right ear of each mouse by topical application of 2.5 $\mu\text{g}/\text{ear}$ of tetradecanoylphorbol acetate (TPA) as phlogistic agent dissolved in 20 μl of acetone (De Young *et al.*, 1989). This was applied by an automatic pipette in 10- μl volumes to both inner and outer surfaces of the right ear. The left ear (blank) received the same volume of vehicle acetone. Test compounds were applied at a dose of 0.5 mg/ear dissolved in acetone or 70% EtOH, before the application of TPA. Dexamethaxone (0.05 mg/ear) and indo-

methacin (0.5 mg/ear) were used as reference compounds dissolved in acetone. Inflammation was allowed to develop for 4 h, after which the animals were killed by cervical dislocation, and a section (6 mm diameter) of the central portion of both ears weighed. The swelling induced by TPA was assessed in terms of the increase in the weight of the right ear punch biopsy over that of the left ear (Carlson *et al.*, 1985). Inhibition percentages were calculated by comparison to the control group that only received the TPA application but none of the treatments.

AA-induced mouse ear edema

Arachidonic acid (AA) (2 mg/ ear) dissolved in 20 μ l of acetone was applied as above into the right ears of the mice. The left ear received acetone only. Test compounds were applied at a dose of 0.5 mg/ear dissolved in acetone or 70%EtOH, 30 minutes prior to the AA administration. Inflammation was allowed to develop for 1 h and animals were then killed for local dislocation and equal sections of both ears were punched out and weighed. Dexamethaxone (0.05 mg/ear) and indomethacin (0.5 mg/ear) were also used as reference compounds. The extent of swelling was calculated as above (Young *et al.*, 1984).

Myeloperoxidase assay

Myeloperoxidase activity was determined in the supernatants from the homogenates of the ear biopsys (from the TPA-induced edema) prepared as described by De Young *et al.* (1989). Briefly, the complete sections of punched ears placed in 1.5 ml of 50 mM sodium phosphate buffer, pH 6.0, containing 0.5% hexadecyltrimethyl-ammonium bromide (HTAB) were homogenized for 45 sec at 0 °C in a motor-driven homogenizer (Polytron PT 1200, Kinematica AG Luzern).

For the assay of myeloperoxidase we have followed the method of Bradley *et al.* (1982) modified for lecturing in a microplate reader. The following reagents were added in the following order to wells of a 96-well microtiter plate: 50 μ l of supernatant, 50 μ l pH 6.0 phosphate buffer containing 0.5% HTAB, 50 μ l o-dianisidine 0.68 mg/ml in distilled water, and 50 μ l freshly prepared 0.003% hydrogen peroxide to start the reaction. The optical density at 450 nm was read immedi-

ately and thereafter at 5 min intervals. The amount of enzyme in the samples was obtained by comparison of the rate of reaction with that in wells containing supernatants from the control group treated with TPA only.

Test compounds and chemicals

The total unsaponifiable fraction was obtained after the saponification of the fat matter of the oil (5 g) with a 2 N KOH solution in ethanol and subsequent extraction of the unsaponifiable compounds with 3 volumes of approximately 70 ml of ethyl ether. Erythrodiol, squalene and β -sitosterol was obtained directly from the unsaponifiable fraction by preparative TLC on silica gel (Merck) using n-hexane: ether (7:3 v/v) as mobile phase. The polar fraction of the oil was obtained using the method described by Vázquez Roncero *et al.* (1976): the oil dissolved in n-hexane was extracted with methanol: water (60:40, v/v, 3 times). The three extracts were then combined and the solvent evaporated to dryness in a flash evaporator (40 °C). Caffeic acid and tyrosol were purchased from Fluka (Buchs, Switzerland); Oleuropein was extracted from the leaves of *Olea europea* (Ruíz-Gutiérrez *et al.*, 1995). Hydroxytyrosol was prepared by acidic hydrolysis of oleuropein, (Graciani and Vázquez, 1980).

12-O-Tetradecanoylphorbol acetate (TPA), arachidonic acid (AA), indomethacin, dexamethasone, hexadecyltrimethylammonium bromide (HTBA), and o-dianisidine were purchased from Sigma-Aldrich Química (Madrid).

Statistical analysis

The data are expressed as mean \pm S. E. M. Analysis of the "Student t test" was used for statistical evaluation.

Results

Topical application of specific minor compounds of virgin olive oil produced an anti-inflammatory effect in TPA- and AA-induced ear edema. The phenolics and the compounds from the unsaponifiable fraction, except squalene, reduced significantly the edema in the TPA-induced inflammation model at a dose of 0.5 mg/ear (Fig. 1, A). The mean increase in ear edema with TPA was $16.0 \pm$

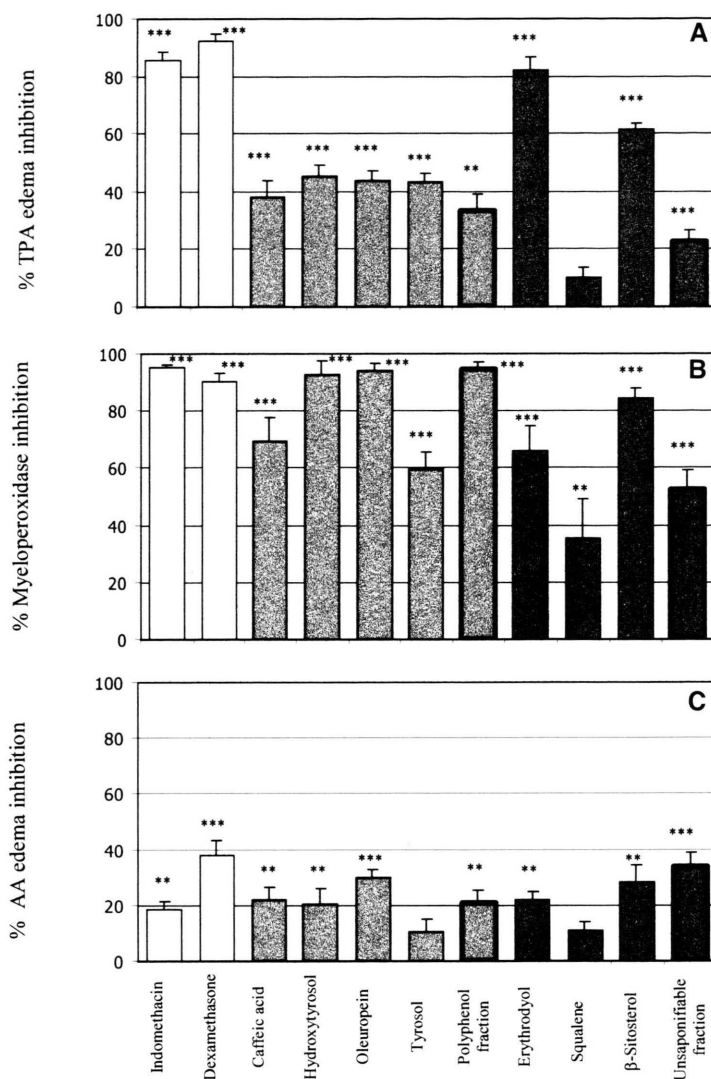


Fig. 1. **A.** Inhibition percentages of TPA-induced edema by virgin olive oil constituents. **B.** Inhibition percentages of MPO activity. **C.** Inhibition percentages of AA-induced edema by virgin olive oil constituents. (mean \pm SEM, $n=6$). ** $p < 0.01$; *** $p < 0.001$ compared to control group. (Student's unpaired t-test).

0.76 mg ($n=6$). β -Sitosterol and erythrodiol showed a potent antiedematous effect with 61.4 and 82.1% inhibition respectively, values close to that of the reference compounds indomethacin (85.6%) and lower to that produced by the phospholipase A_2 inhibitor dexamethasone (92.3%). The four phenolics exerted a similar range of inhibition (33–45%). The minor components from virgin olive oil significantly decreased the MPO activity in the supernatants of the TPA-treated ears (Fig. 1, B). This effect was higher for the complete

polar fraction and the catechol phenolics oleuropein and hydroxytyrosol.

Topical application of AA (2 mg/ear) induced a rapid ear edema, with a maximal effect at 1 h (mean increase in ear edema of 15.31 ± 0.62 , $n=6$). In general the potencies of the compounds in the arachidonate assay, including reference compounds, were much lower when the edema was induced by topical application of TPA (Fig. 1, C), except for the unsaponifiable fraction that produced the highest inhibition (34%) with a similar

effect like dexamethasone tested at 0.05 mg/ear. Among the phenolics, oleuropein was the most effective as inhibitor of the edema (30%), all the other components were less active in this assay.

Discussion

Inflammatory model of several types allow hypothesis testing, evaluation of test compounds, and perhaps more importantly, provide a better understanding of the inflammatory process. AA (arachidonic acid or TPA (12-O-tetradecanoylphorbol-13-acetate; a tumor promoting agent and protein kinase C activator) are widely used agents that induce cutaneous inflammation in experimental animals. Topical application of AA to mouse ears was reported to induce a short-lived inflammatory response that paralleled the generation of prostaglandins and leukotrienes (Chang *et al.*, 1986; Opas *et al.*, 1985). In contrast, topical application of TPA was reported to induce a longer-lasting inflammatory response with a transient increase in prostanoid production (Fustenberger and Marks, 1982; Carlson *et al.*, 1985; Rao *et al.*, 1993).

Although inhibitors of cyclo-oxygenase (COX) or 5-lipoxygenase (5-LO) attenuated inflammatory responses in both assays, the arachidonate assay is an useful model for the rapid "*in vivo*" screening of agents showing selective activity against AA lipoxygenase enzymes (Chang *et al.*, 1986; Young *et al.*, 1984). The effect of the olive oil phenolics in this AA assay agrees with the potent inhibitory action against the leukocyte 5-LO enzyme demonstrated by these compounds in previous studies (Koshihara *et al.*, 1984; Petroni *et al.*, 1997; De la Puerta *et al.*, 1999).

Measurement of myeloperoxidase (MPO), a granular constituent of neutrophils, is an index of neutrophil influx into the inflamed tissue (Bradley *et al.*, 1982). In our experiments we measured the

concentration of MPO in homogenates from the TPA-treated tissues since it has been demonstrated that in this assay the inflammatory response is associated with a marked influx of neutrophils resulting in a gradual increase in MPO levels that persisted up to 24 h. In contrast, topical application of AA resulted in time-dependent increases in mouse MPO levels that lagged behind the edema response (Rao *et al.*, 1993). All minor components of virgin olive oil strongly inhibited the MPO activity. The stronger action of the phenolics on inhibition of the MPO enzyme compared to their antiedematous response could be related to the fact that they are scavengers of hydrogen peroxide, the substrate for this enzyme (De la Puerta *et al.*, 1999). Among all the compounds only erythrodilol and β -sitosterol potentially reduced both the edema, developed either by TPA or AA, and the influx of neutrophils. This may be related to their typical steroidal structure and its possible action at the phospholipase A₂ level.

Our results show that specific minor components of virgin olive oil exert interesting topical anti-inflammatory effects likely inhibiting the prostanoid production and neutrophil influx which might contribute to the potential biological properties reported for this oil. Further research should elucidate the potential application of a virgin olive oil of a high content in these compounds for the treatment of different topical pathological processes.

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