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Review

Protective role of oleuropein and its metabolite hydroxytyrosol on cancer

M.P. Carrera-González*, M.J. Ramírez-Expósito, M.D. Mayas and J.M. Martínez-Martos

Experimental and Clinical Physiopathology Research Group BIO-296, Department of Health Sciences, Physiology, Faculty of Experimental and Health Sciences, University of Jaén, E-23071 Jaén, Spain (Tel./fax: +34 953 21 26 00; e-mail: pcarrera@ujaen.es)

Most therapies do not discriminate between normal and cancerous cells, leading to toxicity and unwanted side effects. In this sense, the oleuropein, minor component of extra virgin olive oil, is an excellent candidate due to its antitumorals estates described so much *in vitro* and *in vivo* experiments in colon, breast and skin cancer, and the capacity to across the blood–brain barrier. The recent results obtained in glia tumoral cells line, tumors characterized by its complicated treatment and worse prognosis; make to the oleuropein an exceptional candidate for the treatment of cancer.

Introduction

References to the olive tree date back to Biblical and Roman times and to Greek mythology. Historically, the products of *Olea europaea* have been used as aphrodisiacs, emollients, laxatives, nutritives, sedatives, and tonics. Specific conditions traditionally treated include colic, alopecia, paralysis, rheumatic pain, sciatica, and hypertension (Gilani, Khan, Shah, Connor, & Jabeen, 2005). Extra-virgin olive oil, the major source of dietary fat in the countries where olives are grown (Visioli, Bellosta, & Galli, 2002; Wahrburg & Assmann, 2001) constitutes part the commonly referred to "Mediterranean diet" of countries that surrounding the Mediterranean Sea. Although there are dietary variations among Mediterranean countries, a common feature is the high consumption of extra-virgin olive oil (Harwood & Yaqoob, 2002).

Among the generally accepted correlations between dietary habits and disease risk, the Mediterranean diet has been recognized as a healthful dietary pattern with preventive effect against chronic diseases, including cancer and cardiovascular diseases (Massaro, Scoditti, Carluccio, & De Caterina, 2010), and it has been suggested that modulation of uncontrolled free radical production and inflammation may be involved in this effect (de la Torre-Carbot *et al*., 2010; Llorente-Cortes *et al*., 2010; Tosetti, Noonan, & Albini, 2009). The established beneficial effects of extra-virgin olive oil on cardiovascular risk in the context of the Mediterranean diet have been mainly attributed to minor though highly bioactive components, including polyphenols, namely compounds with several hydroxyl groups on aromatic ring (Pignatelli *et al*., 2006). Therefore, extra-virgin olive oil is a functional food, which in addition to contain multiple minor components also has a high level of monounsaturated fatty acids (MUFA) (de la Torre, 2008). Minor components are present in about 2% of extra-virgin olive oil weight and include >230 chemical compounds. These minor components are present almost exclusively in virgin extra-virgin olive oil because the refining process expunges these compounds. Thus, the proportions of these minor compounds depend on the manufacturing processes of oil. Because these processes vary by oil mill, it is difficult to quantify the dietary intake of these components but Mediterranean countries tend to consume extra-virgin olive oil, which is much richer in phenolic compounds than refined oils.

The main antioxidants in olives are carotenoids and polyphenolic compounds. The primary polyphenols are oleuropein, 2-(3,4-dihydroxyphenyl)-ethanol (hydroxytyrosol, HT) and α-tocopherol. Oleuropein is the main phenolic compound in olive fruit; its concentration can reach up to 14% of net weight. The concentration of this secoiridoid declines with physiological development of fruit in what is called green maturation phase (Amiot, Fleuriet, &

* Corresponding author.

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Macheix, 1986; Bonoli, Bendini, Cerretani, Lercker, & Toschi, 2004; Huang & Sumpio, 2008).

The oleuropein hydrolyzes to the catechol HT and functions as a hydrophilic phenolic antioxidant that is oxidized to its catechol quinone during redox cycling. Many studies link specific secoiridoids as key minor components in an explanation of the extraordinary nutritional and healthy effects of extra-virgin olive oil (Dell'Agli & Bosisio, 2002; Manna *et al*., 1999; Tuck & Hayball, 2002).

Since the 1800s, the bitter component in olives has been used in humans against malaria-induced fevers, and beginning in the last century, researchers have focused considerable attention on the health benefits of the components of extra-virgin olive oil.

Recently, the biological effects of polyphenols present in extra-virgin olive oil have been further investigated. In particular it has been found that oleuropein is a potent scavenger of the free radicals (Manna *et al*., 2002) and nitrogen species (de la Puerta, Martinez-Dominguez, Ruiz-Gutierrez, Flavill, & Hoult, 2001) as well inducing the production of nitric oxide in macrophages (Visioli, Bellosta, & Galli, 1998). In addition, it plays an important role in the prevention of DNA damage, thus impairing mutagenesis and carcinogenesis (Valko, Izakovic, Mazur, Rhodes, & Telser, 2004). In this sense, Hamdi and Castellon (2005) demonstrated that antitumoral effect of oleuropein exerted by the disruption of actin filament in tumor cells.

Also, the oleuropein has been described as an inhibitor of low density lipoprotein (LDL) oxidation (Carluccio *et al*., 2003; Owen *et al*., 2000; Rietjens, Bast, de Vente, & Haenen, 2007).

In this review, we will describe the anticarcinogenic effects of oleuropein both *in vivo* as *in vitro* studies.

In vivo and *in vitro* studies about oleuropein antitumoral role

Oleuropein is a complex phenol present in large quantities in olive tree leaves and in low quantities in extra-virgin olive oil (Soler-Rivas, Espin, & Wichers, 2000), and it is responsible for the bitter taste and pungent aroma of extra-virgin olive oil. It was discovered by Bourquelot and Vintilesco (1908) and its structure was specified as being that a heterosidic ester of elenolic acid and HT. The chemistry of oleuropein has allowed proposing possible ways (showed in Fig. 1). In the stomach, oleuropein undergoes acid hydrolysis with the formation of different metabolites whose distribution and concentration are strictly related to the pH of the medium and the time of permanence of the molecule in the stomach. The acid-catalyzed hydrolysis cleaves the β-glycosidic bond with the release of glucose and the aglycone moiety, from which two dialdehydes are immediately originated. The dialdehydes are unstable and in the lipid/water interface they are converted into the metabolite known as transposed secoiridoid. This is a stable lipophilic compound and only under drastic and prolonged acid conditions, by cleavage of the two estereal groups, it does lose the HT and/or methanol fragment. The transposed secoiridoid can also be produced by the action of the enzyme β-glucosidase.

When oleuropein is administered as gastro-resistant capsules, it reaches the intestine unchanged and here it may have a different fate. Oleuropein is a high molecular-weight hydrophilic compound and it seems unlikely that

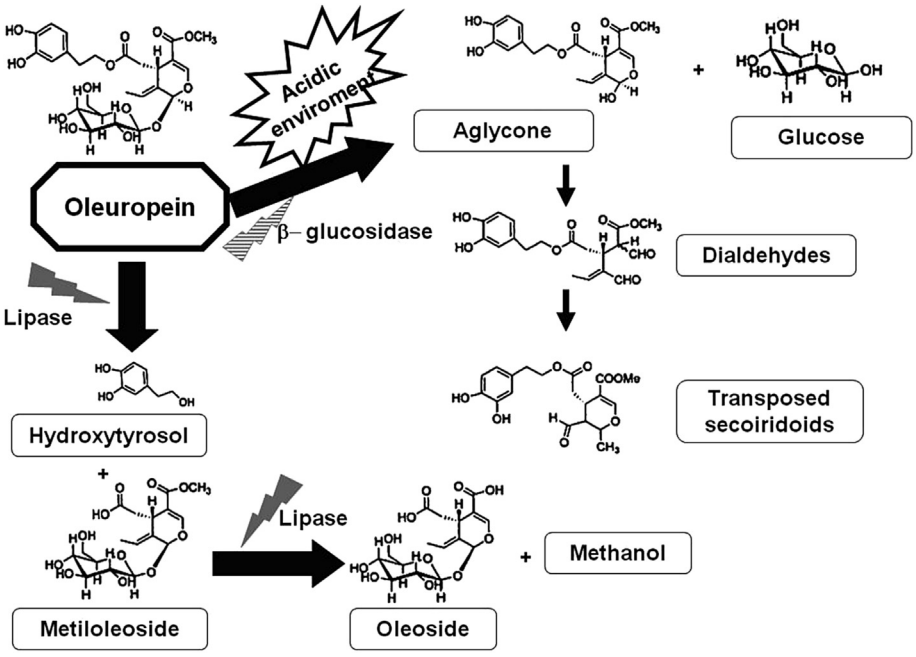

Fig. 1. Metabolism of oleuropein after ingestion.

it could readily diffuse through the lipid bilayer or paracellum junctions. As a glycoside, oleuropein could possibly access a glucose transporter, but no experimental data have till now supported this theory. In the literature there are no conclusive reports that clarify the pharmacokinetics of oleuropein and it is reasonable to believe that only a small amount of unchanged oleuropein reaches the systemic circulation.

In addition, in the intestine, oleuropein can suffer the action of two lipases with the subsequent release of HT and methyl oleoside and from this latter compound; methanol and oleoside are produced (Furneri, Piperno, & Bisignano, 2009). Among these different compound derived from hydrolysis of oleuropein, HT stands out.

During the past few years, thank the availability of pure compound, the biological activities of extra-virgin olive oil phenolic, oleuropein and HT, have been thoroughly investigated (Quiles, Farquharson, Simpson, Grant, & Wahle, 2002).

To date, the antitumoral effect of oleuropein and HT comes from *in vitro* studies, mainly, since few authors have described the anticarcinogenic effect of oleuropein *in vivo* (Hamdi & Castellon, 2005). In this sense, in the present year, Casaburi *et al*. (2013) have summarized the several antioxidant and antitumoral effects of olive oil phenols *in vitro* studies and the putative mechanisms involved.

In relation to HT, the product of hydrolysis of oleuropein in *in vivo* system, it has been testing its antitumor capacity in a breast cancer animal model chemically induced by 7,12-dimethylbenz[α]anthracene (DMBA) (Granados-Principal, Quiles, Ramirez-Tortosa, Sanchez-Rovira, & Ramirez-Tortosa, 2010).

In vitro studies

Numerous lines of evidence demonstrate that antioxidants protect against DNA damage, a major step in oncogenic processes (Valko *et al*., 2004). Oleuropein is a potent antioxidant endowed with antiinflammatory and antineoplastic properties.

Recently, it has been described the effect of oleuropein on LNCAP and DU145 prostate cancer cell line and BPH-1, prostate normal cell. In tumoral prostate cell, the oleuropein reduces cell viability and induces thiol group modification, γ-glutamylcysteine synthetase, reactive oxygen species (ROS), pAKT and heme oxygenase. These results (Acquaviva *et al*., 2012) show that oleuropein decreases prostate cancer cell proliferation and induces necrotic cell death. The study is more interesting since in DU145 cell, oleuropein causes a significant dose-dependent increase in the level of ROS but in BPH-1, oleuropein induced a significant reduction in ROS. Thus, the oleuropein behaves as an antioxidant in BPH-1 and as pro-oxidant in neoplastic cells. However, already in 2005 the antitumor and antiangiogenic capacities of oleuropein were described. Oleuropein inhibits cell growth, motility, invasiveness and completely inhibits cell motility in normal fibroblasts, T-47D breast cancer cell, LN-18 glioblastoma cell and melanoma cells lines. Indeed, in human melanoma cell line can form tubes on hard Matrigel. Unlike the vascular endothelial cell tubes, melanoma tubes collapse by retraction in 1 week. In certain cases, they never retract and cells invade the matrix. When added to the tubing phase of the assay, 0.1% oleuropein disrupted the tubes *in situ* by rounding the cells and preventing tube retraction. This process is relatively fast, occurring within 2 h; this contrasts with tube retraction, which occurs within 1 week (Hamdi & Castellon, 2005). In addition, the same authors have also demonstrated that oleuropein treatment dramatically disrupts the organization of actin filaments within the cells.

The aromatic ring present in oleuropein and HT is a feature common to estradiol (E2). This common structure can lead to suppose a putative mechanism of action of these polyphenols correlated to their capacity to compete with estrogens for estrogen receptor (ER) binding sites. As is well known, the growth of many breast tumors is stimulated by E2 and therefore, polyphenols of natural origin as oleuropein and HT could interfere with tumor cell proliferation. In this context, recently it has been described in human MCF-7 breast cancer cells the presence of oleuropein and HT interferes with E2-dependent MCF-7 proliferation in a dose-dependent manner. However, concentration higher than 100 μM the two compounds show cytotoxic effects (Sirianni *et al*., 2010). Thus, the two molecules possess antiestrogenic-activity. The data point out an inhibition of E2-dependent rapid signaling pathways which are able to induce, within a few minutes, molecules involved in proliferation events, such as mitogen-activated protein kinase (MAPK). These studies are in accordance with recent results obtained by Elamin *et al*. (2013). Oleuropein is cytotoxic and induces apoptosis in breast cancer cells, being this effect more pronounced on ER-negative breast cancer cells than ER-positive cells. Alike, oleuropein is a potent inhibitor of cell proliferation by delaying the cell cycle at S phase and down-regulated two major breast cancer-related onco-proteins, NF-κβ and cyclin D1.

Thereby, these studies shown that these phenolic compounds have potent antibreast cancer properties.

On the other hand, it has been described a new molecular mechanism by which the oleuropein aglycone, through the specific inhibition of HER2 oncogene, may exert protective effects not only in the promotion but further in the progression of human breast cancer (MCF-7 breast cancer line). That is to say, the oleuropein directly regulates the expression and activity of HER2, a proto-oncogene that plays a pivotal role in malignant transformation, tumorigenesis, metastasis, and treatment failure in breast cancer disease. The ability of oleuropein aglycone to exhibit synergistic antitumor effects when concurrently given to breast cancer cells chronically exposed to trastuzumab for several months further underscores the clinical relevance of these findings as they reveal a novel approach capable

to circumvent trastuzumab resistance in breast cancer disease. Thus, Menendez *et al*. (2007) show that oleuropein aglycone is the most potent phenolic compound in decreasing breast cancer cell viability.

As mentioned in the beginning of this section, the oleuropein has antiinflammatory property. In this way, the inflammatory angiogenesis is a key pathogenic process both in cancer and atherosclerosis. Cyclooxygenase (COX-2) is a pro-inflammatory enzyme involved in inflammatory angiogenesis. In fact, recently it has been reported that polyphenols as oleuropein and HT suppressed inflammatory angiogenesis in cultured human vascular endothelial cells via the attenuation of stimulated COX-2 expression suggesting that COX-2 represents a potential molecular target susceptible to polyphenol modulation (Scoditti *et al*., 2012).

In this context, MAPK signaling pathway, which is known to regulate COX-2 activity in a variety of tissues (Tsatsanis, Androulidaki, Venihaki, & Margioris, 2006), has long been viewed as an attractive pathway for anticancer therapies, due to its central role in regulating the growth and survival of cells from a broad spectrum of human cancers (Sebolt-Leopold & Herrera, 2004). MAPK enzymes, including p38 extracellular signal-related kinase (ERK) and c-Jun N-terminal kinase (JNK) have been implicated in the regulation of COX-2 gene expression in the large intestine and are involved in both transcriptional and post-transcriptional regulation of COX-2 (Shao, Sheng, Inoue, Morrow, & DuBois, 2000). The control of COX-2 transcription is regulated by p38 and/or other signaling pathways through the activation of transcription factors, such as cyclic AMP response element binding protein (CREB), NF-κβ, NFAT or AP-1 (Tsatsanis *et al*., 2006). COX-2 is aberrantly overexpressed in many human cancers, most notably of colonic origin, and has been demonstrated to play a role in tumor progression and metastasis (Chu, Lloyd, Trifan, Knapp, & Rizzo, 2003). In colorectal cancer cells, COX-2 is overexpressed and this overexpression has a strong association with colorectal neoplasia, by promoting cell survival, cell growth, migration, invasion and angiogenesis (Chu *et al*., 2003; Trifan & Hla, 2003). Within this context, it has been described that the extra-virgin olive oil polyphenols are capable of down-regulating COX-2 expression in colonic cancer cells by a mechanism involving the early inhibition of p38 and downstream inhibition of the transcription factor CREB (Corona *et al*., 2007).

Multiple studies have revealed a role for COX-2 inhibitors in decreasing the risk of colon cancer development and in suppressing tumor formation and growth (Corona *et al*., 2007; Tsatsanis *et al*., 2006). These observations agree with other studies on human colon cancer cell line, Caco-2 cells, which demonstrate that inhibition of p38 significantly reduces COX-2 expression (Arbabi, Rosengart, Garcia, Jelacic, & Maier, 2001). Therefore, the antiproliferative effects of extra-virgin olive oil polyphenols, representative of those reaching the large intestine, may be mediated, in part, by the inhibition of p38 and CREB phosphorylation/activation leading to a reduction in COX-2 expression (Corona *et al*., 2007).

On the other hand, in nervous tissue, the results about oleuropein effect on nervous cancer are very scarce. In this sense, our laboratory in a preliminary study has analyzed the antitumoral activity of oleuropein on C6 rat glioma cell line by cytotoxicity assay.

To our knowledge, this study provides the first data on the antitumor capacity of oleuropein on glioma cells. This work presents preliminary data that advance the potential of this minority compound present in extra-virgin olive oil against these types of tumors. In this study, we evaluated the inhibitory effects of oleuropein on proliferation of C6 glioma cells and we observed an inhibition of cell proliferation dose-dependent after treatment of glioma cells with this compound (Fig. 2). Specifically, the oleuropein inhibited of cellular growth with micromolar doses by 40%. The calculated IC50 value was 911 μM (Table 1).

These data allow us to foretell the potentiality of this minority compound in extra-virgin olive oil on glioma tumors, especially knowing the current limitations for the treatment of this pathology.

In vivo studies

Despite compelling evidences of the good absorption of polyphenols, their poor bioavailability is a major drawback in our understanding of the properties of these compounds *in vivo* and the potential health benefits derived from their consumption (de la Torre, 2008). We have to bear in

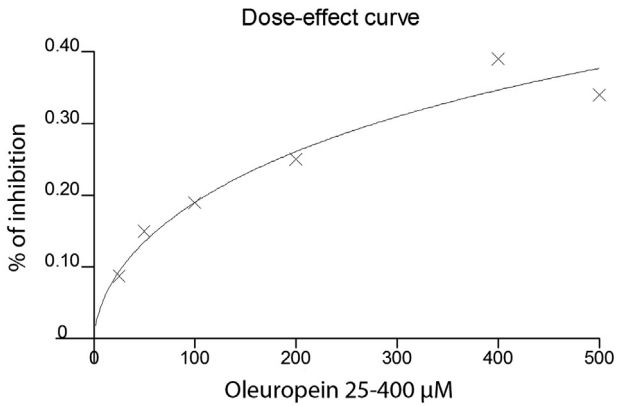

Fig. 2. Dose–response curves about oleuropein effect (25–400 μM) on the proliferation of C6 glioma cells. Cells were treated for 24 h and tested for cytotoxicity. Cytotoxicity assays were repeated three times, using four replicates in each experiment. The cytotoxic method assayed is described by Vichai and Kirtikara (2006), based in the stained with sulforhodamine B (SRB) dissolved in 1% acetic acid of TCA-fixed cells 0.4% (w/v). At the end of staining period, SRB was removed and cultures were rinsed with 1% acetic acid to remove unbound dye. The cultures were air dried and bound dye was solubilized with 10 mM Tris base (pH 10.5). Optical density (OD) was read in a Tecan Genios Plus plate reader at 492 nm. The photometer response was linear with dye concentration and it was proportional to cell numbers counted in parallel with an automatic cell counter (TC-10, Bio-Rad).

Table 1. The table shows the values cellular affect fraction (Fa), curve sigmoidicity coefficient (*m*), the doses that induced 50% inhibition of the cellular growth (Dm), equal IC50 value and lineal correlation value for median-effect curve (*r*), after treatment of glioma cells with several concentrations of oleuropein.

Oleuropein dose (μM)	Fa	*m*	Dm (μM)	*r*
25	0.0870			
50	0.150			
100	0.190			
200	0.250			
400	0.390			
		0.640	911.11	0.989

mind that one of prerequisites for assessing the physiological significance of extra-virgin olive oil phenolic compounds in human beings is the ability to determine their bioavailability. However, several clinical and animal studies have provide evidence that phenolic compound are absorbed, and exert their biological effects in a dose-dependent manner (Visioli *et al*., 2000). After absorption and metabolism of extra-virgin olive oil, the antioxidant actives may display their activity at the cellular level (Omar, 2010). In addition, not all the beneficial effects attributed to the intake of extra-virgin olive oil or its phenolic compounds, such as oleuropein or HT, are related to its antioxidant capacity; other beneficial properties, such as a neuroprotective effect (Mohagheghi, Bigdeli, Rasoulian, Zeinanloo, & Khoshbaten, 2010; Omar, 2010), antihypertensive effect (Carluccio, Massaro, Scoditti, & De Caterina, 2007; Cicerale, Lucas, & Keast, 2010), and including those already mentioned above cardioprotective (Covas *et al*., 2006; Kay, Kris-Etherton, & West, 2006; Omar, 2010; Perona, Cabello-Moruno, & Ruiz-Gutierrez, 2006; Vogel, Corretti, & Plotnick, 2000; Williams *et al*., 2001) and antiinflammatory effects (Cicerale, Breslin, Beauchamp, & Keast, 2009; Groff & Gropper, 2000; Impellizzeri & Lin, 2006) are being studied.

Nevertheless, the phenolic compounds of extra-virgin olive oil and their metabolites are present in biological fluids at very low concentrations and some authors are cautious about attributed a direct relationship between ingestion and the beneficial effects of this minor fraction of extra-virgin olive oil (de la Torre, 2008; Vissers, Zock, & Katan, 2004). The key to the relationship between the ingestion of extra-virgin olive oil and its beneficial effect is probably not to be found in such widely analyzed biological fluids as plasma and urine, but must be sought in those tissues in which the phenolic compounds and their metabolites and other minor extra-virgin olive oil compounds contribute positively to the normal cell metabolic process.

In this sense, it has been recently described (Serra *et al*., 2012) the distribution of extra-virgin olive oil phenolic compounds and their metabolites in plasma and rat tissues after the ingestion of a phenolic extract from olive cake (PEOC). These authors quantified free forms of some phenolic compounds, such as oleuropein derivative in the plasma and brain, luteolin in the kidney, testicle, brain and heart, or HT in the plasma, kidney and testicle. The quantification of metabolites of the phenolic compounds in the plasma and multiple tissues indicate that after an acute ingestion of olive phenolic compounds, they are absorbed, metabolized and distributed though the blood stream to practically all parts of the body, even across the blood–brain barrier.

After ingestion, the secoiridoid derivatives may be partially modified in the acidic environment of the stomach, suffering a non-enzymatic metabolism that considerably decreases their concentration and increases moderately the total phenyl alcohols and the total phenolic acids (Corona *et al*., 2006; Suarez, Macia, Romero, & Motilva, 2008). Nevertheless, this could be explained because the parental form of the secoiridoids are not the major bioavailable form *in vivo* (Pinto *et al*., 2011), which explains the absence of other secoiridoids in the plasma after the ingestion of a secoiridoid-rich extract like PEOC. Tyr, HT and their secoiridoid derivate (e.g. oleuropein glucoside) and the aglycone form of oleuropein make up around 90% of the total phenolic content of extra-virgin olive oil (de la Torre-Carbot *et al*., 2005).

Probably, the first step of the intestine/hepatic metabolism could be related to the ingested dose. High doses could saturate the conjugation metabolism of the olive phenolic compounds and this may allow the detection of free forms in the plasma, these probably being absorbed by passive diffusion. Of special interest is the presence of these free aglycones circulating in blood, probably with biological effects different from those of conjugated metabolites. Equally important is the detection in heart of conjugated forms of HT and the free forms of oleuropein derivative and luteolin (Serra *et al*., 2012). These phenolic metabolites could exert a direct protective effect related to oxidative process in the arterial wall, which have stimulated research on HT potential role in cardiovascular protection (Owen *et al*., 2000).

The increase reported by Serra *et al*. (2012) in some phenolic metabolites in the brain as a consequence of the ingestion of the phenolic extract is of special interest too. In relation, our laboratory has achieved promising results since the treatments with HT, but not oleuropein, in an experimental glioma animal model, during short time periods produce a significant inhibition of tumor growth, through mechanisms that involves endogenous antioxidant defenses and with very low adverse effects (Mayas *et al*., submitted for publication).

Also, it has been suggested that non-absorbable phenolic compound may display local antioxidant activities in the gastrointestinal tract. This idea is supported by the capacity of isolated phenolic compound to scavenge both the free radicals generated by the fecal matrix (Owen *et al*., 2000) and those induced in epithelia cells of the intestine (Manna *et al*., 1999).

As described above, the oleuropein in the organism will be metabolized in its different metabolites. There are several *in vivo* studies about oleuropein effect on cardiovascular disease (Tripoli *et al*., 2005), platelet function (Singh, Mok, Christensen, Turner, & Hawley, 2008) and LDL oxidation (Leenen *et al*., 2002). On the other hand, very little is the bibliography about its effect on cancer.

To combat cancer, medicine relied on toxic compound (Blagosklonny, 2005). Most therapies do not discriminate between normal and cancer cells, leading to toxicity and unwanted side effects. In this sense, some studies were undertaken to determinate the toxicity of oleuropein and its two main metabolites (HT and elenolic acid); all were found to be completely non-toxic in several animal species (D'Angelo *et al*., 2001; Elliott, Buthala, & DeYoung, 1969; Petkov & Manolov, 1972).

Important discoveries have been realized by the researches Hamdi and Castellon (2005). In Swiss albino mice, that spontaneously develop soft tissue sarcomas, a 1% oleuropein in the drinking water (consumed *ad libitum*) induced dramatic tumor regression. This is quite unique among chemotherapeutic agents, since most of them do not induce complete regression in such a short time. This rapid effect of oleuropein on tumors could be explained by its effect on the cytoskeleton inducing cell rounding within the tumors itself without having obvious effects on the vasculature. These data suggest that the principal antitumors mechanism of oleuropein in vascularized tumors involved the direct disruption of tumors cells.

Nevertheless, the *in vivo* antitumoral effect of oleuropein could be carried out by its metabolites, mainly HT. The antitumor effect of HT has been studied as a result of its ability to inhibit the proliferation and promote apoptosis in several tumor cell lines, in addition to being chemopreventive as a result of its high antioxidant activity (Granados-Principal *et al*., 2010). In this way, recently, it has been reported that HT inhibits mammary tumor growth induced in rat by 7,12-dimethylbenz[α]anthracene (DMBA). This antitumor capacity of HT is supported by the decrease in cell proliferation, associated with a less nuclear Ki-67 immunostaining, demonstrating a powerful antiproliferative activity similar to that of doxorubicin (Granados-Principal *et al*., 2011). This study also shows a better mammary histopathologic outcome after HT administration, providing slightly better results than in the doxorubicin-treated group.

Conclusions

The benefits of the Mediterranean diet are worldwide known, but it has been recently when the useful effects of minor compounds of extra-virgin olive oil, in particular of oleuropein and its metabolites as HT, it has been reported. The discovery of the cytostatic and antiangiogenic roles in different tumors types, together with the absence of toxic effects; make the oleuropein and its metabolites as HT, excellent candidates for testing their antitumoral capacity in dept. In particular, their putative applications on nervous tumors, since across the blood–brain barrier, make these compounds worthy of subsequent studies.

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