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Leishmanicidal activity assessment of olive tree extracts

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ABSTRACT

Leishmaniasis, a protozoan parasitic disease that remains a major worldwide health problem with high endemicity in developing countries, is prevalent around the Mediterranean basin. High cost, systemic toxicity, and diminished efficacy due to development of parasite resistance are the serious drawbacks of current treatment options. Thus, identifying new, effective, and safer anti-leishmanial drug(s) is of paramount importance. Here we tested the anti-promastigote and anti-amastigote activity of five natural products, including oleuropein and hydroxytyrosol, present in olive tree leaves and olive mill wastewater. These products are recognized as low-cost starting materials rich in bioactive compounds, particularly biophenols. Oleuropein and hydroxytyrosol exhibited the best inhibitory effect among the natural products tested in both stationary and middle logarithmic phase promastigotes of *L. infantum*, *L. donovani*, and *L. major*. Similarly, oleuropein and hydroxytyrosol demonstrated the highest selectivity index ratio against *L. donovani* amastigotes that parasitize J774A.1 macrophages. Moreover, oleuropein was tested *in vivo* in an experimental visceral leishmaniasis model. *L. donovani*-infected BALB/c mice received intraperitoneal oleuropein a total of 14 times at intervals of every other day. Three days after treatment termination, the spleen parasitic burden was reduced >80%. Of interest, this effect of oleuropein persisted and was even enhanced 6 weeks after the termination of the treatment, as determined by parasite depletion of >95% in liver and spleen. These findings contribute to the potential development of natural products as effective drugs against parasites of the *Leishmania* genus, with low cost and diminished cytotoxicity.

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Introduction

Leishmaniasis is a vector-borne protozoan disease caused by species of the genus *Leishmania*. *Leishmania* spp. are responsible for parasitic diseases with a wide range of symptoms and considerable impact on public health worldwide. Leishmaniasis is still one of the world's most neglected diseases, accounting for 1.5–2 million new cases and 50,000 deaths attributable to the visceral form annually (World Health Organization, Leishmaniasis Control home page). The persistence of and increase in leishmaniasis is mainly due to factors such as the AIDS epidemic, climatic changes, international travel, migration flows, lack of effective vaccines, problems with vector control, and development of drug resistance (Dujardin et al., 2008).

Treatment and control of leishmaniasis involve a limited number of drugs with various adverse reactions, effectiveness, and long duration of treatments. So far, chemotherapy includes pentavalent antimonials, numerous forms of amphotericin B, paromomycin (aminosidine), pentamidine isethionate, hexadecylphosphocholine (HePC; miltefosine), and azoles such as ketoconazole, fluconazole, and itraconazole (World Health Organization, Leishmaniasis Control home page). Despite the numerous treatments, none is fully satisfactory because of the toxicity of the treatment itself, a tendency to host relapse, and the development of resistant parasite strains (Kappagoda et al., 2011). All of these disadvantages are assisted by the fact that many people worldwide have no access to conventional pharmacological treatments (den Boer et al., 2011). These patients prefer to focus their efforts for therapy on folk remedies (alternative treatments) that consist basically of natural products that are considered non-toxic, but their safety and effectiveness require evaluation (Sen and Chatterjee, 2011). Screening natural products for potential use in the therapy of leishmaniasis is essential to satisfy the urgent need for alternative treatments (Rocha et al., 2005).

Olives and olive oil are considered the cornerstone of the Mediterranean diet and are recognized for their overall health

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benefits (Cicerale et al., 2009). Olive tree (*Olea europaea*) leaves and olive mill wastewater (OMWW), which is a high-polluting by-product of olive oil production, are regarded as low-cost sources that are rich in phenolic compounds with strong antioxidant properties (Cardinali et al., 2010). The majority of the compounds, at least 30, are present in olive oil, and recent data have suggested that many of the constituents may have more health benefits than previously thought (Cicerale et al., 2009).

Oleuropein obtained from olive leaves and hydroxytyrosol obtained from OMWW have multiple biological properties (Tuck and Hayball, 2002; Waterman and Lockwood, 2007). Oleuropein, the main ingredient of olives and olive leaves, is a secoiridoid glucoside and belongs to the polyphenol group. Hydroxytyrosol is basically found in olives and in olive oil in the form of its elenolic acid ester oleuropein, and especially after hydrolysis during degradation, in its plain form. The major activity of oleuropein and hydroxytyrosol is to scavenge free radicals to protect cells from oxidation. Especially, the anti-oxidant ability of oleuropein may slow ageing and reduce the risk of coronary disease (Andreadou et al., 2006; Katsiki et al., 2007). Moreover, oleuropein enhances the response of macrophages to bacterial lipopolysaccharide via an increase in inducible nitric oxide synthase activity whereas oleuropein by itself does not enhance nitric oxide production by macrophages (Visioli et al., 1998). Finally, oleuropein and hydroxytyrosol have been reported to have antitumor, antiviral, antibacterial, and antiparasitic action (Bisignano et al., 1999; Granados-Principal et al., 2010; Jiang et al., 2008; Lee-Huang et al., 2007a,b; Tuck and Hayball, 2002; Waterman and Lockwood, 2007) whereas, in the year 2000, oleuropein was claimed in a U.S. patent (Fredrickson, Num: 6,117,844) to have potent antiviral activity against numerous viral species.

In this study, we evaluated five natural products, two pure constituents and three crude extracts, derived from olive leaves and OMWW, as potent *in vitro* and *in vivo* anti-leishmanial agents. For this purpose, they were tested against promastigotes of three *Leishmania* species and against *L. donovani* intracellular amastigotes. Finally, oleuropein was also tested *in vivo* against experimental visceral leishmaniasis caused by *L. donovani* parasites.

Materials and methods

Crude extracts and pure compounds

Air-dried and pulverized leaves (5 kg) from *Olea europaea* var *koroneiki* collected in Crete (Greece) were extracted by mechanical stirring for 12 h with acetone (2× 2.5 l). The extract was evaporated completely and washed with a mixture of CH_2Cl_2/MeOH:98/2 (3 l). The insoluble material (360 g) was separated and dried, producing a yellow powder containing 60% oleuropein. Ten grams of this yellow powder was subjected to countercurrent chromatography, using a fast centrifugal partition chromatograph (FCPC) apparatus (Kromaton). The system of solvents used in this procedure was EtOAc/EtOH/H_2O:10/1/10 (6 l). The capacity of the column was 1 l, the rapidity of the rotation was 900 rpm, and the flow rate was 15 ml/min. A total of 5.0 g of oleuropein (purity 90%) was isolated by the above-mentioned process.

Olive leaf water extraction (OLWE) and olive leaf decoction (OLD) were derived from the same collection of *Olea europaea* leaves. Olive leaf powder was extracted using the method of accelerated solvent extraction (ASE) performed on an ASE 300 system (Dionex, USA). The extraction was conducted with deionized water at 80 °C and the OLWE was finally lyophilized. For the other portion, fresh leaves were blended using a general-purpose electric blender. The blended material was added to water and boiled for 3 min.

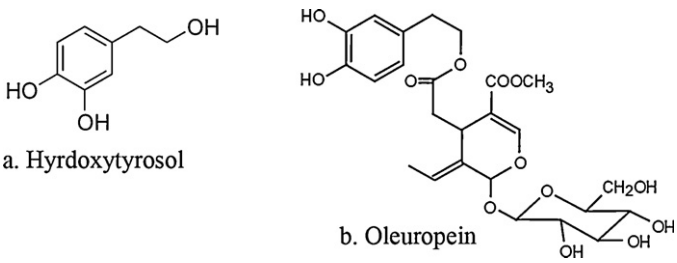

Fig. 1. Molecular formulas of (a) hydroxytyrosol and (b) oleuropein.

Then, the hot mixture was filtered through a filter paper, and the filtrate was extracted and evaporated to dryness under reduced pressure to yield a solid residue.

Hydroxytyrosol was extracted from OMWW resulting from olive oil production using olive fruits of *Olea europaea* var *koroneiki*. During the process, the material was filtered using filter paper. Then filtered wastewater was applied to Amberlite XAD-4 resin and the column eluted with 96% ethanol. Then ethanol was evaporated and a phenolic fraction was received. The final step aimed at the purification of hydroxytyrosol. The above polyphenolic fraction was submitted to countercurrent chromatography using the FCPC apparatus (Kromaton). The system of solvents used in this procedure was c-hexane/EtOAc/MeOH/H_2O:4/6/4/6 (Agalias et al., 2007). The capacity of the column was 1 l, the rapidity of the rotation was 900 rpm, and the flow rate was 15 ml/min. Hydroxytyrosol (purity > 90%) was isolated using the above-mentioned process.

The purity of oleuropein, hydrotyrosol (Fig. 1) and olive leaf decoction was established by high performance liquid chromatography (HPLC) (Fig. 2) and nuclear magnetic resonance (NMR). Significant was, the presence of oleuropein and hydroxytyrosol alongside with other unidentified compounds in the olive leaves decoction. Prior to use, pure oleuropein, pure hydroxytyrosol, and the oleuropein 60% mixture were diluted in distilled water while the other two crude extracts, OLWE and OLD, were diluted in dimethyl sulfoxide (DMSO). All extracts were Millipore filtered with a 0.45 μm filter and stored at 4 °C until use.

Parasites

Three reference *Leishmania* strains were used in this study: *Leishmania infantum* (zymodeme MON-1, strain MCAN/PT/98/IMT 244), *Leishmania donovani* (zymodeme MON-2, strain MHOM/IN/1996/THAK35), and *Leishmania major* (zymodeme LV39, strain MRHO/SU/59/P). The promastigotes were cultured in complete medium consisting of RPMI-1640 (with low content of phenol red) (Biochrom AG, Berlin, Germany) supplemented with 2 mM L-glutamine, 10 mM HEPES, 24 mM $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, Paisley, UK) at 26 °C. Cultures were estimated daily for parasite growth, and promastigotes were collected at the middle of the logarithmic or at the beginning of the stationary phase.

Cell cultures

Macrophages of the J774A.1 murine macrophage cell line from ATCC (American Type Culture Collection, Rockville, MD, USA) were cultured in complete medium consisted of RPMI-1640 medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 24 mM $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS, at 37 °C in a 5% CO_2 environment. Subcultures

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