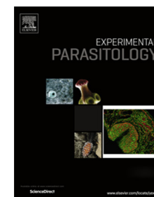
Experimental Parasitology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr



Bioassay guided isolation and identification of anti-*Acanthamoeba* compounds from Tunisian olive leaf extracts

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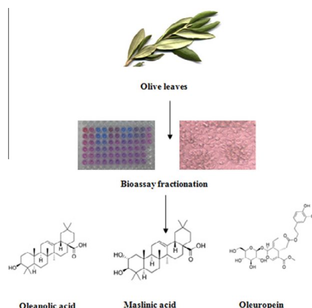
HIGHLIGHTS

- Olive leaf extracts contain an interesting anti-*Acanthamoeba* activity.
- The bio-guided fractionation of the extract yielded three known molecules: oleanolic acid, maslinic acid and oleuropein.
- To the best of our knowledge the activity of the isolated molecules has not been previously reported against amoebae.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 30 November 2013
Received in revised form 10 February 2014
Accepted 21 February 2014
Available online xxxx

Keywords:
Olive leaf extract
Acanthamoeba
Chemotherapy
Bioassay fractionation

ABSTRACT

Pathogenic *Acanthamoeba* strains are causative agents of Granulomatous Amoebic Encephalitis (GAE) and *Acanthamoeba* keratitis (AK) worldwide. The existence of the cyst stage complicates *Acanthamoeba* therapy as it is highly resistant to antibiotics and physical agents. The aim of this study was to investigate the activity of Limouni olive leaf cultivar against the trophozoite stage of *Acanthamoeba*. The ethyl acetate and methanol extracts of this variety were tested against *Acanthamoeba castellanii* Neff. The ethyl acetate extract of olive leaf was the most active showing an IC_{50} of 5.11 ± 0.71 μg/ml of dry extract. Bio-guided fractionation of this extract was conducted and led to the identification of three active compounds namely oleanolic and maslinic acids and oleuropein which could be used for the development of novel therapeutic approaches against *Acanthamoeba* infections.

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1. Introduction

Acanthamoeba species are ubiquitous free-living amoebae which dwell in several habitats, including air, soil, and water environments. However, these amoebae can also act as opportunistic pathogens causing Granulomatous Amoebic Encephalitis (GAE) and *Acanthamoeba* keratitis. The therapy of these diseases has been undermined by resistance, variable efficacy between strains or species, toxicity, and requirement for long courses of treatment. A need for identifying alternative natural and safe sources of molecules, especially of plant origin, to treat these diseases has notably increased in recent years.

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http://dx.doi.org/10.1016/j.exppara.2014.02.018
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Please cite this article in press as: Sifaoui, I., et al. Bioassay guided isolation and identification of anti-*Acanthamoeba* compounds from Tunisian olive leaf extracts. Exp. Parasitol. (2014), http://dx.doi.org/10.1016/j.exppara.2014.02.018

Among these plants, olive leaves have been used from the past in traditional medicine to cure many infections such as malaria and ulcers. More recently, pharmaceutical and food industries started to use several olive products considering their richness in bioactive molecules. Phytochemical investigations of olive leaves led to the isolation of various secoiridoid and triterpenes, some of which were found to possess several pharmacological properties (Sifaoui et al., 2013). Oleuropein and related derivatives constitute the major class of molecules in olive leaf extracts. Several studies have reported antioxidant, hypoglycemic, antihypertensive, antimicrobial, antitumoral, antiatherosclerotic, antiparasitic and antiviral, including anti-HIV, properties of olive leaves (Lee-Huang et al., 2003; Somova et al., 2003; Goulas et al., 2009; Sudjana et al., 2009). Oleanolic and maslinic acids, a natural penta cyclic triterpene, are widely present in dietary plants, especially in olive product. Those compounds have attracted much interest due to their biological activities, such as anti-viral (Saija and Uccella, 2000) antidiabetogenic (Jemai et al., 2009) and anticancer functions (Sánchez-Tena et al., 2013). Our preliminary work with Limouni variety showed that ethyl acetate fraction of it had interesting amoebicidal activity. Thus, the objectives of this work were to isolate and identify the major molecules responsible for this effect.

2. Materials and methods

2.1. Plant material

Plant material (leaves) of Limouni variety was harvested from the southeastern part of Tunisia, 'Ain el Maaguel, Douiret', during the maturing fruit season 2010/2011. The olive leaves collected were ground to a fine powder using a mill. Each shell powder sample (0.25 g) was macerated with 20 ml of extraction solvents (first with ethyl acetate than with methanol) in a capped glass tube on an agitating plate at a constant stirring rate (280 rpm) for 1 h and under 55 °C. Afterwards, a rotary vacuum evaporator at 40 °C was used in order to remove solvent.

2.1.1. Bioassay guided fractionation of Limouni ethyl acetate extract

The fractionation of the olive leaf extract was guided by inhibitory activity against *Acanthamoeba* Neff. Initially, the crude extract was fractionated by to 14 fractions by chromatography on silica gel (Hexane/AceOH/MeOH). The 14 fractions were concentrated *in vacuo*. Based on the observed antiprotozoal activity and similarities in TLC profiles, we procedure to combined some fractions. Nine fractions was further recovery. The most active fractions against the tested parasite were subjected to silica gel flash chromatography or Sephadex gel as illustrated in Fig. 1.

2.2. In vitro drug sensitivity assay

2.2.1. Amoebic strain

Acanthamoeba castellanii Neff (ATCC 30010), a type strain from the American Type Culture Collection was used in this study. This strain was axenically grown in PYG medium (0.75% (w/v) proteose peptone, 0.75% (w/v) yeast extract and 1.5% (w/v) glucose) containing 40 μg gentamicin ml^{-1}(Biochrom AG, Cultek, Granollers, Barcelona, Spain) previous it use for the assays.

2.2.2. In vitro effect against the trophozoite stage of Acanthamoeba

The anti-*Acanthamoeba* activities of the assayed extracts were determined by the Alamar Blue® assay as previously described (McBride et al., 2005; Martín-Navarro et al., 2008). Briefly, *Acanthamoeba* strains were seeded in duplicate on a 96-well microtiter plate with 50 μl from a stock solution of 10^4 cells ml^{-1}. Amoebae were allowed to adhere for 15 min process which was checked using a Leika DMIL inverted microscope (Leika, Wetzlar, Germany). After that, 50 μl of serial dilutions of the fraction or the pure compound was added to each well, (In all tests, 1% dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, Mo.), a concentration that was used to dissolve the highest dose of the compounds but that had no effect on the parasite). Finally the Alamar Blue Assay Reagent® (Biosource, Europe, Nivelles, Belgium) was placed into each well at an amount equal to 10% of the medium volume. Test plates containing Alamar Blue were then incubated for 120 h at 28 °C with a slight agitation.

Subsequently the plates were analyzed, during an interval of time between 72 and 144 h, on a Microplate Reader Model 680 (Biorad, Hercules, CA) using a test wavelength of 570 nm and a reference wavelength of 630 nm. Percentages of growth inhibition, 50% inhibitory concentrations (IC_{50}) were calculated by linear

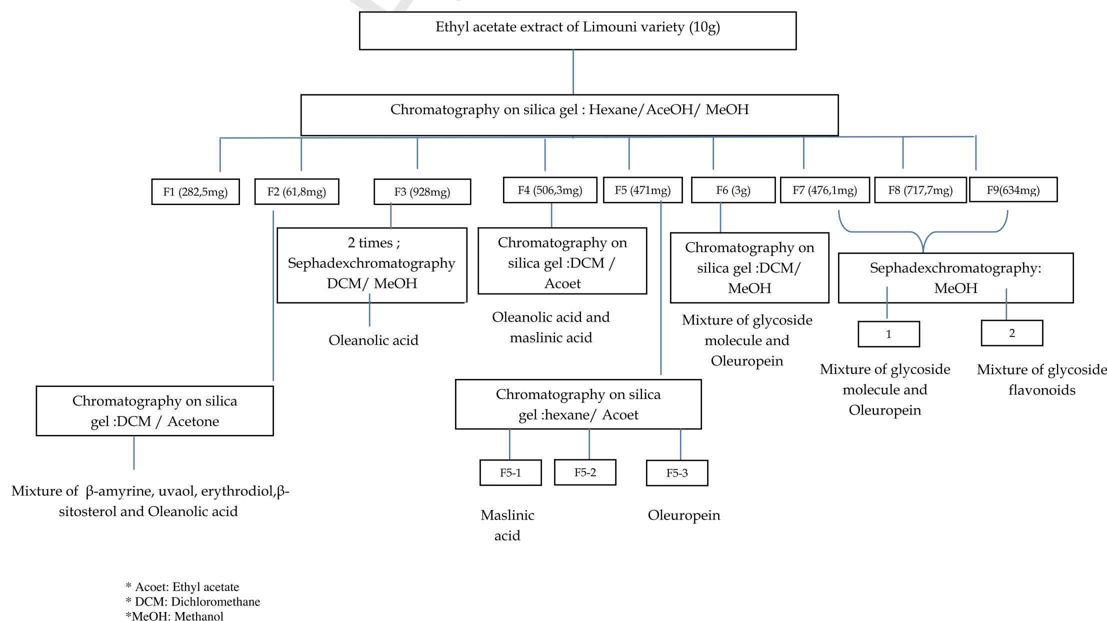

Fig. 1. Bioassay guided isolation of anti-*Acanthamoeba* compounds olive leaf extracts.

Please cite this article in press as: Sifaoui, I., et al. Bioassay guided isolation and identification of anti-*Acanthamoeba* compounds from Tunisian olive leaf extracts. Exp. Parasitol. (2014), http://dx.doi.org/10.1016/j.exppara.2014.02.018

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