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Original Article

Isolation of natural products with anti-ageing activity from the fruits of *Platanus orientalis*

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ABSTRACT

Background: Ageing is defined as the time-dependent decline of functional capacity and stress resistance resulting in increased morbidity and mortality.
Hypothesis/Purpose: Reportedly, these effects can be delayed by mild genetic or pharmacological activation of the main modules of the proteostasis network.
Study Design-Methods: By employing advanced phytochemical methods we isolated natural products from the fruits of *Platanus orientalis* and studied (via a bio-guided approach) their effects in *Drosophila* flies, as well as in normal human fibroblasts.
Results: We report herein that dietary administration in *Drosophila* flies of a phenolics-enriched methanol extract from the fruits of *Platanus orientalis* exerted antioxidant effects; activated proteostatic mechanisms and mildly extended flies' longevity. We then isolated the two major compounds of the extract, namely Platanoside and Tiliroside and found that enrichment of the total extract with these compounds decreased oxidative stress and (in the case of the Tiliroside enriched extract) activated proteostatic mechanisms. Administration of purified Tiliroside in flies activated proteostatic genes, enhanced proteasome and lysosomal-cathepsin activities and decreased tissues' oxidative load; moreover, it delayed the rate of age-related decrease in flies' locomotion activity and increased flies' longevity. Notably, Tiliroside also activated proteasome in normal human fibroblasts and delayed progression of cellular senescence indicating that it may also impact on human cells rate of senescence.
Conclusion: Our presented findings highlight the potential anti-ageing activity of naturals products derived from the fruits of *P. orientalis*.

Introduction

Ageing is a multifactorial process being characterized by (among others) accumulation of cellular damage that is promoted by both genetic and environmental factors (Argyropoulou et al., 2013). Cellular damage occurs in spite of numerous pathways of maintenance and repair and it also (among others) causes proteotoxic stress that appears to represent a major hallmark of either cellular senescence or *in vivo* ageing (Trougakos et al., 2013). This is not surprising, especially after the emerging realization that most (if not all) of the critical cellular functions depend on the functionality of highly sophisticated protein machines, that makeup a modular, yet integrated system; the functionality of which, is ensured by proteome quality control or as it is otherwise called the proteostasis (proteome homeodynamics) network (PN) (Labbadia and Morimoto, 2015; Trougakos et al., 2013).

Central to the PN functionality and proteome damage responses are the molecular chaperones; the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signalling pathway that (among others) mobilizes anti-oxidant

Abbreviations: ALS, Autophagy Lysosome System; ARE(s), Antioxidant Response Element(s); ET, Tiliroside enriched fraction; EP, Platanoside enriched fraction; EPME, Enriched in Phenolics Methanol Extract; ESI-HRMS, Electrospray Ionisation-High Resolution Mass Spectrometry; FCPC, Fast Centrifugal Partition Chromatography; HPLC, High Performance Liquid Chromatography; Hsp, Heat shock protein; NMR, Nuclear Magnetic Resonance; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; PN, Proteostasis Network; ROS, Reactive Oxygen Species; TLC, Thin Layer Chromatography; UPS, Ubiquitin Proteasome System

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defense pathways, and the two main proteolytic systems, namely the autophagy lysosome- (ALS) and the ubiquitin proteasome- (UPS) systems (Trougakos et al., 2013). ALS is mostly involved in the degradation of long-lived or aggregated proteins, as well as in the recycling of damaged organelles (Sridhar et al., 2012). On the other hand, UPS is the main site of protein synthesis quality control and it is also involved in the recycling of both normal short-lived proteins and of non-repairable unfolded proteins (Glickman and Ciechanover, 2002; Tsakiri and Trougakos, 2015). Reportedly, the Nrf2 signalling pathway, along with the ALS and UPS functionality decline during ageing (Rahman et al., 2013; Tsakiri et al., 2013a; Tsakiri and Trougakos, 2015) indicating that these pathways are actively involved in the molecular processes that are linked with the appearance and, likely, the progression of the ageing phenotypes. In line with this notion, UPS, ALS or Nrf2 genetic activation has been linked to enhanced cellular defenses and efficient clearance of damaged biomolecules (Lionaki et al., 2013; Sykiotis and Bohmann, 2008; Vilchez et al., 2012); thus it is anticipated that their activation will, likely, induce anti-ageing effects.

Notably, lifespan can also be extended by dietary interventions. More specifically, several studies in model organisms have demonstrated that longevity can be increased by reduction of the nutrients sensing pathways activity, *e.g.* through caloric restriction (CR) (Argyropoulou et al., 2013). In addition, it has been found that dietary natural products (NPs), including extracts or pure compounds, can induce longevity at *in vivo* experimental models by either modulating nutrients sensing pathways or by increasing resistance to stressors. More specifically, it was shown that extracts from rose petals (Jafari et al., 2008), *Rhodiola rosea* (Bayliak and Lushchak, 2011), oregano and cranberry (Zou et al., 2012) can increase lifespan or promote resistance to stress in yeast and *Drosophila melanogaster*. In relation to small molecules or peptides, resveratrol, rapamycin, spermidine or a yeast-derived hexapeptide were found to delay cellular senescence or *in vivo* ageing in various models (Argyropoulou et al., 2013; Baur et al., 2006; Eisenberg et al., 2009; Harrison et al., 2009; Sklirou et al., 2015). Despite these encouraging findings regarding the potential bioactivity of NPs towards the delay of cellular senescence and/or *in vivo* ageing, the greatest part of world's plant, marine or microbial bio- and chemodiversity is uninvestigated; also, in most cases the mechanism of NPs action remains elusive.

In order to identify NPs that can modulate healthspan and/or lifespan by activating (at the whole organism level) modules of the PN, we are exploiting as an *in vivo* experimental screening platform the premier animal genetic system of *Drosophila melanogaster*. *Drosophila* is well suited to this line of investigation due to its powerful genetics and similarities in key metabolic and ageing pathways with mammals. Moreover, the Nrf2 pathway along with all main PN modules are conserved in *Drosophila* and engage in the same regulatory interactions as in vertebrates (Tsakiri and Trougakos, 2015); thus, *Drosophila* is an excellent model for studying the effects of NPs and/or PN modules activation on ageing.

In the presented study we deployed a bio-guided approach to study the potential anti-ageing bioactivity of NPs derived from the fruits of *Platanus orientalis*. Platanus, also known as plane tree, is a unique living member of Platanaceae family that is used in traditional medicine for the treatment of dermatological, gastrointestinal or rheumatic pathologies (Ebn-e Sina, 1988; Tonekaboni, 2007); also, NPs of *P. orientalis* have also been associated with anti-inflammatory, antimicrobial, antiseptic and analgesic properties (Haider et al., 2012; Hajhashemi et al., 2011). Recently, we analyzed the chemical composition of the *P. orientalis* fruits and found that it is characterized by the presence of fatty acids, terpenoids, coumarins and especially flavonoids and flavonoid glycosides (Thai et al., 2016). We report herein that NPs isolated from the fruits of *P. orientalis* exert antioxidant effects, activate proteostatic mechanisms and delay flies' ageing or human cells senescence.

Materials and methods

Reagents and instrumentation for phytochemical analyses

The reagents and instrumentation used for NPs isolation and characterization throughout this study are described in details in Supplementary Materials and Methods.

Extraction, characterization and isolation of P. orientalis fruits compounds

The fruits of *P. orientalis* were collected in Euboea Island in March 2012 and were authenticated by Dr. Eleftherios Kalpoutzakis at the Division of Pharmacognosy and Natural Products Chemistry of the University of Athens, where a voucher specimen is conserved (specimen No. 203 ATPH). An amount of 9691.4 g of the dried and pulverized plant material was sequentially extracted with dichloromethane (DCM) (65 l, 48H × 3), MeOH (50 l, 48H × 3), and H_2O (40 l, 48H × 2) and the derived solvent extracts were evaporated *in vacuo* to dry. Therefore 350.1 g (3.6%) of DCM extract, 600.09 (6.2%) of MeOH extract and 337.4 g (3.4%) of water extract were afforded. From the 600.09 g of the MeOH crude extract obtained, a pre-treatment of 589.2 g was performed using an Amberlite XAD-7 column in order to obtain the phenolics-enriched extract (163.18 g) (Fig. 1). The enriched extract was profiled using Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC)-DAD and found that the major compounds were Platanoside and Tiliroside, both belonging to coumaroyl-glucosides of kaempherol (Mitrocotsa et al., 1999); this preparation was then forwarded for biological evaluation.

In order to isolate the major constituents of the enriched extract, Fast Centrifugal Partition Chromatography (FCPC) technology was employed using a previously reported method. More specifically, a two-phase solvent system composed of cyclohexane - ethyl acetate - methanol - water: (1:2:0.8:0.9, v/v/v/v) was used for the separation and *K* values were calculated using both TLC and HPLC-DAD (Thai et al., 2016). The *K* values were found to be 2.6 and 0.3 for Platanoside and Tiliroside, respectively. Dual-mode was used and was as follows: the column was filled with the stationary phase (upper phase) at a flow rate of 15 ml/min; then the enriched extract (10 g) was loaded, and the mobile phase (lower phase) was pumped into the column at a flow rate of 10 ml/min from tail-to-head (descending mode). The apparatus was rotated clockwise at 650 rpm; and 30 ml fractions were collected immediately after the injection of the extract every 3 min. This process allowed the isolation of 644.2 mg of a fraction enriched in Tiliroside. After 300 min the mobile phase was changed to upper phase and pumped in at the flow rate of 10 ml/min from head-to-tail (ascending mode) leading to the isolation of 239.6 mg of a fraction enriched in Platanoside. Both fractions enriched in either Tiliroside or Platanoside were forwarded for biological evaluation following a bio-guided isolation concept (see below).

Pure Tiliroside (97% HPLC *vs*. pure Platanoside 95% HPLC) was obtained after recrystallization of its enriched fraction using hot water and its structure was elucidated by direct interpretation of its spectrum data using ESI-HRMS in negative mode, as well as 1D and 2D NMR (1H-1H COSY, 1H-1H NOESY, HSQC-DEPT 135, and HMBC) and literature comparison.

Drosophila lines

Flies stocks were maintained at 23 °C, 60% relative humidity on a 12-h light:12-h dark cycle and were fed standard medium with the addition of either the NPs solvent (control) or the studied NPs at the indicated concentrations (Trougakos and Margaritis, 1998). The *D. melanogaster* flies used in this study were the Oregon R strain, the ARE (Antioxidant Response Elements)–green fluorescent protein (GFP) transgenic reporter line of glutathione S-transferase Delta (gstD)-ARE:GFP (ARE-containing the gstD1 gene enhancer) and the gstD-