Journal of Functional Foods 22 (2016) 270–277

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Phenolic compounds and *in vitro* immunomodulatory properties of three Andalusian olive leaf extracts

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ARTICLE INFO

Article history:
Received 2 December 2015
Received in revised form 15 January 2016
Accepted 18 January 2016
Available online

Keywords:
Olive leaf extracts
Phenolic compounds
HPLC-DAD-TOF-MS
In vitro assays

ABSTRACT

Phenolic compounds in extracts of three olive leaf cultivars were determined using HPLC-DAD-TOF-MS. The determination of the immunomodulatory properties of the whole phenolic extracts was then carried out on RAW 264 mouse macrophages as a preliminary *in vitro* study. Twenty-eight phenolic compounds were determined in the olive leaf extracts and high contents of total phenolics were shown, particularly for 'Picual' cultivar. In addition, all olive leaf extracts inhibited the release of the pro-inflammatory mediator nitric oxide in LPS-stimulated RAW264.7 cells revealing their immunomodulatory properties. As a preliminary result, it could be deduced that the inhibition of NO by the olive leaf extract may depend on the type of phenolic compounds rather than on the total phenolic contents. This is the first time that whole phenolic extracts of olive leaves have been used in *in vitro* study of their anti-inflammatory properties.

Contents

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http://dx.doi.org/10.1016/j.jff.2016.01.037
1756-4646/

1. Introduction

Olive leaves (*Olea europaea*) have been used as folk medicine throughout the history of civilization in the Mediterranean area. However, they became important when olive leaf extract was reported to be effective in treating fever and malaria in 1854 (Altinyay & Altun, 2006). Current scientific research has shown that olive leaves contain phenolic compounds responsible for several biological activities, including antioxidant and anti-inflammatory, antimicrobial, antiviral, anti-carcinogenic, as well as beneficial cardiovascular effects (Lee & Lee, 2010; Lee-Huang, Zhang, Huang, Chang, & Huang, 2003; Poudyal, Campbell, & Brown, 2010; Taamalli et al., 2012). The majority of studies attribute biological activities of olive leaf extracts to total phenols or individual phenolic compounds such as secoiridoids and, particularly, oleuropein (Al-Azzawie & Alhamdani, 2006) and hydroxytyrosol (Bouallagui, Han, Isoda, & Sayadi, 2011), and to flavonoids such as diosmetin, quercetin, luteolin, apigenin and their derivatives (Goulas, Papoti, Exarchou, Tsimidou, & Gerothanassis, 2010; Poór et al., 2014).

The major classes of phenolic compounds in olive leaf extract are phenolic acids, phenolic alcohols, flavonoids and secoiridoids, and include mainly vanillic acid, caffeic acid, hydroxytyrosol, tyrosol, rutin, verbascoside, luteolin, quercetin, oleuropein, demethyloleuropein and ligstroside (Talhaoui, Taamalli, Gómez-Caravaca, Fernández-Gutiérrez, & Segura-Carretero, 2015c).

When considering the potential effects of these active compounds as anti-inflammatory agents, it is interesting to note that nitric oxide (NO) has been proposed to play a key role in the pathogenesis of the inflammatory response. NO is a free radical messenger molecule with both intra- and extracellular regulatory functions for many cells. Endogenous NO is generated from L-arginine by oxidation of terminal nitrogen in the guanidine group in reaction catalysed by the enzyme nitric oxide synthase (NOS) (Calcerrada, Peluffo, & Radi, 2011). Different functional forms of NOS can be recognized: constitutive and inducible forms. NO synthesis by the constitutive isoforms, endothelial (eNOS) and neuronal (nNOS), generate low levels of NO under normal physiological conditions (Calcerrada et al., 2011). In the gastrointestinal tract (GIT), constitutive isoforms found in the endothelial cells (eNOS) and certain nerve terminals (nNOS) innervating the colon regulate blood flow and bowel motility by promoting muscle relaxation of the vessels and the bowel, respectively (Kolios, Valatas, & Ward, 2004). The inducible isoform, iNOS, is highly expressed in cells involved in the inflammatory response like macrophages and neutrophils, as well as in endothelial and smooth muscle cells, upon different stimuli, like endotoxine and/or cytokines, whose production is increased in the inflammatory environment (Palatka et al., 2005). After its induction, iNOS generates high, sustained levels of NO that may be toxic to the healthy tissue. Thus, tissue injury may result from the interaction of NO with superoxide anion, one of the reactive oxygen species (ROS), resulting in a formation of peroxynitrite that further contributes to tissue damage and up-regulation of the inflammatory response (Kolios et al., 2004). All the above support the important role ascribed to NO in chronic inflammatory conditions, including Crohn's disease (CD) and ulcerative colitis (UC), the major forms of inflammatory bowel diseases (IBD) (Roediger, Lawson, Nance, & Radcliffe, 1986). Therefore, those compounds or product able to down regulate an exacerbated NO production could represent an important therapeutic tool in the management of inflammatory conditions, like IBD.

Thus, the aim of this study was, firstly, to determine the phenolic compounds in olive leaf extracts from three different olive cultivars grown in the same experimental orchard under the same agronomic and environmental conditions and collected in December, 2014, and, secondly, to evaluate for the first time the anti-inflammatory properties of the whole phenolic extracts of olive leaves by determining the inhibitory effect towards NO production in LPS-stimulated RAW 264.7 cells of the three olive leaf cultivars and compare the results among them. December is the period in which olive leaves of the cultivars studied had the highest phenolic contents during the year (Talhaoui et al., 2015b).

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical reagent grade and used as received. HPLC-grade acetonitrile and acetic acid were purchased from Labscan (Dublin, Ireland) and Fluka (Switzerland), respectively. Standard compounds such as hydroxytyrosol, tyrosol, luteolin, and apigenin and all chemicals for MTT test and Griess assay were obtained from Sigma-Aldrich (St. Louis, MO, USA). Oleuropein was from Extrasynthèse (Genay, France). Distilled water with a resistance of 18.2 MΩ was deionized in a Milli-Q system (Millipore, Bedford, MA, USA). The stock solutions containing these analytes were prepared in methanol (Panreac, Barcelona, Spain). All the solutions were stored in a dark flask at –20 °C until use within three days.

2.2. Extraction of phenolic compounds from olive leaf extracts

Samples of olive leaves (*O. europaea* L.) were from 'Arbequina', 'Picual' and 'Sikitita' cultivars, cultivated in the same orchards in IFAPA of Cordoba (Spain) under the same agronomic