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Original article

Assessment of *Olea europaea* L. fruit extracts: Phytochemical characterization and anticancer pathway investigation

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ABSTRACT

Olea europaea L. has been widely used as an advantageous rich source of bioactive compounds of high economic value leading to its use in pharmaceutical, cosmetic, and agriculture industries. Ethanolic extracts of olive fruits from three different cultivars (OFE) were studied for their phytochemical contents and were investigated for antioxidant activities and anticancer potential. Major polyphenols detected in these extracts were tyrosol, hydroxytyrosol, oleuropein, rutin, quercetin and glucoside forms of luteolin and apigenin. All these compounds have shown to significantly contribute to the antioxidant activity of OFE, which was evaluated by DPPH and ABTS assays. Proliferation of hepatic and colon cancer cells, HepG2 and Caco-2, were shown to be sensitive to OFE with IC_{50} less than 1.6 mg/ml for all tested extracts. Moreover, flow cytometry analysis showed that OFE induced cell cycle arrest in the S-phase within both HepG2 and Caco-2 cells. This has triggered a cell death mechanism as shown by DNA fragmentation, expression of p53 and phosphorylation level of Akt and Erk proteins. Interestingly, these extracts could be further used as a potential source of natural compounds with both antioxidant and anticancer effects.

1. Introduction

The olive tree is known as one of the oldest plants cultivated in the Mediterranean countries where more than 95% of the worldwide olive production is concentrated [1,2]. Indeed, The beneficial impacts of olive products on human health have been the subject of increasing scientific interest [3]. These products, in particular, olive oil, are rich sources of promising nutritional and bioactive molecules, such as phenolic compounds. Thanks to their potent antioxidant activity, polyphenols exhibit several biological activities, including cardioprotective and anti-inflammatory properties [4]. Moreover, these compounds are believed to offer a protection against the development of some diseases, including different forms of cancer. Indeed, a positive relationship between the consumption of olive fruits and the reduction of cancer proliferation has been previously reported [5]. Nonetheless, studies dealing with the effect of olive fruits in both colon and hepatic carcinoma cells have been scarcely investigated.

Unfortunately, mortality rates related to cancer incidence are interestingly increasing in the world. Hepatocellular carcinoma, the most common primary liver cancer, is the fifth most frequent cancer globally and the third-leading cause of cancer death. In this context, HepG2 cells have been often used as a good model to study the *in vitro* toxicity to the liver, since they characterized the normal human hepatocytes [6]. On the other hand, colon adenocarcinoma is the most common histopamthological type of colorectal cancer [7]. Such type of cancer, causing about 700,000 deaths per year is the fourth leading cause of cancer death [8].

Previous studies reported the effectiveness of some dietary components such as isoflavones and polyphenols as inhibitors of cancer cell growth by modulating cell signaling pathways. Interestingly, these components activate cell death signals and induce apoptosis in cancer cells, resulting in the prevention of cancer development [9,10]. On this line, MAPKs (mitogen-activated protein kinases) have received increasing attention in cancer prevention and therapy. In addition, Akt (Protein kinase B), known as a serine/threonine-specific protein kinase, has been also considered as an attractive target for cancer treatment [11] since several components of the PI3K–Akt pathway were dysregulated in a wide spectrum of human cancers [12]. Moreover, the tumor suppressor and transcription factor p53 was considered as a critical regulator of many cellular processes including cellular response to DNA-damage, cell cycle control, and apoptosis [10]. It has been demonstrated that functional p53

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activated the transcription of some genes such as p21 and Bax to induce the apoptotic process, thus inhibiting the growth of cancer cells [13,14].

In this study, we have focused on the investigation of the phytochemical composition and the antioxidant potential of olive fruit extracts from three different cultivars, namely Jerboui (JFE), Marsaline (MFE) and Ouesleti (OuFE). Based on the phytochemical characterization, only one extract was selected for further study dealing with the evaluation of its antiproliferative and apoptotic effects against liver and colon cancer cells (HepG2 and Caco-2).

2. Material and methods

2.1. Material

Dulbecco's modified Eagle medium (DMEM) and Fetal bovine serum (FBS) were purchased from Gibco (Life Technologies, UK). Penicillin (10.000 IU/ml)-Streptomycin (10.000 IU/ml) solution was purchased from eurobio (France). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT), trypan blue, Dimethylsulfoxide (DMSO), 1,1-phenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), catechin, gallic acid, Follin Cicalteu, acetonitrile and hexane were obtained from Sigma-Aldrich (France).

2.2. Olive fruit extracts preparation

Olive fruits from three different olive cultivars, namely Jerboui, Ouesleti and Marsaline, were studied. Olive trees were grown in Tunisia and fruit samples with a maturation index of 2 were collected. Fruit samples from five different trees were harvested for each cultivar. First, fresh fruits (200 g) were manually milled to obtain a homogeneous paste which was subsequently extracted twice with 350 ml of ethanol (70%) under agitation (200 rpm in an orbital shaker) for overnight at room temperature. The extract was then concentrated and washed with hexane to remove the lipid fraction. Finally, the resulting olive fruit ethanolic extract (OFE) was freeze-dried and stored for further analyses. The extraction yield was in the order of 10%.

2.3. Total phenols content

Total phenols were measured using the method described by Slinkard and Singleton [15], with some modifications. Appropriately diluted extract (25 μl) or standard (gallic acid in the range of 0–400 μg/ml) were mixed with 25 μl Folin–Ciocalteu reagent and incubated for 6 min. Later, 100 μl of Na_2CO_3 (75 g/l) were added to the mixture which was allowed to stand for 90 min at room temperature before recording the absorbance at 765 nm. Total phenols content was expressed as mg gallic acid equivalent GAE per gram of OFE.

2.4. Total flavonoïds content

Total flavonoïds content was measured as previously described by Meda et al. [16] with slight modifications. In a 96-well microplate, 25 μl of sample or standard solution and 10 μl of $NaNO_2$ (50 g/l) were mixed and incubated for 5 min. Thereafter, 15 μl of $AlCl_3$ (10%) were added to the reaction mixture and further incubated for 6 min. Finally, 50 μl of NaOH (1 M) and 50 μl distilled water were added and the absorbance was measured at 510 nm. A blank sample and a reference curve using catechin (5–160 μg/ml) were prepared under same conditions. Total flavonoïds in OFE were expressed as mg of catechin equivalent (CE) per gram of extract.

2.5. High-performance liquid chromatography analysis

Chromatographic analyses were performed according to Souilem et al. [17]. The instrument consists of an Agilent series 1260 HPLC-DAD (Agilent. Waldbronn. Germany). Compounds separation was carried out on a ZORBAX Eclipse XDB-C18 column (4.6 mm I.D. × 250 mm × 3.5 μm particle size). The mobile phase was made of phase A (0.1% acetic acid in water) and phase B (100% acetonitrile). The elution conditions were: flow rate set at 0.5 ml/min, injection volume of 10 μl and operating temperature of 40 °C. The running gradient was as follows: 0–22 min, 10–50% B; 22–32 min, 50–100% B; 32–40 min, 100% B; 40–44 min, 100-10% B. Re-equilibration duration lasted 6 min. The DAD detector scanned from 190 to 400 nm and detection was achieved at 254, 280 and 330 nm. Compounds were identified according to their UV, retention times and mass spectra recorded using an ion trap mass detector MSD trap XCT.

2.6. LC–MS/MS analysis

Experiments were performed with an Agilent 1100 LC system according to Bouallagui et al. [18]. Briefly, the chromatographic separation was carried out using a Zorbax 300 Å Extend-C-18 Column (2.1 × 150 mm). The column outlet was coupled to an Agilent MSD Ion Trap XCT mass spectrometer supplied with an ESI ion source. Data acquisition and mass spectrometric evaluation were accomplished on a personal computer with data Analysis software (Chemstation).

2.7. Antioxidant activity assay

The antioxidant activity of OFE was determined using DPPH and ABTS methods. The DPPH radical scavenging activity was determined based on the method previously described by Chang et al. [19] with slight modifications. Briefly, In a 96 well plate, 25 μl of OFE solution were mixed with 200 μl of DPPH ethanolic solution (150 μM). The reaction was incubated for 1 h at room temperature in the dark and the absorbance was recorded at 517 nm. For the ABTS assay, we applied the method previously reported by Bouaziz et al. [20]. In both methods, a reference curve was prepared with Trolox (25–800 μM) under same conditions. The antioxidant activity was expressed as Trolox equivalent (TE) per gram of extract.

2.8. Cell lines and culture conditions

Two continuous human carcinoma cell lines, HepG2 and Caco-2, were used for the anti-proliferative effect of OFE. HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS and 1% PS. This culture medium was supplemented with 1% non-essential amino acid (NEAA) when used to grow Caco-2 cells. Cultures were maintained at a 5% CO_2 atmosphere.

2.9. Viability assessment

Cell proliferation was assessed using the MTT assay following treatment of cells (3×10^4 live cells/ml) with different concentrations of OFE (0–2000 μg/ml) for different incubation times (24, 48 or 72 h). Cell viability was evaluated spectrophotometrically as previously reported by Bouallagui et al. [18].

2.10. Flow cytometry analysis

Cell cycle distribution and multicaspases expression were determined using a Guava PCA instrument. Staining of cells was performed using a cell cycle and a multicaspases kits from Mersk-