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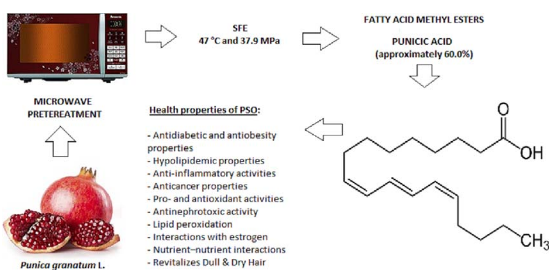
Antioxidant and cytotoxic activity of fatty oil isolated by supercritical fluid extraction from microwave pretreated seeds of wild growing *Punica granatum* L.

Sanja Đurđević[a,*], Katarina Šavikin[b], Jelena Živković[b], Volker Böhm[c], Tatjana Stanojković[d], Ana Damjanović[d], Slobodan Petrović[a]

[a] *University of Belgrade, Faculty of Technology and Metallurgy, Karnegijeva 4, 11000 Belgrade, Serbia*
[b] *Institute for Medicinal Plants Research "Dr Josif Pančić", Tadeuša Koćuška 1, 11000 Belgrade, Serbia*
[c] *Institute for Genetics, University of Cologne, Zuelpicher Straße 47a, 50674 Cologne, Germany*
[d] *Institute of Oncology and Radiology of Serbia, Pasterova 14, 11000 Belgrade, Serbia*

GRAPHICAL ABSTRACT



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ABSTRACT

Pomegranate seeds are an excellent source of compounds with biological activity. In the present study, the pomegranate seed oil (PSO) was tested for antioxidant and cytotoxic potential. PSO was extracted by supercritical fluid extraction with previous treatment of seeds by microwaves and analysed for its fatty acid profile, carotenoid and tocopherol contents. PSO demonstrated to be a good source of polyunsaturated fatty acids (PUFAs) (83.5%), followed by monounsaturated fatty acid (MUFA) and saturated fatty acid (SFA) (9.5% and 7.0%, respectively). Concerning fatty acid profile, punicic (C18:3), oleic (C18:1) and linoleic (C18:2) acids were the predominant ones. Carotenoids and tocopherols were identified and quantified by HPLC. Results showed that the PSO was a good source of γ- tocopherol as dominant among investigated compounds (128.6 mg/100 g). The extract was screened for its antioxidant activity using α-TEAC assay. Cytotoxic effect was determined by MTT test against malignant HeLa, LS174 and A549 cells (IC50 = 49.51–91.54 μg/mL), and against normal MRC-5

Abbreviations: ABTS, 2,2-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid); α-TEAC, α-tocopherol equivalent antioxidant capacity assay; HeLa, Caski, SiHa, Hep2c, cervix adenocarcinoma cell; LS174, HT-29, colon carcinoma cell; CLnA, conjugated α-linolenic acid; ELISA, enzyme-linked immunosorbent assay; FAME, fatty acid methyl esters; FBS, fetal bovine serum; GC/FID, gas chromatography/flame ionization detector; GC/MS, gas chromatography/mass spectrometry; HPLC, high performance liquid chromatography; A549, H1299, MRC-5, lung carcinoma cell; MTBE, methyl *tert*-butyl ether; MTT, microculture tetrazolium test; MUFA, monounsaturated fatty acids; PME, pomegranate fruit extract; PSO, pomegranate seed oil; PC-3, prostate cancer cells; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SDS, sodium dodecyl sulfate; scCO2, supercritical carbon dioxide; SFE, supercritical fluid extraction; EA.hy926, umbilical vein endothelial cell

* Corresponding author.
E-mail address: sanyadjurdjevic@gmail.com (S. Đurđević).

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and EA.hy926 cells (IC50 > 200 μg/mL). The best effect was exhibited on cervix adenocarcinoma cell line while no cytotoxicity was found for normal cells pointing out PSO safety.

1. Introduction

Pomegranate seeds (*Punica granatum* L.) are often referred as a notable industrial waste. For that reason, finding solutions for using this residue would constitute an excellent opportunity. Extraction of oil from pomegranate seeds is a good option as numerous health benefits were associated with its ingredients, mainly vitamin E and polyunsaturated fatty acids. Vitamin E has neuroprotective properties, it is able to lower the cholesterol levels and has antioxidant activity [1], while the fatty acids are referred in the literature as protectors of cardiovascular system [2].

The pomegranate seed oil (PSO) consists of approximately 80% conjugated octadecatrienoic fatty acids, with a high content of *cis* 9, *trans* 11, *cis* 13 acid *i.e.* punicic acid [3]. Punicic acid, also known as trichosanic acid, is an omega-5 long chain polyunsaturated fatty acid and an isomer of conjugated α-linolenic acid (CLnA) with structural similarities to conjugated linoleic acid (CLA) and α-linolenic acid (LnA) [4]. Researchers had demostrated that conjugated fatty acids have several potential health benefits [5] including their antioxidant, immunomodulatory, anti-atherosclerotic and serum lipid-lowering activities [6]. Also, CLA isomers may prevent diseases such as cancer [7,8].

Technologies that are used today for the production of fatty oils are well established, but they use methods that are energy demanding and solvents produced from fossil fuels that often need to be controlled with respects to residual levels [9]. The aim of green extraction processes application is process intensification through faster extraction rate and more efficient energy use [10]. In our study we have performed supercritical fluid extraction with microwave pretreatment of seeds as two green extraction technologies in order to increase oil yield.

Périno-Issartier et al. [11] pointed out that influence of microwave energy on chemical or biochemical reactions, as well as, on extraction is strictly thermal. The quantum energy of microwave photons is in the range 0.00001–0.001 eV and these energies are much lower than the usual ionization energies of biological compounds (13.6 eV), of covalent bond energies like OH (5 eV), hydrogen bonds (2 eV), Van der Waals intermolecular interactions (lower than 2 eV) and even lower than the energy associated to Brownian motion at 37 °C ($2.7\ 10^{-3}$ eV). From this scientific point of view, direct molecular activation of microwaves should be excluded. Some kind of stepwise accumulation of the energy, giving rise to a high-activated state should be totally excluded due to fast relaxation [12]. Peterson [13] wrote in many of his articles: 'The question and the debate of the non-thermal effect of microwave give a lot of damage for the reputation of this technology and its application in industry'. Microwaves are only absorbed by dipoles, transforming their energy into heat.

Supercritical carbon dioxide is considered as a safe solvent having in mind principles of the green chemistry. It can generally penetrate faster into solid sample compared to liquid solvent due to its high diffusion rate. Also because of its low viscosity, it can rapidly transport dissolved solvents from the sample matrix [9]. One of the main advantages of the application of microwave pretreatment of raw material is shortening of the extraction time. According to Yedhu Krisnan and Rajan [14] microwave radiation facilitates the disruption of plant material and intensifies the leakage of active compounds.

Due to several disadvantages associated with conventional extraction techniques, use of ultrasound assisted extraction increased in recent decades. UAE is an environment-friendly extraction technology and it is possible to obtained several classes of food components such as antioxidants, proteins, pigments and also, essential oils. The major advantages of UAE is enhancing extraction yields. It is possible to apply ultrasound extraction to enhance extraction rates with or without using solvents. Also, application of ultrasound provided opportunity to use alternative Generally Recognized as Safe (GRAS) solvents, which may provide environmental and health and safety benefits.

In some cases, solvents that have poor extractability using conventional methods, ultrasound can increased the extraction efficiency of that solvent. For example, Albu et al. [15] improved extractability of antioxidants from *Rosmarinus officinalis* using ethanol, allowing scope for solvent choice, leading to a more economic process and a step towards green extraction [16].

Studies found that pomegranate seed oil contain significant levels of tocopherols [17]. The best known form of vitamin E is α-tocopherol, found in the largest quantities in blood and tissue. It is critical, however, for anyone supplementing with vitamin E to make sure they are also getting adequate γ-tocopherol each day. Gamma-tocopherol has the ability to reduce inflammatory threats, which are major cause of all degenerative diseases. Also, research supports the significance of γ-tocopherol in preventing degenerative diseases associated with aging [18,19].

The object of this work was the waste of pomegranate processing industries, *i.e.* pomegranate seeds. The aim was to obtain seed oil using supercritical fluid extraction with pretreatment of seeds by microwaves in optimized conditions described in our previous work [20] and to analyse chemical composition as well as antioxidant and cytotoxic activity of the obtained pomegranate seed oil.

2. Materials and methods

2.1. Plant material

Wild growing fruits of *Punica granatum* L. were collected from the private property in Bosnia and Herzegovina in the village Do, during 2015, at the full ripeness stage. Pomegranate seeds were separated from their juice sacs, washed several times with distilled water and air-dried at ambient temperature (4–6 days). Afterwards, the dried seeds were ground with a high-speed mill (MMB 1000/05, Bosch). Ground seeds were further fractionated by a series of sieves (0.2, 0.5, and 1 mm) to obtain a uniform particle size distribution. Particles with size distribution of 0.2 mm were used for the extraction. Moisture content was determined by drying of the seed samples (8 g) at 105 ± 0.5 °C until constant mass. Determined moisture value was 2.58 wt.% for ground pomegranate seeds, respectively. Prior to extraction, ground pomegranate seeds were placed in Petri dishes inside the microwave oven (NN-GD 469 M, Panasonic) and were treated at a frequency of 2450 MHz at 250 W for 6 min [20].

2.2. Chemicals and reagents

All solvent/chemicals used were of analytical grade and obtained from Carlo Erba Reagents (France) and Messer-Tehnogas (Serbia). HPLC grade water (18 MΩ) was prepared using a Millipore Milli-Q purification system (Millipore GmbH, Schwalbach, Germany). MTBE (methyl *tert*-butyl ether) was bought from Sauerbrey (Reinhardshagen, Germany). All tocopherols were purchased from Calbiochem (Darmstadt, Germany) and all tocotrienols from Davos Life Science (Singapore). Carotenoids (97–99% analytical grade) were purchased from CaroNature (Ostermundigen, Switzerland). 2,2-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) was obtained from Sigma–Aldrich (Taufkirchen, Germany).

All cell lines were obtained from the American Type Culture