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Solid state fermentation of pomegranate husk: Recovery of ellagic acid by SEC and identification of ellagitannins by HPLC/ESI/MS

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ABSTRACT

2, 3, 7, 8-Tetrahydroxy-chromeno [5, 4, 3-cde] chromene-5, 10-dione, commonly named ellagic acid was successfully separated by size-exclusion chromatography (SEC) from pomegranate husk ellagitannins purified by solid-state fermentation. Recovery of metabolites from fermented mash was carried out by the addition of ethanol and manual pressing. Culture extracts were loaded on a Sephadex LH-20 column, three fractions were separated, and second fraction was identified and evaluated by high performance liquid chromatography followed by electro spray ionization and mass spectrometry (HPLC/ESI/MS). It was possible to obtain a yield of 47 mg/g of pomegranate husk polyphenols. The analysis of HPLC/ESI/MS allowed to identify punicalagin (1082.97 m/z), punicalin (780.99 m/z) and ellagic acid (300.80 m/z). After the fermentation process, the main compound was ellagic acid. The present study describes a rapid and effective fermentation/chromatographic separation process for ellagic acid production.

1. Introduction

Polyphenols are secondary metabolites distributed in the kingdom Plantae, as part of the bark, leaves, stems, flowers and fruits. Currently polyphenols according to their structural characteristics are classified into: flavonoids, condensed tannins, phlorotannins, hydroxystilbenes and hydrolyzable tannins (Quideau, Deffieux, Douat-Casassus, & Pouységu, 2011). Within the latter group are the ellagitannins. Ellagitannins are esters of hexahydroxydiphenic acid (HHDP) usually linked to glucose, when the ester link is hydrolyzed by enzymes or acid-basic methods, the HHDP group is released and undergoes molecular rearrangement to form ellagic acid (EA) (Ascacio-Valdés et al., 2014).

EA is a dilactone and potent bioactive compound presented in berries, pomegranate, grapes, and walnut; also consumed frequently along with fruit juices, jams and other beverages (Landete, 2011). As EA is having great benefits to human health, it highly important to produce and purify this secondary metabolite. For example, the EA has antiepileptic activity in mice, it's probably through the increase of GABAerig transmission in brain (Dhingra & Jangra, 2014). The inhibition of aldo reductase activity by EA present in many dietary sources reported was evaluated and concluded that the EA holds a therapeutic promise to prevent or treat complications of diabetes (Akileshwari et al., 2014). Recently a study of ellagic acid was encapsulated in chitosan particles prepared by ionotropic gelation and characterized for its physicochemical properties. The results suggest that the encapsulation of ellagic acid favors thrombosis due to synergistic action of chitosan and ellagic acid on same molecular targets and highlighted the potential of ellagic acid–chitosan as an effective system for anti-hemorrhagic activity (Gopalakrishnan, Narashimhan-Ramana, Sethuraman, & Maheswari-Krishnan, 2014). It has been reported that EA has a renal protective effect in diabetic induced rats (Ahad, Ganai, Mujeeb, & Siddiqui, 2014). The hydroalcoholic flower extract of *D. volubilis* with a presence of EA (208.8 μg/g) was evaluated for antidiabetic activity in vitro. The results showed a therapeutic potential of this plant to its application in postprandial hyperglycemia control (Das, De, Das, Das, & Samanta, 2017). EA contained in higher plants has diverse uses in traditional medicine for treatment of tuberculosis and bacterial infections (Fyhrquist, Laakso, Garcia-Marco, Julkunen-Tiitto, & Hiltunen, 2014). Antimicrobial activity of EA obtained from blueberry (*Vaccinium corymbosum* L.) extracts was reported (Shen et al., 2014). Moreover, formulations with pomegranate peel extract (12% EA), added with nanostructure lipid carriers showed antioxidant and anti-tyrosine activity with possible application on cosmetics (Tokton, Ounaroon, Panichayupakaranant, & Tiyaboonchai,

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2014). Further, study the potential artheroprotective effects of urolithins, ellagitannins derived gut microbiota metabolites, on different key factors in artherosclerosis development. Results indicated that EA was able to reduce the adhesion of TPH-1 monocytes to human umbilical vein endothelial cells, the secretion of a cellular adhesion molecule and pro-inflammatory cytokine (Mele et al., 2016). Besides, the ellagitannins and ellagic acid, were evaluated for its anticancer effects against colon cells. The effect against colon cancer cells is through a common CDKN1A upregultory mechanism (González-Sarrías et al., 2015). Due to these biological properties, it is necessary develop new methodology of production and recovery of EA from agro-industrial waste like pomegranate husk.

Most of the EA products in the market are produced by acid hydrolysis and solvent extraction. This is linked to pollution and besides recovery and purification costs are increased. Recently, EA production has been carried out by exploiting agroindustrial wastes using fungal strains and fermentation process. For example, a continuous system for EA production was developed using solid state culture (SSC) with ellagitannase as biocatalyst, which was attached to polyurethane foam (Buenrostro-Figueroa et al., 2014). Low cost agroindustrial wastes such as that generated during tea production was employed for EA production using *Aspergillus niger* MTCC 281 strain under SSC (Paranthaman, Kumaravel, & Singaravidel, 2013). The factors influencing of EA production from pomegranate husk agroindustrial waste by solid state culture (SSC) using *Aspergillus niger* GH1 have been addressed (Sepúlveda et al., 2012). The aim of present work is the separation EA using molecular-size exclusion chromatography from pomegranate husk polyphenols produced by SSC fermentation process and subsequent identification using HPLC/ESI/MS.

2. Materials and methods

2.1. Chemicals

Standard EA was purchased from Sigma-Aldrich® Chemistry, S.L. (Toluca, México); methanol, acetonitrile, acetic acid HPLC grade, Tween 80, KCl and KH_2PO_4 from J. T. Baker® (México, D.F.). Ethanol absolute was obtained CTR scientific (Saltillo, Coahuila, México). $NaNO_3$ and $MgSO_4$ Analityka® (México D.F.). PDA BD-Bioxon (México, D.F.). Sephadex™ from Amersham Biosciences, El Crisol S.A. de C.V. (Monterrey, Nuevo León, México) and Amberlite XAD-16 from Rohm and Haas (México. D.F).

2.2. Microorganism and raw material

A strain of *Aspergillus niger* PSH (collection DIA-UADEC, Saltillo, Coahuila), was used. The fungal strain was maintained at −40 °C in a mixture of glycerol and skimmed milk (30/10 v/v). Spores were reactivated on 30 ml PDA medium at 30 °C for 5 days. The new culture spores were collected with a sterile solution of 0.01% Tween-80 and counted in a Neubauer® chamber. Pomegranate fruit ('Wonderful' variety) was purchased from a supermarket in San Juan de Sabinas, Coahuila, México. Husk was manually removed from the fruit and dehydrated at 60 °C for 48 h. The dried husk was pulverized in a mill (PULVEX® mini 100) and particle size 600 µm were obtained.

2.3. Polyphenols extraction of pomegranate husk and culture media

Pomegranate husk total polyphenols (PHP) were obtained from pomegranate husk powder by extracting with water at 60 °C in the ratio of 1:5 (w/v) for 30 min. This was filtered through Whatman no. 41 filter and PHP were obtained using Amberlite XAD-16, first distilled water was used as the eluent to discard undesirable compounds, such as low and high molecular weight sugars, and then ethanol was employed as the eluent to obtain a total PHP fraction (Ascacio-Valdés et al., 2014). Solvent was evaporated from the fractions and PHP was recovered as a fine powder. Czapeck-Dox medium for SSC was used, which had the following composition (g/L): $NaNO_3$ 7.65, KCl 6.08, KH_2PO_4 3.04 and $MgSO_4$ 3.04. Final pH of the culture media was adjusted to 6.0. Culture medium was sterilized at 121 °C for 15 min. PHP were added at a final concentration of 7.5 g/L as sole carbon source.

Table 1
Extraction yield of PHP.

Yield (g/kg pomegranate husk)	Reference
58	Seeram, Lee, Hardy, and Heber (2005)
53	Aguilera-Carbó (2009)
60	Ascacio-Valdés (2012)
70	Buenrostro-Figueroa (2013)
47	Present study

2.4. Preparation to SSC

EA production was performed by SSC. Polyurethane foam was used as support in tray reactor (40 × 30 × 6 cm) and washed three times hot waters for 10 min (Mussatto, Aguilar, Rodrigues, & Teixeira, 2009). Polyurethane foam was impregnated with culture medium for initial moisture of 70%. Inoculum (2×10^7 spores/g) was added to the culture medium. Support was homogenized and spread, with a height of 1.5 cm around the tray reactor. SSC was carried out at 30 °C for 18 h (time of maximal EA production) (De la Cruz et al., 2015). After 18 h, ethanol was added and the biomass was compressed manually. Culture broth was placed in recipient and stored (4 °C) until further analysis.

2.5. Separation by Size-exclusion Chromatography (SEC)

Culture broth was used for EA purification. The broth was air-dried in an oven at 80 °C for 12 h to obtain culture powder. Culture powder solution was prepared (300 mg/5 ml ethanol-water, 1:1). Culture powder solution was loaded into a Sephadex LH-20 column (Bioscience). Previously, column was washed and equilibrated with MilliQ water for 1 h at flow of 1 ml/min and pressure at 80 psi. The column was coupled to a pump (Varian Prostar 210) and a UV–Vis detector (Sanki UVIS 200) was used at 270 nm. First, the sample was eluted with water to remove hydro soluble compounds. Finally, ethanol was used to elude the fraction rich in EA. The fraction was concentrated in an oven at 80 °C for 6 h. The fraction was dissolved in ethanol for further analysis.

2.6. HPLC/ESI/MS analysis

Recovered EA was analyzed by HPLC (Varian Prostar, Santa Clara, California, USA) using a photodiode array detector at 253 nm. Fractions were filtered (0.45 µm). Separation was performed with a Grace Denali C-18 column (5 µm, 250 × 4.6 mm) at 30 °C. Mobil phase A methanol (wash phase), B acetonitrile and C acid acetic solution (3%). The gradient program was initial 3% B and 97% C, 0–5 min 9% B and 91% C, 5–15 min 16% B and 84% C, 15–30 min 33% B and 67% C, 30–33 min 90% B and 10% C, 33–35 min 90% B and 10% C, 35–42 min 3% B and 97% C. The flow rate was 1.2 ml/min and sample injection volume was10 µL. Fractions were analyzed by Varian 500-MS ion tramp instrument equipped with an electrospray ionization (ESI) interface. The capillary voltage was 90 v. Spectra were recorded in a negative ion made between m/z 100 and m/z 2000.

3. Results and discussion

3.1. PHP extraction

In this work, the PHP was used a sole carbon source and energy for

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