Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Document heading doi:

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Antifungal ellagitannin isolated from *Euphorbia antisyphilitica* Zucc

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PEER REVIEW

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Comments

This is a good study in which the authors isolated, identified and characterized a new ellagitannin. The result is interesting and suggests that candelitannin can be used for the fungal control.
(Details on Page 45)

ABSTRACT

Objective: To study antifungal activity of a new ellagitannin isolated from the plant residues of *Euphorbia antisyphilitica* (*E. antisyphilitica*) Zucc in the wax extraction process. **Methods:** An extract was prepared from dehydrated and pulverized residues and fractionated by liquid chromatography on Amberilte XAD-16, until obtained an ellagitannin-rich ethanolic fraction which was treated by rotaevaporation to recover the ellagitannin as fine powder. An aqueous solution was prepared and treated through ionic exchange liquid chromatography (Q XL) and gel permeation chromatography (G 25). The ellagitannin-rich fraction was thermogravimetrically evaluated (TGA and DTA) to test the thermo-stability of ellagic acid (monomeric unit). Then ellagitannin powder was analyzed by infrared spectrospcopy to determinate the functional groups and, also mass spectroscopy was used to determine the molecular ion. **Results:** The principal functional groups of ellagitannin were determined, the molecular weight was 860.7 g/mol; and an effective antifungal activity against phytopathogenic fungi was demonstrated. **Conclusions:** It can be concluded that the new ellagitannin (860.7 g/mol) isolated from *E. antisyphilitica* Zucc is an effective antifungal agent against *Alternaria alternata*, *Fusarium oxyzporum*, *Colletotrichum gloeosporoides* and *Rhizoctnia solani*.

KEYWORDS

Isolated, Ellagitannin, *Euphorbia antisyphilitica* Zucc, Antifungal activity

1. Introduction

Polyphenols are widely distributed in the plant kingdom and are important components of common foods including tea, red wine, fruits, beverages and various medicinal plants. The importance of polyphenols arises from their effects on sensory properties, including astringency and colour, and possible health effects that they may have. Ellagic acid (EA) is a dimeric derivative of gallic acid which mainly exists in higher plants, including fruits and nuts, combined with its precursor, hexahydroxydiphenic acid or bound in the form of ellagitannins[1].

EA was studied in the 1960s, mainly for its effects on blood clotting, its hemostatic activity and its effects on whitening of the skin. However, effects of EA on carcinogenesis were reported in the following decades. Interests in EA have increased during the past few years due to its possible antimutagenic, antiviral and anticarcinogenic effects, proved by several studies, especially in laboratory animals, while a few works were reported in humans[2-5]. Some

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Foundation Project: Supported by CONACYT's program of Food Science and Technology (Grant No. CONACYT-CONAFOR-2008-91633).

Article history:
Received 2 Sep 2012
Received in revised form 9 Oct, 2nd revised form 15 Oct, 3rd revised form 22 Oct 2012
Accepted 12 Dec 2012
Available online 28 Jan 2013

ellagitannins have also been shown to possess anti-tumor-promoting activity, antibacterial and antiviral properties and host-mediated antitumor effects[6]. EA has also shown antioxidant activity as an inhibitor of *in vitro* lipid peroxidation and, because of its combined actions, it is used in the food industry. Extracts from red raspberry leaves or seeds, pomegranates, or other sources are said to contain high levels of EA. EA are also available as dietary supplements in capsule, powder, or liquid forms. A recent profusion of pomegranate nutraceutical products, "standardized to 40% EA" has appeared in the marketplace[7].

EA was reported to occur in 46 fruits, including raspberries, strawberries and cranberries, in abundant quantities and also in nuts, walnuts, pecans, pomegranates and other plant foodstuffs[1]. Plants produce EA to protect themselves from microbial infection and pests. *Euphorbia antisyphilitica* Zucc stalks (*E. antisyphilitica*) (commonly known as "candelilla") are rich in polyphenol components, mainly ellagitannins which help to protect the trees against predators and pathogens[8,9]. Candelilla stalks are of great economic importance in Northern Mexico. They are used for wax extraction which is used for various purposes in the food industry and also for traditional use in ornaments and therapeutic applications. However, candelilla wax extraction generates 140 tons of waste per year. Previous reports showed that EA is present in candelilla stalks[10].

In order to assess the importance of candelilla stalks as a source of EA, it is important to characterize the isolation of the EA precursors (ellagitannins) in this plant.

2. Materials and methods

2.1. Vegetal material

Candelilla stalks were collected in the Mexican semidesert in Coahuila state, México. The plant was cut from the base in order to avoid damage in the root (the Mexican official norm NOM-018-RECNAT-1999 recommends it)[11], and then the stalks were stored in the laboratory.

2.2. Candelilla wax extraction

The candelilla stalks were placed in stainless steel containers and a 20% sulfuric acid solution was added to the wax extraction[12]. The mixture was placed in a stove until boiling point. The wax began appear as gray foam and was collected in a beaker (500 mL), the stalks without wax were collected and dehydrated in a stove at 60 °C for 48 h, then were pulverized.

2.3. Ellagitannins extraction and separation

A hundred grams of pulverized candelilla stalks was placed in a steel container and 500 mL of water were added[2]. With this, the ellagitannin extraction process was carried out at 60 °C for 30 min, and then the mix was percolated[8]. After that, obtained extract was filtered using filter paper (Whatman No. 41) to eliminate the biggest residue particles. Column chromatography of the candelilla extract was performed using an Amberlite XAD-16. First, water was used as the eluent to discard undesirable compounds, and then, ethanol was employed as the eluent to obtain a total polyphenols of candelilla (TPC) fraction[13]. Solvent was evaporated from the fractionated extract and TPC was recovered as a fine powder.

2.4. HPLC test

A sample of TPC powder was analyzed by HPLC, 200 mg of sample was placed in precipitate glass and 8 mL of metanol were added. HPLC was performed according to the method reported by Chapman *et al*[14]. This solution was filtered by 0.45 μm membrane and was placed in 1.5 mL vials to HPLC analysis in order to find interesting compounds. A varian HPLC was used for the EA determination under the following operation conditions: 5 μm column Optisil ODS, 250.0 m×4.6 mm, flow rate of 1 mL/min, sample volume of 10 μL, with the following solvents to the analysis solvent methanol, acetonitrile, and 3% acetic acid.

2.5. Thermogravimetric and thermodifferential analysis

For this section the TPC powder was used, 20 mg of powder were used to carry out a thermogravimetric analysis in order to demonstrate the EA thermostability, the analysis was carried out by 87 min, monitoring the sample degradation. Also 200 mg of powder were used to carry out a thermodifferential analysis to determinate the sample decomposition in function of the energy liberated with increase of temperature during the analysis (the analysis was carried out by 78 min). These studies were made to demonstrate the EA thermostability as an equivalents of ellagitannins in TPC powder.

2.6. Ellagitannins separation

A 100 mg/mL TPC solution was prepared to carry out a liquid chomatography in FPLC equipment (Fast Protein Liquid Chromatography). First an ionic interchange column (Hi-Trap Q XL) was used, 2 mL of sample was injected and the analysis conditions were shown in square 1, each recollected fraction with this chromatography was hydrolyzed in order to determinate the EA concentration as a ellagitannins equivalents. A total of 250 μL of each fraction were placed in assay tubes with a screw top with

a plastic ring for hermetic closing, then 1.5 mL of sulfuric acid/methanol and the tubes were placed in stove at 80 °C by 30 h. The EA quantification was carried out according the Aguilera-Carbó *et al.* method in HPLC equipment[10].

After the EA quantification, the Q XL fraction with the richest EA content was chosen to make a chromatography with a desalting column (Hi-Trap G 25) using a FPLC equipment. The analysis conditions are showed in Table 1. The final volume of Q XL fractions was 20 mL and the final volume of G 25 fractions was 50 mL.

Table 1
FPLC analysis conditions.

column conditions	Q XL	G 25
Pressure (MPa)	1.0	1.0
Flow (mL/min)	1.0	1.0
Volume of wash (mL)	15.0	15.0
Elution volume (mL)	40.0	15.0
Sample volume (mL)	2.0	1.4
Fraction volume (mL)	2.0	2.0
First and second wash (mL)	10.0	-

2.7. Infrared spectroscopy

With the Q XL column 60 fractions were obtained and 250 μL of each fraction were placed in assay tubes with screw top with a plastic ring to hermetic closing and 1.5 mL of sulfuric acid/methanol were added to quantify EA as an equivalent of ellagitannin with a HPLC equipment. The fraction number 10 was chosen due to has the highest concentration of EA (0.27 mg/g). Using this fraction a G 25 column chromatography was performed and 12 fractions were obtained. The fraction 2 was chosen and hydrolized as above mentioned and an EA concentration of 0.21 mg/g was obtained; this fraction was liofilized and hydrolized with the methodology mentioned above and an infrared analysis was carried out and it was compared with a comercial EA estándar (Sigma). A Spectrum GX Perkin-Elmer for the infrared analysis was used.

2.8. Mass spectroscopy analysis

The lyophilized fraction 2 was analyzed by mass spectroscopy; 3 mg of powder was mixed with 10 mL of 90% methanol and filtered by 0.45 μm nylon membrane. A solution of formic acid 0.01% (in acetonitrile) was prepared to be used as eluent during the analysis. A Varian mass spectrum model 500-MS was used to mass analysis with flow 1 mL/min, mass rate 400-2000 by 10 min.

2.9. In vitro antifungal activity assay

The fungi used in this analysis belong to DIA-UAdeC collection, and they are microorganisms previously purified, identified and crio-conserved at -20 °C. The microorganisms used were *Fusarium oxysporum* F. sp. lycopersici, *Colletotrichum gloeosporoides*, *Rhizoctonia solani* y *Alternaria alternata*. Potato dextrose agar plates were prepared and several aliquots of purified ellagitannin were added (0, 2000, 4000, 6000 mg/L). The radial growth was measured every 24 h.

3. Results

In this work, we described the isolation of an ellagitannin from "candelilla" stalks and following the results are shown. Figure 1 shows the chromatogram of the HPLC analysis carried out by 72 min in order to reach a correct separation of the compounds. It shows four peaks; at 8.06 min an intense signal in TPC is obtained, after at 15 min it presents a second signal and two more signals (25.4 and 30.4 min).

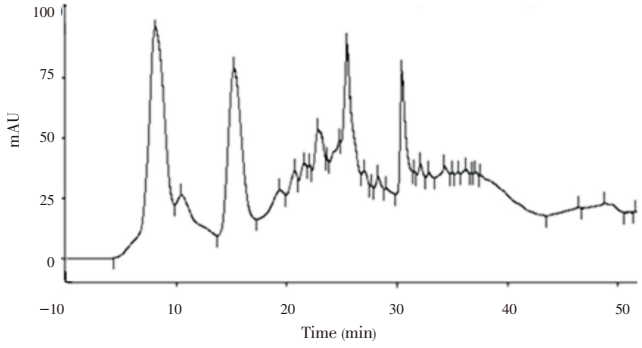

Figure 1. HPLC chromatogram of TPC.

Ionic interchange chromatogrphy (Q Sepharose XL) from TPC permitted to separate several fractions after the EA quantification as an equivalent of ellagitannin content (Figure 2).

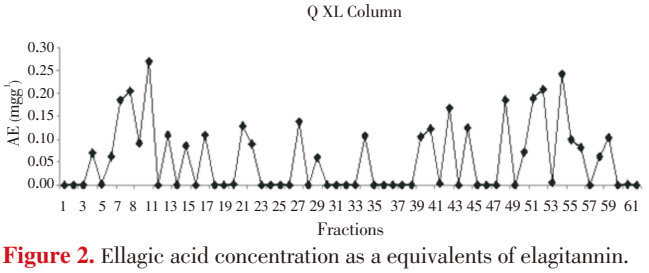

Figure 2. Ellagic acid concentration as a equivalents of elagitannin.

With the fraction 10 from Q XL chromatography a G 25 chromatography was performed, the fractions were recovered and the EA was quantified as a equivalents of ellagitannins. The principal action of G 25 column (sephadex) is the compounds separation by molecular weight. The low molecular weight compounds are retained in the column, while the high molecular weight compoumnds are liberated due to there is no interaction between the column and the compounds, for this the high molecular weight are obtained

in the first fractions. In Figure 3, the EA concentration is showed, the fraction number 2 is the fraction with the highests concentration of ellagic acid, this meant that the purified ellagitannin had a high molecular weight.

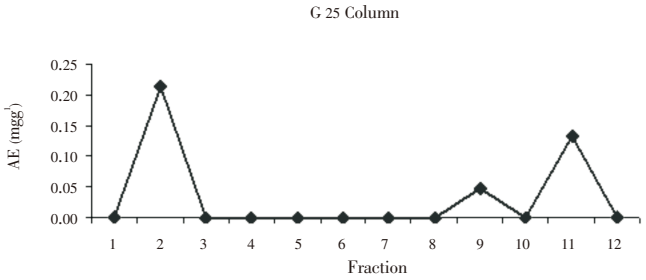

Figure 3. Ellagic acid concentration as equivalents of ellagitannins.

To reinforce the study the fraction number 2 was hydrolyzed in order to obtain an EA infrared spectrum and was compared with a comercial EA spectrum. These results coincide with the results reported by Robledo-Olivo *et al.*[15] and Abo-Moch *et al*[16]. The results are shown in Figure 4. Once determined the functional groups of ellagitannin by infrared spectroscopy, HPLC-mass spectroscopy analysis was performed using the purified fraction 2 in order to demonstrate this was the purified ellagitannin (Figure 5).

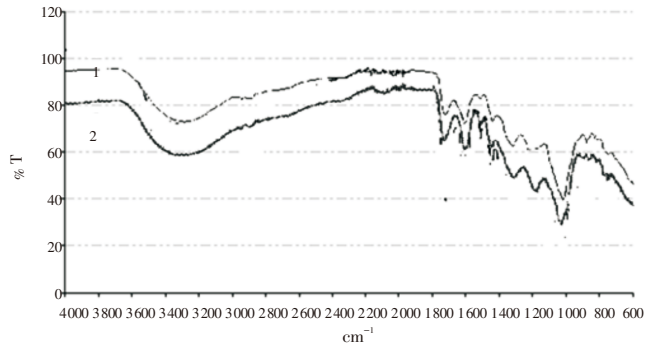

Figure 4. Ellagic acid infrared spectrum.
1: Hydrolized fraction; 2: Commercial ellagic acid.

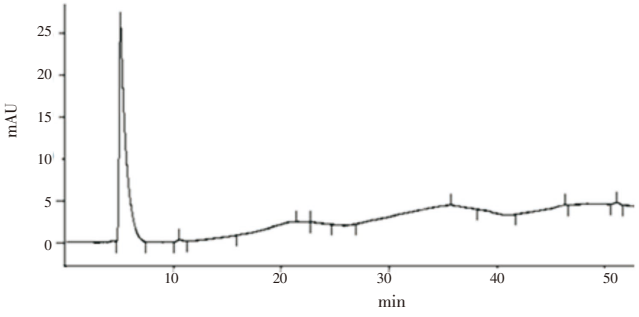

Figure 5. HPLC cromatogram from the purified fraction that contains an ellagitannin.

In Figure 6, it was observed the mass signal obtained by mass spectrometry, the molecular weight of the purified ellagitannin was 860.7 g/mol, this indicated that the purified ellagitannin was a high molecular weight compound and the analysis was reinforced by the infrared spectroscopy results. Taking into account the obtained results in this work and considering the protocol reported by Chapman *et al.* using MS/MS[14], in this work it was possible to consider the putative structure shown in Figure 7, which has been called candelitannin.

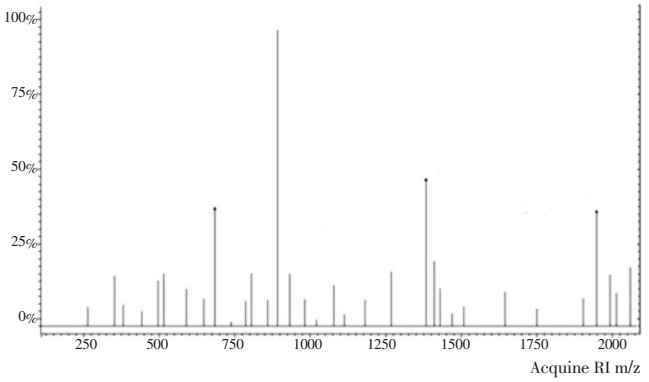

Figure 6. Mass spectrum from the purified ellagitannin.

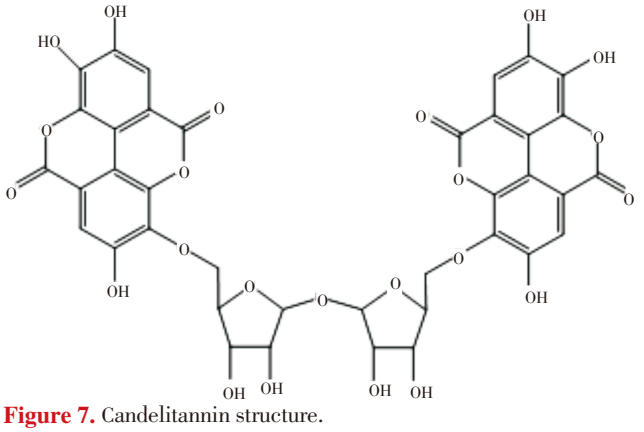

Figure 7. Candelitannin structure.

Once purified the candelitannin, an antifungal activity assay was carried out against 4 important phytopathogenic fungus (*Alternaria alternata*, *Fusarium oxysporum* F. sp. *lycopersici*, *Colletotrichum gloeosporioides* and *Rhizoctonia solani*), and the results of their mycelial growth were shown in Figure 8.

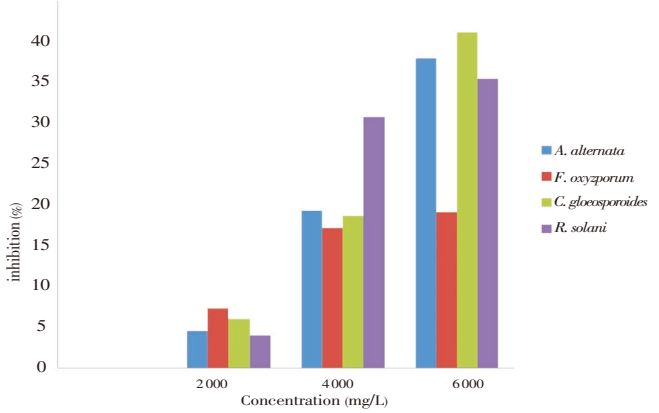

Figure 8. Antifungal capacity of the candelitannin against four phytophatogenic fungi.

4. Discussion

Chapman *et al.* determined by HPLC five different compounds from oak wood; this study proposes the use of Q XL and G 25 liquid chromatography to purify ellagitannins[14]. Vekiari *et al.* and Yoshimura *et al.* purified ellagitannins from chestnut with a similar methodology[17,18].

It was observed that the ellagaitannins from TPC were retained in the c-18 ODS column due to the negative charges[19]. In some fractions there was not EA concentration after hydrolysis, this means that in this fractions there were not ellagitannins. The fraction number 10 was chosen due to its EA concentration was the highest, besides it was verified the relevant ellagitannins content in candelilla stalks using this methodology.

The liquid chromatography is important due to its use in separation of molecules with biological properties, like ellagitannins. Hernández-Rivera *et al.* used this chromatography (Q XL and G 25 columns) to separate enzimes with biological activity with positive results[20]. In others studies the polyphenols has been successful with the use of liquid chromotography; Lei *et al.* carried out the purification of two ellagitannins[21], vascalagin and castalagin) from oak wood using a Sephadex LH-20 column. Hussein *et al.* purified elagitannins from berries with chromatography using a similar method like used by Lei *et al.*[21] and Khadem *et al*[22,23].

Haidari *et al.* isolated an ellagitannin, punicalin, from pomegranate husk with a molecular weight of 781 g/mol[24], this molecular weight is approximate to the molecular weight of the ellagitannin purified in this work. In other works[25-27], a ellagitannin glucoside (HHDP-glucopiranoside) was isaloted with a molecular weight of 481 g/mol, medium value regard to the mass value obtain in this work. Around 500 ellagitannins molecules have been characterized and one of the most important parameters is the molecular weight, it has been reported the mass of important molecules as a tellimagrandin II (983), el casuarictin (936), y pedunculagin (748).

Regarding the antimicrobial activity of ellagitannins, punicalagin has been previously reported as efficient agent against *Staphylococcus aureus, Escherichia coli, Candida albicans, Cryptococcus neoformans* and *Aspergillus fumigatus*[28-30]. In our study, the inhibition percentage increase with the amount of the ellagitannin concentration due to the antigungal potential of ellagitannins[26], in this case we can indicate candelitannin is an efficient antifungal activity.

In conclusion, a new ellagitannin was purified from TPC powder and called candelitannins. It was demonstrated the antifungal potential of this ellagitannin againts phytopathogenic.

Conflict of interest statement

We declare that we have no interest conflict.

Acknowledgements

Ascacio-Valdes wants to thank CONACYT for the scholarship assigned to his postgraduate study in the program of Food Science and Technology, UAdeC. Authors thank the financial support provided by the program CONACYT-CONAFOR-2008-91633.

Comments

Background

There is a strong interest for isolation and characterization of phytochemicals, particularly of those with high bioactivity. Ellagitannins are considered as one of the most important secondary metabolites with important biological activities, for this reason, the purification and identification is nowadays of interest.

Research frontiers

This paper describes for the first time an ellagitannin isolated from Candelilla (*E. antisiphylitica* Zucc).

Related reports

No other phytochemical has been previously reported in *E. antisiphylitica* Zucc.

Innovations and breakthroughs

A new ellagitannin, named candelitannin which exhibits high antifungal activity.

Applications

By isolating Ellagitannins from the *E. antisiphylitica* Zucc, the authors provide a potential alternative for food by controlling phytopathogenic fungi.

Peer review

This is a good study in which the authors isolated, identified and characterized a new ellagitannin. The result is interesting and suggests that candelitannin can be used for the fungal control.

References

[1] Klein G, Kim J, Himmeldirk K, Cao Y, Chen X. Antidiabetes and anti-obesity activity of *Lagerstroemia speciosa*. *Evid Based Complement Alternat Med* 2007; **4**: 401-407.

[2] Aguilera-Carbó A, Augur C, Prado-Barragán LA, Favela-Torres E, Aguilar CN. Microbial production of ellagic acid and biodegradation of ellagitannins. *Appl Microbiol Biotechnol* 2008; **78**: 189-199.

[3] Reddy MK, Gupta SK, Jacob MR, Khan SI, Ferreira D. Antioxidant, antimalarial and antimicrobial activities of tannin-rich fractions, ellagitannins and phenolic acids from *Punica granatum* L. *Planta Medica* 2007; **73**: 461-467.

[4] Faria A, Monteiro R, Mateus N, Azevedo I, Calhau C. Effect of pomegranate (*Punica granatum*) juice intake on hepatic oxidative stress. *Eur J Nutr* 2007; **46**: 271-278.

[5] Panichayupakaranant P, Tewtrakul S, Yuenyongsawad. Antibacterial, anti-inflamatory and anti-allergic activities of standardised pomegranate rind extract. *Food Chem* 2010; **123**: 400-403.

[6] Madrigal-Carballo S, Rodríguez G, Krueger CG, Dreher M, Reed JD. Pomegranate (*Punica granatum*) supplements: authenticity, antioxidant and polyphenol composition. *J Funct Foods* 2009; **1:** 324-329.

[7] Lansky EP. Beware of pomegranates bearing 40% ellagic acid. *J Med Food* 2006; **9**(1): 119-122.

[8] Ascacio-Valdés JA, Aguilera-Carbó AF, Martínez-Hernámdez JL, Rodríguez-Herrera Aguilar CN. *Euphorbia antisyphilitica* residues as a new source of ellagic acid. *Chem Pap* 2010; **64**: 528-532.

[9] Bianco MA, Handaji A, Savolainen G. Quantitative analysis of ellagic acid in hardwood samples. *Sci Total Environ* 1999; **222**(1-2): 123-126.

[10] Aguilera-Carbo AF, Augur C, Prado-Barragan LA, Aguilar CN, Favela-Torres E. Extraction and analysis of ellagic acid from novel complex sources. *Chem Pap* 2008; **62**: 440-444.

[11] The Mexican Ministry of Enviroment and Natural Resources. *Mexican official norm: Procedures, criterions and technical and administrative specifications for sustainable use of candelilla shrub, transportation and storage*. Tlalpan: The Mexican Ministry of Enviroment and Natural Resources; 1999.

[12] De Leon-Zapata MA, Saucedo-Pompa S, Contreras-Esquivel JC, Jasso-Cantú D, Sáenz-Galindo A, Rodríguez R, et al. Technological improvements for candelilla (*Euphorbia antisyphilitica* Zucc.) wax extraction. In: Soto-Cruz O, Peggi MA, Gellegos-Infante A, Rodriguez-Herrera R, (edirs.) *Advances in food science and food biotechnology in developing countries*. New Delhi: Asiatech Publishers Inc; 2010, p. 272-280.

[13] Seeram N, Lee R, Herber D. Rapid large scale purification of ellagitannins form pomegranate husk, a by-product of the commercial juice industry. *Sep Purif Technol* 2005; **41**: 49-55.

[14] Chapman JM, Nast JR, Scholes C, Niemann S. Application of LC/ESI/MS and LC/EI/MS to the characterization of tannins and flavonoids from the acorns of Quercus macrocapra. Poster presented at the 57th Pittsburg Conference on Analytical Chemistry and Applied Spectroscopy 2007 March, Orlando, Florida.

[15] Robledo-Olivo A, Aguilera-Carbó A, Rodríguez R, Martínez JL, Aguilar CN. Ellagic acid production by *Aspergillus niger* in solid state fermentation of pomegranate residues. *J Ind Microbiol Biotechnol* 2008; **35**: 507-513.

[16] Abo-Moch F, Saadi I, Holland D, Mansour F. The potential of pomegranate peel and heartwood extracts as a source of new bioacaricides to control the carmine mite *Tetranychus cinnabarinus*. *Israel J Plant Sci* 2010; **58**: 13-17.

[17] Vekiari SA, Gordon MH, García-Macías P, Labrinea H. Extraction and determination of ellagic acid content in chestnut bark and fruit. *Food Chem* 2008; **110**: 1007-1011.

[18] Yoshimura M, Ito H, Miyashita K, Hatano T, Taniguchi S, Amakura Y, et al. Flavonol glucuronides and C-glucosidic ellagitannins from *Melaleuca squarrosa*. *Phytochemistry* 2008; **69**: 3062-3069.

[19] Feldman KS. Recent progress in ellagitannin chemistry. *Phytochemistry* 2005; **66**: 1984-2000.

[20] Hernandez-Rivera JS. *Producción, purificación y caracterización de la enzima de Aspergillus Níger GH1 responsable de la hidrólisis del grupo HHDP de los elagitaninos (Master's dissertation)*. México: Universidad Autónoma de Coahuila; 2008.

[21] Lei Z. Monomeric ellagitannins in oaks and sweetgum (Doctoral dissertation). Faculty of Virginia, Politechnic Institute and State University fulfilment. Virginia: Virginia Polytechnic Institute and State University; 2002.

[22] Hussein SAM, Ayoub NA, Nawwar MA. Caffeoyl sugar esters and an ellagitannin from *Rubus sanctus*. *Phytochemestry* 2003; **63**: 905-911.

[23] Khadem S, Marles RJ. Monocyclic phenolic acids; hydroxy- and polyhydroxybenzoic acids: ocurrence and recent bioactivity studies. *Molecules* 2010; **15**: 7985-8005.

[24] Haidari M, Ali M, Casscells SW, Madjid M. Pomegranate (*Punica granatum*) purified polyphenol extract inhibits influenza virus and has a synergistic effect with osealtamivir. *Phytomedicine* 2009; **16**: 1127-1136.

[25] Madhumitha G, Saral AM. Preliminary phytochemical analysis, antibacterial, antifungal and anticandidal activities of successive extracts of *Crossandra infundibuliformis*. *Asian Pac J Trop Med* 2011; **4**(3): 192-195.

[26] Kaponen JM, Happonen AM, Mattila PH, Törrönen AR. Contents of anthocyanins and ellagitannins in selected foods consumed in Finland. *J Agric Food Chem* 2007; **55**: 1612-1619.

[27] Balakumar S, Rajan S, Thirunalasundari T, Jeeva S. Antifungal activity of *Ocimum sanctum* Linn. (Lamiaceae) on clinically isolated dermatophytic fungi. *Asian Pac J Trop Med* 2011; **4**(8): 654-657.

[28] Lavanya R, Veerappan N. Pharmaceutical properties of marine macroalgal communities from gulf of mannar against human fungal pathogens. *Asian Pac J Trop Dis* 2012; **2**(Suppl 2): S320-S323.

[29] Ueda-Yamaguchi M, Pelegrin-Garcia F, Garcia-Cortez DA, Ueda-Nakamura T, Dias-Filho BP, Vataru-Nakamura C. Antifungal effects of ellagitannins isolated of *Ocotea odorifera* (Lauraceae). *Antonie van Leeuwenhoek* 2011; **99**(3): 507-514.

[30] Soni N, Prakash S. Entomopathogenic fungus generated Nanoparticles for enhancement of efficacy in Culex quinquefasciatus and *Anopheles stephensi*. *Asian Pac J Trop Dis* 2012; **2**(Suppl 2): S356-S361.