

Production of Antioxidant Nutraceuticals by Solid-State Cultures of Pomegranate (*Punica granatum*) Peel and Creosote Bush (*Larrea tridentata*) Leaves

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Summary

In this work, creosote bush (*Larrea tridentata*) leaves and pomegranate (*Punica granatum*) peels were characterized for their use as a source of antioxidants and a support in solid-state fermentation. This work focuses on the kinetic evaluation of physicochemical changes during the fungal fermentation of two tannin-rich plant materials, mainly on the polyphenolic content. *Aspergillus niger* GH1 was used in the fermentation processes, the cultures were monitored for 96 h. The content of proteins, crude fibres, lipids, reducing and total sugars was evaluated following the AOAC methods. Tannins were analyzed using a spectrophotometric method. Biodegradation of ellagitannins was monitored kinetically and the accumulation of gallic and ellagic acids was determined by HPLC. Results demonstrated that pomegranate peel and creosote bush leaves are excellent supports for solid-state fermentation and sources of antioxidants.

Key words: pomegranate peel, creosote bush, antioxidants, solid-state fermentation

Introduction

Tannins are high molecular mass plant polyphenols divided into two chemically and biologically distinct groups: condensed tannins or proanthocyanidins (*e.g.* from tea, grapes, cranberries, *etc.*), and hydrolysable tannins: ellagitannins (ETs) (*e.g.* from raspberries, strawberries, pomegranates, *etc.*) and gallotannins (GTs). Tannins gained original popularity in the commercial tanning industry where animal hides were converted into leather by using plant extracts but have attracted much atten-

tion recently due to their numerous biological activities and implications in potential benefits to human health.

Larrea tridentata (Sessé & Moçño ex DC.) Cov., also known as larrea, chaparral, or creosote bush, is a shrub-by plant belonging to the family Zygophyllaceae, which dominates some areas of the desert in the southwest of the United States and northern Mexico, as well as some desert areas of Argentina (1). Tea brewed from the leaves of *L. tridentata* has been used in traditional medicine to treat digestive disorders, rheumatism, venereal disease, sores, bronchitis, chickenpox, and the common cold (1). Phytochemical studies carried out on *L. tridentata* showed

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that it contains a series of lignans (2,3), flavonoids (4–6), condensed tannins (7), triterpene saponins (8), and naphthoquinones (9). The extracts of the constituents of *L. tridentata* have been reported to possess antioxidant (10), anti-HIV (11,12), antimicrobial (13), enzyme inhibitory (1,13), antitumour (13), and antihyperglycemic (9) activities. The plant contains a powerful antioxidant nordihydroguaiaretic acid (NDGA) (1).

Pomegranate (*Punica granatum* L.) is grown mainly in the Near East, India, Spain (southeastern), Israel and the United States (California), and is of significant economic importance since the fruits are either consumed fresh or used commercially in the juice, jam and wine industries (14,15). Pomegranate husk is rich in ETs such as punicalagin and its isomers (2,3-hexahydroxydiphenoyl-4,6-gallagylglucose) (14), as well as smaller amounts of punicalin (4,6-gallagylglucose) (15), gallic acid (GA) (16), ellagic acid (EA) (17) and EA-glycosides (hexoside, pentoside, rhamnoside, *etc.*) (14–18). These ETs are extracted in significant levels into the juice during industrial hydrostatic processing treatment of the whole fruits (14). Commercial pomegranate juices exhibit potent antioxidant properties that have been attributed to their high content of polyphenols including punicalagin, which can reach levels >2 g/L juice, depending on the fruit cultivar and processing methods (14,15). Tannins have also been identified as the active antiatherosclerotic compounds in pomegranate juices responsible for the ability of this juice to protect human low-density lipoprotein cholesterol from oxidation *in vivo* (15).

In this work, a fungal strain, previously reported as excellent tannin-degrading microorganism and tannase producer (19), has been used to evaluate its ability to convert pomegranate and creosote bush tannins to ellagic acid, gallic acid and catechin in solid-state cultures. The changes in the chemical content of plants during fermentation, the accumulation of antioxidants and the enzymes produced in fermentation media of monocultures of each strain were estimated and compared.

Materials and Methods

Microorganism and plant material

Aspergillus niger strain GH1 (culture collection of the DIA-UAdeC, Mexico) was used in this study. Fungal strain had previously been isolated, identified and characterized (19). Creosote bush leaves and pomegranate peels were collected from the Desert region of Coahuila State in North Mexico and transported to the Microbiology Laboratory of the Food Research Department, where the material was cleaned, dried at 60 °C for 48 h, pulverized in a homogenizer LP12 Series 600 Maquinaria® and stored at room temperature in black bags.

Solid-state culture

Two sets of reactors (5 flasks of 250 mL of each plant material) were inoculated with *Aspergillus niger* GH1 spores at $2 \cdot 10^7$ spores/g of plant material and the plant material was impregnated with culture broth. Reactors were maintained at 30 °C and the duration of fermentation was 96 h. Culture broth was composed of (in g/L): KH_2PO_4 4.38, NaNO_3 8.79, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.88,

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.088, $\text{MnCl}_2 \cdot 6\text{H}_2\text{O}$ 0.018, $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ 0.0088 and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.012. Each fermentation process was done in triplicate. Samples were collected daily and the composition changes were monitored using standard methods.

Physicochemical characterization

Fresh and fermented plant materials were analyzed for their chemical composition. Analysis of reducing and total sugars, protein and crude fibre were carried out using the methods described by AOAC (20). Total polyphenolic content was determined using the method reported by Makkar *et al.* (21). Condensed and hydrolysable tannin contents were determined using spectrophotometric method described by Waterman and Mole (22). Catechin was determined spectrophotometrically (HCl-butanol method), while gallic and ellagic acids were determined by the HPLC method based on the technique reported by Matějčíček *et al.* (23) and modified by Aguilera-Carbo *et al.* (24). All determinations were made in triplicates and mean values and standard deviations were calculated and reported.

Results and Discussion

In this study the changes of chemical composition of creosote bush leaves and pomegranate peels were evaluated during their fermentation. Table 1 shows the initial and final values of the chemical composition of plant materials. Major changes in the chemical composition were registered for reducing sugars and protein content. Reducing sugar content decreased 71 and 88 % for creosote bush leaves and pomegranate peels, respectively, after 96 h of cultivation, while in the same time period the protein mass fraction increased 14 and 19 times, respectively.

Table 1. Chemical composition of dried plant material before and after the fermentation

Component	Creosote bush leaves		Pomegranate peels	
	w/%			
	Initial	Final (96 h)*	Initial	Final (96 h)*
Total solids	94.1	97.5	94.50	98.10
Moisture	5.9	–	5.40	–
Total sugars	9.3	9.9	17.70	12.10
Reducing sugars	9.1	2.6	4.34	0.50
Protein	4.3	5.0	4.90	6.10
Crude fibre	18.8	19.8	16.30	17.60
Fat content	2.6	2.6	1.26	1.27
Ash	8.0	9.9	3.40	4.30

*Time of cultivation

Table 2 shows the main changes in the tannin mass fraction. After 96 h of culture, the fungus reduced the high tannin mass fraction in creosote bush leaves and pomegranate peels demonstrating the ability of the fun-

Table 2. Polyphenolic composition of plant material before and after the fermentation

Component	Creosote bush leaves		Pomegranate peels	
	<i>w</i> /%			
	Initial	Final (96 h)*	Initial	Final (96 h)*
Total phenols	55	5.3	8.10	1.9
Hydrolysable tannins	13	0.5	6.11	0.9
Condensed tannins	41	2.3	1.20	0.6

*Time of cultivation

gal strain to degrade condensed and hydrolysable tannins. Creosote bush has a high tannin level and it is not a limiting factor of the growth of *A niger* GH1, which has the ability to convert the condensed tannins to monomers of catechin (Fig. 1) releasing up to 14 % in 96 h. In the fermentation of pomegranate peels, the fungus did not modify the catechin level because it always remains constant. Similar to these results, the conversion from hydrolysable tannins to gallic acid was very significant in creosote bush (Fig. 2), reaching an accumulation of 6 % at 72 h of cultivation, while for pomegranate peels the fungal fermentation allowed a scarce accumulation of gallic acid.

Ellagic acid was accumulated considerably in both plant materials after fungal fermentation; however, this accumulation was higher in pomegranate peels than in creosote bush leaves, reaching up to 0.9 and 0.5 %, respectively (Fig. 3). These results demonstrated that the high level of hydrolysable tannins in creosote bush leaves corresponds to gallotannins, while in pomegranate peels the hydrolysable tannins are mainly ellagitannins. Considerable progress has been made in recent years in the study of polyphenols from several sources. In this case, the tested plant materials have been poorly studied.

There is published information on physicochemical characterization of creosote bush leaves and pomegranate peels (25–29), however, this study reports the most complete information to date, and it is relevant because it includes the polyphenolic content characterization. Results demonstrate a high tannin fraction in creosote bush leaves, mainly of condensed tannins, and the fraction in pomegranate peels corresponds to ellagitannins. It is important to observe that the solid-state fermentation permits the release and recovery of potent phenolic antioxidants from pomegranate peel and creosote bush leaves.

In our study, the percentage yield of phenolic antioxidants was calculated based on the fermented material and not on the corresponding tannin mass fraction. The fungal fermentation process resulted in a high accumulation of gallic acid and catechin level in creosote bush leaves, and ellagic acid in pomegranate peels. Research of microbial production of catechin has not been published until today, in contrast to the microbial production of gallic acid, about which there is a lot of information available, and about ellagic acid the information is more scarce and deficient.

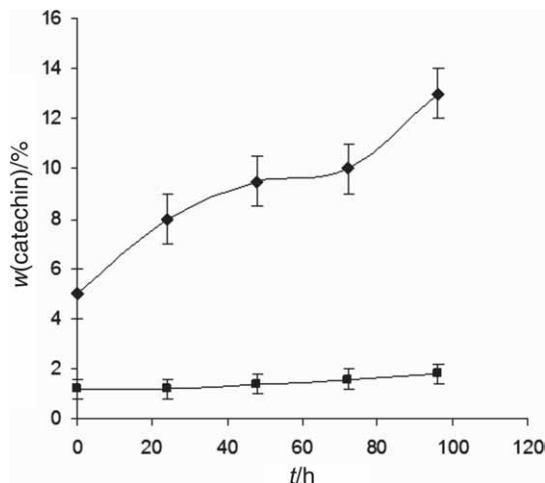


Fig. 1. Catechin accumulation during the fungal fermentation of creosote bush leaves (◆) and pomegranate peels (■) in solid-state fermentation

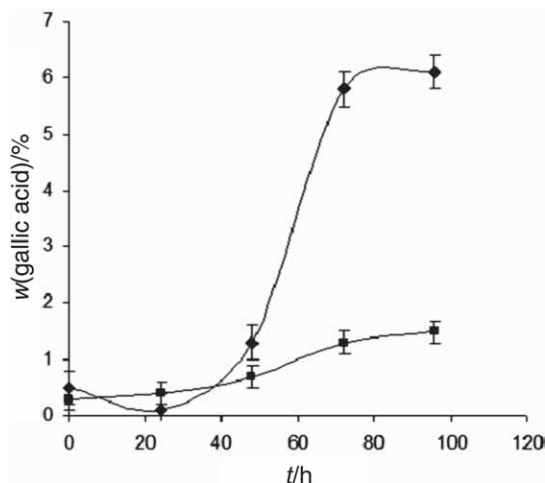


Fig. 2. Gallic acid accumulation during the fungal fermentation of creosote bush leaves (◆) and pomegranate peels (■) in solid-state fermentation

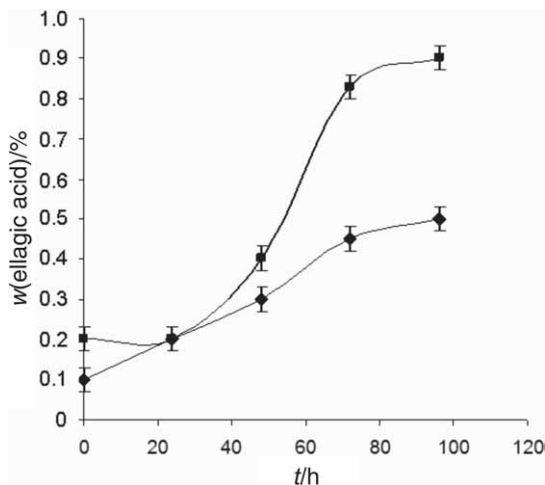


Fig. 3. Ellagic acid accumulation during the fungal fermentation of creosote bush leaves (◆) and pomegranate peels (■) in solid-state fermentation

Other phenolic antioxidants have been produced in solid-state fermentation, mainly ferulic acid and *p*-coumaric acid from corn cobs enzymatically treated and fermented with *Sporotrichum thermophile* (30).

High yields of gallic acid recovery associated with high tannase activities were reported by Kar and Banerjee (31) and Kar *et al.* (32) during the fermentation of a tannin-rich forest residue (gilo seeds, *Caesalpinia digyna*) or powdered *Terminalia chebula* fruits using the fungal strains *Rhizopus oryzae* and *Aspergillus foetidus* (33,34). Recently, gallic acid accumulation yields after fungal fermentation of creosote bush leaves have been improved significantly in comparison with those reported by Treviño-Cueto *et al.* (29), who published an incipient stage on gallic acid accumulation by biodegradation of creosote bush tannins giving as a result the possibility of a commercial exploitation of creosote bush as a source of phenolic compounds.

Chemical extraction processes for ellagic acid production have been widely applied in several plants. However, these methodologies are contaminant, expensive and give low yields. For this reason, alternative biological methods have been explored. Vatterm and Shetty (35) reported the solid-state production of phenolic antioxidants (rosmarinic acid, ellagic acid and resveratrol) from cranberry pomace using the fungus *Rhizopus oligosporus*, and they also reported a maximum ellagic acid production of 400 mg per 100 g of pomace after 12 days of fungal fermentation.

Using valonea tannins from fruit shell of *Quercus aegilops*, Shi *et al.* (36) reported high levels of conversion to ellagic acid (21 %) when a coculture of *Candida utilis* and *Aspergillus niger* was employed. In this study, when pomegranate fermented with *A. niger* GH1, the ellagic acid recovery was twice higher than the values reported by Vatterm and Shetty (35) and 1.25 times higher when creosote bush leaves were fermented.

Conclusions

Although numerous studies have been conducted on biodegradation of tannins and on the degradation mechanism of some simple tannins such as gallotannins, little is known about the pathways and the enzymes involved in breaking of complex tannins, especially about the accumulation mechanism of some intermediates by fungi and yeasts. This work is an initial effort in the accumulation of the antioxidant phenolic compounds by biodegradation of tannins present in creosote bush leaves and pomegranate peels, and further studies should be carried out to optimize the process in solid-state fermentation.

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