

Fermented *Flourensia cernua* Extracts and Their *in vitro* Assay Against *Penicillium expansum* and *Fusarium oxysporum*

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Summary

The antioxidant activity and efficiency of aqueous extracts of fermented tarbush (*Flourensia cernua* DC) in the inhibition of two phytopathogenic fungi, *Penicillium expansum* and *Fusarium oxysporum*, have been evaluated. A solid-state fermentation of tarbush leaves by *Aspergillus niger* GH1 was performed to enhance the biological activities of the obtained extracts. Fungal culture conditions were: initial moisture of 60 %, pH=5.5 and temperature of 30 °C during 96 h. Aqueous extracts were obtained every 12 hours during the culture time. Antioxidant activity was evaluated by DPPH method. The aqueous extract of fermented tarbush had the highest antioxidant activity at 12 h, which was 63 % higher than the control (unfermented material). Fermentation enhanced the fungicidal effect against both phytopathogenic microorganisms at a concentration of 0.5 g/L. This study demonstrated that fungal fermentation of tarbush increased the biological activities of the aqueous extracts.

Key words: *Flourensia cernua*, fermentation, aqueous extracts, antioxidants, antifungal activity

Introduction

Currently, the control of fungal phytopathogenic microorganisms in industrial crops requires new alternative techniques because the use of some traditional synthetic agrochemicals represents serious human health risks such as toxicity to users and consumers (1), environmental damage and negative impact on beneficial organisms (2). Another important aspect is that phytopathogenic organisms have generated resistance to the active ingredient of some synthetic fungicides (3,4) in response to high doses and continuous applications, leading to great economic losses.

The study of biological activity of some compounds found in plants offers an opportunity to discover new

and effective bioactive compounds for pest control (5,6). Some of these phytochemical compounds are tannins, flavonoids, lignans and terpenes, which play an important role in the defense mechanisms of fruits and vegetables (7–9) and could be considered as potential promoters of the safety of fresh fruits when applied exogenously, or promote their activity through controlled abiotic stress mechanisms.

Fruit production around the world is affected especially during harvest, because frequently the fruit must withstand long periods of storage prior to their arrival to the market. This can cause great losses due to the decline in quality or quantity of the produced fruit. Post-harvest diseases are attributable mainly to fungi such as *Penicillium*, *Fusarium*, *Botrytis*, *Nectria* and *Alternaria* (10).

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In Mexico there is a variety of widely distributed plants which have not been chemically evaluated to determine their biological activity against phytopathogenic fungi. One of them is tarbush (*Flourensia cernua* DC), which is abundant in arid and semiarid regions of Mexico, where the tea brewed from the leaves of this plant is used in traditional medicine to treat digestive disorders, rheumatism, venereal diseases, herpes, bronchitis, varicella and common cold (11). It has been reported that components of tarbush extracts have antioxidant (12), anti-HIV (13), antimicrobial, antitumour (14) and antidiabetic properties (15).

As antioxidants, polyphenols may protect cells against oxidative damage and therefore reduce the risk of several degenerative diseases associated with oxidative stress caused by free radicals (16). Oxidative stress is commonly defined as the balance between oxidizing and reducing species at the cellular level in an organism (17). Among the most important phenolic compounds are flavonoids (18), which have a wide range of therapeutic effects, such as cardiogenic, anti-inflammatory, hepatoprotective, anti-neoplastic and antimicrobial activities.

Earlier, our group (19) reported that the extracts of *F. cernua*, *Jatropha dioica*, *Turnera diffusa* and *Euphorbia anti-syphilitica* are effective against some fungi such as *Penicillium purpurogenum*, *Fusarium* spp., *Alternaria* sp., *Aspergillus flavus* and *Rhizoctonia* sp. Other authors reported that the *F. cernua* leaves contain substantial levels of phenolic compounds (20). Also, there are some reports on growth inhibition of phytopathogenic fungi such as *Rhizoctonia solani* and *Phytophthora infestans* with methanol extracts of tarbush (21) and *Colletotrichum* spp. with hexanes, diethyl ether and ethanolic extracts against termites (22).

We have previously demonstrated the presence of high levels of phenolic antioxidants during the fermentation of several tannin-rich shrubs, mainly derivatives from the biodegradation of the polyphenols present in leaves; so these plants can be used to produce potent antioxidants by microbiological methods (23). However, microorganisms used for this should be able to produce the necessary enzymes to hydrolyze this type of specific antimicrobial molecules.

These studies have shown that fungal fermentation of these plant materials can be used to increase their value and use, which have until now been used only empirically for the preparation of infusions to treat certain diseases in the rural population of northern Mexico (24).

In the present work, a new bioprocess to enhance the biological activity of tarbush aqueous extracts is described. *Flourensia cernua* was used as a substrate for cultivation of *Aspergillus niger* GH1 to obtain the aqueous extracts and evaluate their antioxidant activity and antifungal capacity to inhibit the growth of *Fusarium oxysporum* and *Penicillium expansum*, which are two of the most important phytopathogenic microorganisms.

Materials and Methods

Collection and pretreatment of tarbush

Tarbush plant was proportioned by Fitokimica Mexicana SA de CV (Saltillo, Coahuila, Mexico). Plant material was dehydrated in an oven at 60 °C for 48 h. The

leaves were separated from the stems, and pulverized in a mill. Powder was stored in black plastic bags for later use.

Fungal fermentation of the plant

Spores of the strain *Aspergillus niger* GH1 (DIA collection, UAdeC, Saltillo, Mexico), isolated and characterized as tannin-degrading fungus (25), were propagated in 250-mL Erlenmeyer flasks containing sterile potato dextrose agar, which was inoculated with 200 µL of spores and incubated at 30 °C for five days. Culture conditions of fungal fermentation of tarbush were: Czapek-Dox medium (Oxoid, Hampshire, UK) previously sterilized in 50-gram tray reactors, initial moisture of 60 %, initial pH=5.5, an inoculum of 2·10⁷ spores per gram of dehydrated plant and finally incubated at 30 °C for 96 h. Samples were collected every 12 h in aluminium foil. Samples were dehydrated in an oven at 60 °C for 24 h.

Preparation of the aqueous fermented tarbush extract

About 50 g of ground fermented tarbush were homogenized in 500 mL of deionized water. This mixture was stirred vigorously for 60 s, then centrifuged for 20 min at 1500×g, with the help of a vacuum pump; the supernatants of the extracts were filtered using Whatman filter paper no. 1 (GE Healthcare, Pittsburg, PA, USA), the filtrate was stored in plastic containers lined with aluminium foil and subjected to refrigeration until use. Control sample was unfermented tarbush. All extracts were lyophilized prior to evaluation of their biological activities.

Quantification of total polyphenols

For quantification of polyphenols as condensed tannins, the HCl-methanol method was used (26). Total flavanoids (compounds containing *meta*-oriented di- or trihydroxy substituted benzene rings, with a single bond at the 2,3 position of the ring C, such as flavan-3-ols, flavan-4-ols, flavan-3,4-diols, flavanones and derivatives) were estimated in the plant extracts using the chromogen *p*-dimethylaminocinnamaldehyde (DMACA; Sigma, St. Louis, MO, USA), following the protocol proposed by Delcour and Janssens de Varebeke (26). All determinations were made in triplicate and values were calculated from a calibration curve obtained with catechin (Sigma-Aldrich, Mexico, DF). Final results were expressed as milligram of catechin equivalent (CE) per gram of tarbush.

Determination of antioxidant activity by DPPH method

The ability of the extracts to capture free radicals was determined using the solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) as reference according to the method reported by Brand-Williams *et al.* (27). The DPPH radical is characterized by an unpaired electron, which is a free radical stabilized by resonance. A solution of DPPH radical at a concentration of 60 µM by diluting with methanol was prepared, and 2.9 mL of DPPH solution were then placed in test tubes covered with foil. After that, 100 µL of the sample were added and left to stand for 30 min. The absorbance was recorded at a wavelength of

517 nm. The percentage of inhibition of DPPH radical was determined by the following equation:

$$\text{Inhibition} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \cdot 100 \quad /1/$$

Antifungal activity assay

For this study, two phytopathogenic fungi belonging to DIA-UAdc collection (*Penicillium expansum* and *Fusarium oxysporum*) were tested. Both fungal strains were incubated on potato dextrose agar at 30 °C for five days. To conserve the fungi, a cryoprotectant system based on a milk solution with glycerol (9:1) was used. Antifungal activity was assayed on Petri dishes containing potato dextrose agar. In this case, the lyophilized extracts of fermented tarbush and control were prepared at 4 concentrations (0.1, 0.3, 0.5 and 1 g/L) in distilled water. An aliquot of 200 µL of cryoprotectant solution with the fungus was placed on agar and spread evenly with a sterile glass rod. Circles of Whatman no. 1 filter paper (2 cm in diameter) were completely moistened in the unfermented and fermented extracts for 12 h and then placed in the previously labelled boxes. Plates were subsequently incubated at 30 °C for 48 h and monitored every 24 h. The diameter of the zone of fungal growth was measured in millimeters in four directions and the average value was taken. This assay was made in triplicate, determining the percentages of fungal growth and the inhibition as the effect of the lyophilized extract of tarbush, evaluated at their respective concentrations according to Eqs. 2 and 3:

$$\text{Growth} = \frac{\text{Diameter of fungal growth}}{\text{Diameter of negative control}} \cdot 100 \quad /2/$$

$$\text{Inhibition} / \% = 100 - \text{Growth} \quad /3/$$

To measure the impact of the fermentation of tarbush on the antimicrobial capacity against phytopathogenic microorganisms, a potentiation constant was defined, which was calculated according to Eq. 4:

$$P_{\text{AF}} = \frac{AF_{\text{EF}}}{AF_{\text{ENF}}} \quad /4/$$

where P_{AF} is the potentiation of antifungal activity by the application of the fermentation process, AF_{EF} is the inhibition of fermented extract and AF_{ENF} is the inhibition of unfermented extract expressed in %.

Experimental design and data analysis

A factorial design with fixed factors was employed. The obtained results were analyzed with ANOVA and where it was needed, the mean values were compared using Tukey's multiple range test. Treatments were considered as different at $p < 0.05$.

Results

Fig. 1 shows the kinetic fermentation process of tarbush. An increment in the release of free phenols expressed as equivalents of catechin was observed at 12 h of fermentation, coinciding with an increase in the antioxidant activity. This increment can be explained by the

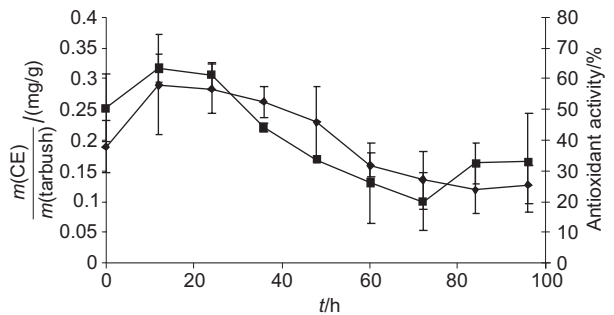


Fig. 1. Effect of fermentation time on antioxidant activity (■), and release of phenols expressed as catechin (◆) present in aqueous extracts of *Fluorensia cernua*

fungal degradation of cell components with the corresponding release of phytochemicals contained in tarbush cells. At 84 h of fermentation, there was an increase in the antioxidant activity because the fungi used enzymes to polymerize and depolymerize phenolic monomers and even reach a dimerization and trimerization of these monomers.

In this assay, the antifungal effect of aqueous extracts of lyophilized *Fluorensia cernua* during the fermentation process at concentrations of 0.1, 0.3, 0.5 and 1 g/L against two phytopathogenic microorganisms of apple such as *Penicillium expansum* and *Fusarium oxysporum* was found.

Fig. 2 shows the percentage of growth inhibition of *Penicillium expansum*, noting that the aqueous fractions of unfermented and fermented extracts showed an inhibition below 20%. The unfermented extracts showed an inhibition above 5% and the fermented extracts below 5% at a concentration of 0.1 g/L. Starting at the concentration of 0.3 g/L, any increase in the concentration was directly proportional to the inhibition up to 1 g/L in both extracts, which shows that the inhibition of fermented extract was higher than of the unfermented extract, but there was no significant difference in the inhibition of growth of *Penicillium expansum* in both extracts, except for those that occurred at the concentrations of 0.1 and 0.3 g/L. There was an increasing inhibition of the fungus in all extracts, except when the unfermented extract was used at a concentration of 0.1 g/L. The fermented extract showed the highest percentage of growth inhibition of *Penicillium expansum*, which was 18.64% at a concentration of 1 g/L.

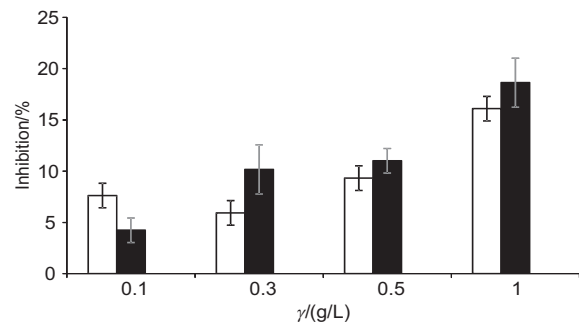


Fig. 2. Effect of concentration of the extracts of unfermented (□) and fermented (■) tarbush on the inhibition of *Penicillium expansum*

Fig. 3 presents the percentage of growth inhibition of *Fusarium oxysporum*, noting that the aqueous fractions of unfermented and fermented extracts show an inhibition near and above 20 % at a concentration of 1 g/L, respectively. Unfermented extract had an inhibition of 5 % and the fermented extract below 5 % at a concentration of 0.1 g/L. It is also seen that at the concentration of 0.3 g/L, there was a decrease in the inhibitory activity of the fermented extract and an increase in the unfermented extract, while above the concentration of 0.5 g/L the increased inhibition of both extracts was observed more

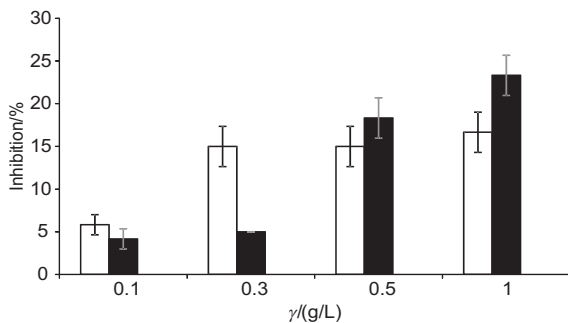


Fig. 3. Effect of concentration of the extracts of unfermented (□) and fermented (■) tarbush on the inhibition of *Fusarium oxysporum*

clearly, so that the fermented extract had a higher percentage of the inhibition of *Fusarium oxysporum* than the unfermented extract. No significant difference in the percentage of the inhibition of both extracts was observed, with the exception of the extracts at concentration of 0.3 and 1 g/L. In both extracts there was an increasing inhibition of the fungus as the concentration increased. The extract fermented for 12 h showed the highest growth inhibition of *Fusarium oxysporum*, which was 23.33 % at a concentration of 1 g/L, but the unfermented extract had more inhibitory activity than the fermented extract at concentrations of 0.1 and 0.3 g/L. The aqueous extract of fermented *Fluorensia cernua* showed an inhibition of *Fusarium oxysporum* above 20 % at a concentration of 1 g/L.

Since the inhibitory effect of the two extracts on fungal growth was similar, the percentages of inhibition of both extracts were divided, giving a value close to 1 or even 1, which indicates a very general view of the potential effect of the antifungal activity during fermenta-

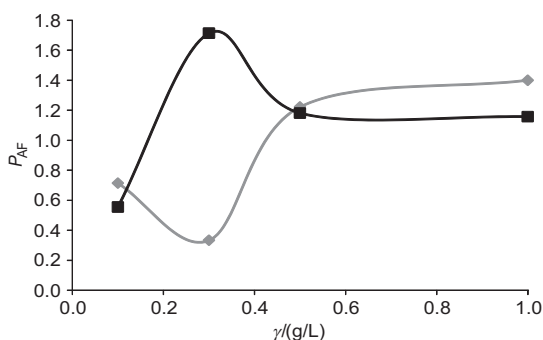


Fig. 4. Enhancement of the antifungal activity (P_{AF}) of aqueous extracts of fermented and unfermented tarbush against *Fusarium oxysporum* (■) and *Penicillium expansum* (◆)

tion of the extract. Taking the value 1 as the reference value, we obtained an interpretation of the concentration at which the greatest potential effect of inhibitory activity on the growth of *Penicillium expansum* and *Fusarium oxysporum* was reached, as shown in Fig. 4. These results show that the greatest effect of the inhibition of growth of *Fusarium oxysporum* is obtained at a concentration of 0.3 g/L, while at a concentration of 0.5 g/L the inhibition of *Penicillium expansum* and *Fusarium oxysporum* is similar.

Discussion

It is important to consider the antioxidant properties of polyphenols rather than the individual compounds, because of the possible antagonistic or synergistic interaction between all the compounds present in the culture broth (28). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which play an important role in absorbing and neutralizing free radicals. The antioxidant potential of polyphenolic compounds depends on the number of hydroxyl groups and the degree of conjugation of the structure, for example in flavonoid compounds and phenolic acids the activity improves as the number of hydroxyl groups increases (29). Our results are in agreement with these reports, because the high correlation between the antioxidant activity and the concentration of polyphenols in *Fluorensia cernua* is clearly visible. The increase in the antioxidant activity and quantification of free phenols as equivalents of catechin in the early stages of fungal growth is attributed to those proteins found in plant material that may have been attached to the polyphenolic compounds, causing an increase in the quantification of condensed tannins (30) and therefore in the antioxidant activity, due to the neutralization of free radicals by a large number of hydroxyl groups present in condensed tannins.

All polyphenols are capable of sequestering simple oxygen and free radicals, OH and NO, through the donation of electrons, generating a relatively stable radical phenoxyl. The protection mechanism of polyphenols occurs in the initial state and more effectively during the actual oxidation by capture of free radicals, thereby inhibiting chain reaction (31). In most of proanthocyanidins, the site of the radical is on the B ring and the replacement of the A ring has got only a limiting influence on the potential production of free radicals (32). Catechins belong to the group of flavonoids that exhibit biological and pharmaceutical effects including antioxidant activity (33).

In the present work condensed tannins displayed a higher entrapment of DPPH radicals at the beginning of fermentation and therefore had higher antioxidant activity, due to the increased availability of hydroxyl groups, since the condensed phenolic compounds are often proanthocyanidins, which may contain 50 or more units of flavonoids (34), compared to fractional molecules, as in this case the catechin. Also for this reason, the antioxidant activity may have been affected due to fungal degradation of condensed tannins in monomer equivalents of catechin, which occurs as a result of the aerobic breakdown of the catechin ring (flavan-3-ol) (35). Our results are in agreement with the results reported in the literature that

A. niger GH1 and *A. niger* PSH are able to produce enzymes that act on the links of condensed tannins, particularly catechin (36). Vattem and Shetty (37) reported a similar trend of the decrease of antioxidant activity which was attributed to the dimerization and trimerization of monophenols during the early stages of fungal growth. This process can lead to initiation of polymerization with other molecules present during fungal growth, which are products of stress induced in the fungus, due to nutrient depletion. In this way, its antioxidant capacity is reduced because the hydroxyl groups are no longer available and cannot perform the free radical trapping.

Most phenolic compounds found in plants are conjugated with sugars (mainly glucose) and glycosides. This combination reduces their ability to function as good antioxidants, since the availability of the free hydroxyl groups in phenolic rings is potentially important for the stabilization of resonance of free radicals (37).

This polymerization may be related to the action of fungal enzymes such as phenoloxidases. These can lead to degradation and polymerization of polyphenolic substrates (38), which could have resulted in polymerization. There are reports that indicate the effectiveness of enzymes such as laccase or *p*-diphenol oxygen reductase, which play an important role in the oxidative binding of ellagitannin monomers to form derived dimers and trimers (39). The content of phenolic compounds from different plant sources has been reported as responsible for the antioxidant activity (28), for which a high content of phenolics in the first instance could suggest a good antioxidant capacity (40). The antioxidant activity found in the Mexican semidesert plants is greater than that reported in *Allium sativum* L. (41), *Coriandrum sativum* L. (42) and several varieties of raspberries rich in ellagitannins (43). Since polyphenols may have unique protective roles as antioxidants, research has focused not only on food and beverages as a source of polyphenols, but also on structurally well defined crude extracts and polyphenolic compounds (44).

In the present work low yields of tannins were obtained compared to those reported by Belmares *et al.* (24), who evaluated the quantification of tannins from methanol and acetone extracts of *Fluorensia cernua*, obtaining a higher yield of hydrolyzable and condensed tannins, possibly due to the influence of the solvent used. Guyot *et al.* (45) reported that the use of solvents such as methanol allows the release of low molecular mass compounds, and subsequent acetone extraction releases the high molecular mass compounds. In addition, extracts were subjected to constant stirring under reflux for 7 h, applying heat to expedite the removal of these compounds (46). It is noteworthy that the tannin content is affected by climate, plant collection season, phenological stage and part of the plant used (leaves, stems, roots, *etc.*) (20).

Currently there are few studies reported about the quantification of tannins in aqueous extract of fermented *Fluorensia cernua*, since most have focused on tannin extraction using different solvents. Methanol and acetone are the most efficient in obtaining these compounds, but tend to be more polluting than water, so in the present work we chose to work with aqueous extracts, in addition to performing a less drastic process for the tannin

extraction and in shorter time. Chemical extraction processes for recovery of ellagic acid have been widely applied in many plants; however, these methodologies are polluting, costly and give low yields. For this reason, bio-logical alternative to this method has been studied.

Other phenolic antioxidants have been produced by solid-state fermentation, mainly ferulic acid and *p*-coumaric acid from fermented corn cobs (47). Vattem and Shetty (48) reported the use of solid-state fermentation for the production of phenolic antioxidants (rosmarinic acid, ellagic acid and resveratrol) of cranberry pulp using *Rhizopus oligosporus*. Regarding the use of tannins from the fruit peel of *Quercus aegilops*, Shi *et al.* (49) reported high levels of ellagic acid conversion by using a coculture of *Candida utilis* and *Aspergillus niger*.

The effectiveness of tarbush extracts in the inhibition of fungal growth of *Penicillium* species has been demonstrated in several works investigating cheaper alternatives and reduced environmental impact, but with the same activity, for handling this type of pests and diseases (50). Guerrero-Rodríguez *et al.* (51) reported the use of ethanol extracts of tarbush, which were more effective in inhibiting the growth of *Penicillium digitatum*, showing an average inhibition of 94.2 and 96.3 % at concentrations of 0.5 and 1 g/L, respectively. These results show higher effectivity compared to those obtained in this work, where the inhibition was about 20 % due to the high polarity of ethanol, which can extract phenolic compounds that inhibit the growth of *Penicillium*.

López-Benites *et al.* (52) reported that aqueous extracts of tarbush (5 and 10 %) had an inhibitory activity on the growth of *Fusarium oxysporum* slightly above 30 %. It is important to note that the used fractions were higher compared to those assessed in this work, where the inhibition above 20 % was achieved. Unfermented extract showed an inhibition above 5 % and the fermented extract below 5 % at a concentration of 0.1 g/L, thus showing that the use of tarbush extracts at low concentration (0.1 g/L) had fungicidal properties against species such as *Rhizoctonia solani*, *Fusarium oxysporum* and *Pythium* spp., representing a high potential as an agricultural fungicide (53).

Most of Mexican semidesert plants use high levels of polyphenols as a defense mechanism against microorganisms and natural repellents against animals, among other essential functions to their survival. These compounds show strong antifungal activity based on the ability of these molecules to form complexes with polysaccharides and proteins in the outer layers of fungal cells, destabilizing the cell wall and membrane function (54). Recently, our group (55) has reported that polyphenolic extracts of creosote bush represent one of the most powerful fungicides of the Mexican semidesert, as well as pecan nut shell (*Carya illinoensis*) and pomegranate peel (*Punica granatum*) extracts, demonstrating a high efficiency in the antifungal activity against some phytopathogenic fungi such as: *Pythium* spp., *Colletotrichum truncatum*, *Colletotrichum coccodes*, *Alternaria alternata*, *Fusarium verticillioides*, *Fusarium solani*, *Fusarium sambucinum* and *Rhizoctonia solani*, highlighting the growth inhibition of several *Fusarium* strains with the methanolic extract of pecan nut.

Guerrero-Rodríguez *et al.* (51) reported the inhibitory activity of unfermented tarbush ethanolic extracts against

important postharvest pathogenic fungi: *Alternaria alternata* (Fr.:Fr.) Keissl., *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. and *Penicillium digitatum* (Pers.:Fr.). Our present study demonstrates that this inhibitory activity can be enhanced when tarbush is previously subjected to fungal fermentation under controlled conditions.

Regarding the phytochemical changes and safety of fungal fermentation process of tarbush, our group reported earlier that the growth of *A. niger* strain was directly related to the biodegradation of tannins (hydrolysable and condensed tannins), degrading more than 77 % of total tannins of tar bush, improving its digestibility (*in vitro*) around 13.98 % and producing very low levels of aflatoxins (B₁ 182 ppt, B₂ <160 ppt and G₁ <160 ppt), eliminating any health risk by the consumption of this kind of fermented product, which conformed to the official norms (56).

Conclusions

The aqueous extract obtained from tarbush fermented for 12 h at 60 % of moisture and pH=5.5 with *Aspergillus niger* GH1 had the highest antioxidant activity of all fermented and unfermented extracts. This extract showed high inhibitory activity against the growth of *Penicillium expansum* and *Fusarium oxysporum* at an initial concentration of 0.5 g/L. Mexican semidesert plants represent an alternative for obtaining extracts with antioxidant and antifungal properties, which can be enhanced through a fermentation process to increase the biological activity of the substances with high potential bioactivity.

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