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Antifungal activity *in vitro* of ethanol and aqueous extracts of leaves and branches of *Flourensia* spp. against postharvest fungi

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ABSTRACT

Fusarium and *Rhizopus* rots occur in stored fruits and cause high post-harvest losses. The most common fruit affected by these fungi is tomato, which is the principal exportation product of Mexico. Chemical treatment is one of the postharvest techniques that are normally used to prolong shelf life and reduce food spoilage. However, the indiscriminate use of pesticides in horticultural products allows the resistance of plagues and also affects human health. The use of natural products, such as plant extracts for the management of fungal diseases in plants, is considered an alternative method to synthetic fungicides. The objectives of this research were to evaluate the antifungal activity *in vitro* of ethanol and aqueous extracts of leaves and branches of *Flourensia cernua*, *F. microphylla* and *F. retinophylla* against *Fusarium oxysporum* and *Rhizopus stolonifer*, which cause diseases in postharvest fruits and high economic losses in tomato; and to obtain information on the chemical composition of the bioactive components of the extracts. The chemical profile identified in ethanol extracts of leaves and branches of *F. cernua, F. microphylla* and *F. retinophylla*, was: 17 and 10 compounds; 7 and 9 compounds and 7 compounds in both tissues, respectively. *Flourensia* spp. ethanol extracts showed high effectiveness in the inhibition of mycelial growth of *F. oxysporum* and *R. stolonifer*. *F. cernua* inhibited *F. oxysporum* at MIC_{90} of 2163 mg/L for leaves ethanol extract and 4240 mg/L for branches ethanol extract. Also, *F. cernua* inhibited *R. stolonifer* at MIC_{90} of 1692 for both tissues. *Flourensia* spp. ethanol extracts represent an alternative to be used as a botanical fungicide, which could replace the use of synthetic fungicides.

1. Introduction

In the development of the agro-food chain, fruits and vegetables are affected by the incidence of diseases caused mainly by fungi during post-harvest stage (Ramos-García et al., 2010). In some cases, the fungi remain dormant or are acquired during the harvest, transportation and/or handling of the product (Ogden et al., 2005). For example, the rot of *Fusarium* and *Rhizopus* appear during the post-harvest, producing high losses (Hahn, 2002). These fungi are the main cause of diseases in tomato crop (Ascencio-Álvarez et al., 2008). Mexico is one of the largest tomato producers in the world and ranks first in the export of this fruit (SAGARPA, 2015). However, this crop presents high losses, of up to 50% of the total production (Bombelli and Wright, 2006). In order to prolong the post-harvest life of horticultural products, different technologies have been implemented (Quezada et al., 2003). However, these practices do not always guarantee the integrity of the product, forcing producers to apply different treatments during post-harvest conservation (Yahia et al., 2005). Synthetic fungicides have been used intensively for control of post-harvest fruit diseases (Jasso De Rodríguez et al., 2011). Nevertheless, lack of regulation in less developed countries allows the indiscriminate use of pesticides causing pathogen resistance, and harmful effects on the environment and on human health (Calvo et al., 2007).

Currently, herbal extracts from several endemic plants of the Mexican semi-desert have been evaluated as an alternative method against post-harvest fungi, due to their minimal harmful effects (Cueto Wong et al., 2010; Hernández-Castillo et al., 2011).

The plants of *Flourensia* genus, *Heliantheae* tribe, *Asteraceae* family, grow exclusively through the American continent from the south of the United States to Argentina (Dillon, 1984). These plants adapt perfectly

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to arid, semi-arid and desert regions, characterized by stress, drought, high temperatures and high UV radiation (Silva et al., 2012). This genus is composed of 40 species of aromatic and resinous shrubs that are characterized by the surface of the leaves, covered with an oil-fatty layer and abundant yellow flowers (Rios et al., 2013). Their identified metabolic products have interesting biological properties, that include: allelochemicals and herbicides (Diaz Napal and Palacios, 2013), anti-feedant, inhibitors of germination and plant growth (Diaz Napal et al., 2009; Silva et al., 2012), insecticides (García et al., 2007), antibacterial (Joray et al., 2011), antialgal, antitermite and antifungal (Tellez et al., 2001). The antifungal effect of ethanol extracts from *Flourensia* spp. against *Alternaria sp.*, *Rhizoctonia solani* and *F. oxysporum* has been reported (Jasso de Rodríguez et al., 2007); as well as, the aqueous extracts of *F. cernua* against *R. stolonifer, B. cinerea*, and *C. Gloeosporioides* (De León-Zapata et al., 2016); and, *F. cernua* with alternative organic solvents (lanolin, cocoa butter) against *R. solani* (Castillo et al., 2010). In *Flourensia* genus, chemical compounds with important biological activity have been identified: eudesman sesquiterpenes, chrysin, galangin, apigenin, kaempferol, galetin, quercetin derivatives, flavanones, 8-prenyl-flavanones, 8-prenyl-flavonols and 5-Acetyl benzofuranes (Rios et al., 2013).

In recent years, research has focused on the search for biofungicides to maintain the sustainability of the environment, crops and foods, reducing the use of synthetic chemicals (Valero Galván et al., 2014).

For the above, and continuing with the search and identification of natural compounds of the *Flourensia* genus in order to generate knowledge of its bioactive metabolites that might be the basis for the production of biofungicides, the present study is proposed, with the objectives of: evaluating the inhibitory activity *in vitro* of ethanol and aqueous extracts from leaves and branches of *F. cernua, F. microphylla,* and *F. retinophylla*, against *F. oxysporum* and *R. stolonifer*, fungi causing post-harvest diseases and significant economic losses in fruits of commercial interest, such as tomato, and to obtain information about the chemical composition of the bioactive components of the extracts.

2. Materials and methods

2.1. Plant material

Samples of leaves and branches of shrubs of *F. cernua, F. microphylla*, and *F. retinophylla* (20 shrubs per species) were identified *in situ* by the herbarium curator Dr. José Ángel Villarreal Quintanilla, and randomly collected in wild sites of the semi-arid region located in the southern part of the Coahuila State, Mexico (Jasso de Rodríguez et al., 2007). The samples were deposited in plastic bags and transported to the Laboratory of Phytochemistry of the Universidad Autónoma Agraria Antonio Narro (UAAAN), and immediately were dried in a stove (Mapsa, Mexico) at 60 °C for 48 h, and grounded using a mill (Thomas Wiley, Swedesboro, NJ, USA) with a 2 mm sieve screen. Plant vouchers (*F. cernua* No. 96035, *F. microphylla* No. 43362 and *F. retinophylla* No. 82956) were deposited in the Herbarium (ANSM) of the UAAAN.

2.2. Extraction of phytochemical compounds

The extraction of the plants was carried out using a soxhlet extractor, with ethanol and water as solvents; using a standardized method in the Phytochemical Laboratory. Briefly, each solvent (200 mL) was mixed with 14 g of each plant sample. Extraction was maintained for 72 h. The solvents from the extracts were removed under low vacuum, using a rotary evaporator (Yamato Scientific Co., Ltd., Tokyo, Japan). The remaining solvent was removed by placing the flasks on a drying oven at 50 °C for 24 h. The extracts were preserved in a desiccator at 25 °C and 0% of relative humidity (RH) until its use in the bioassays.

2.3. Quantification of total phenols and antioxidant capacities of Flourensia spp. extracts

In order to obtain the phenolic content and antioxidant activity, *F. cernua, F. microphylla* and *F. retinophylla* extracts were diluted in ethanol in a concentration of 10 mg/mL (v/v).

2.3.1. Total phenolic content (TPC)

The TPC of *Flourensia* spp. leaves and branches ethanol and aqueous extracts were evaluated by the Folin-Ciocalteu assay, following the IFC method (Commission Regulation, EEC NO 2676/90). Briefly, 20 µL of the *Flourensia* spp. dilutions, blank and calibration curve of gallic acid (GA) were mixed with 120 µL of NA_2CO_3 (15% w/v), 30 µL of Folin-ciocalteu reagent and 400 µL of water. The reaction was performed at 50 °C for 5 min. Absorbances were measured at 700 nm.

2.3.2. Antioxidant activity

The antioxidant activity of *Flourensia* spp. extracts was performed by 2 methods: 1,1diphenyl-1-picrylhydrazyl (DPPH·) and Ferric reducing antioxidant power (FRAP).

2.3.2.1. DPPH· scavenging capacity assay.. The DPPH· assay was carried out according to the method reported by Rodríguez-Jasso et al. (2014). Briefly, a solution of DPPH· reagent (60 µM) was prepared. A volume of 2950 µL of DPPH· was added to 50 µL of sample. The solution was shaken and incubated in the dark for 30 min at room temperature. The reduction of the DPPH· radical was measured by continuous monitoring of the decrease of absorption at 517 nm. The control was 100 µL of distilled water. The results were showed as percent of inhibition, calculated by the following equation:

$$\%\text{Inhibition} = \left[\frac{1 - \text{Abs}_{\text{sample}}}{1 - \text{Abs}_{\text{control}}}\right] x100$$

2.3.2.2. FRAP assay. The assay was performed according to the method described by Benzie and Strain, (1996). The FRAP reagent was prepared by mixing 300 mM acetate buffer (3.1 g sodium acetate trihydrate and 16 mL glacial acetic acid, pH 3.6), 10 mM TPTZ (2,4,6-tri(2 pyridyl)-*s*-triazine) solution in 40 mM HCl, and 20 mM ferric chloride hexahydrate solution (10:1:1, v/v/v) and incubating at 37 °C for 10 min. The working solution of FRAP reagent was always freshly prepared prior to use. Samples of 10 µL of *Flourensia* spp. extracts were placed in a microplate. Subsequently, 290 µL of FRAP reagent were added to each well containing the extracts, and then the microplate was incubated at 37 °C for 15 min to finally read the absorbances of the colored products (Ferrous-TPTZ complex) at 593 nm in a microplate reader (Synergy HT, BioTek, Winooski, VT, USA). Results were expressed as milliequivalents of trolox per 100 milligrams of extract (mEqTrolox/100 mg extract).

2.4. Chemical composition analysis of Flourensia spp. extracts by gas chromatography–mass spectrometry (GC–MS)

GC–MS analysis was carried out in Agilent Technologies 6580 GC attached to a 5975 B MSD in Full Scan mode. The separation of metabolites of the extracts was performed using a HP-5MS column (30 m × 0.25 mm 1D × 0.25 µ) and an ionization system with energy of 70 eV. Helium was used as a carrying gas, with a constant flow at 1.1 mL/min and an injection volume of 1 µL were used, the injection temperature was 250 °C. The warming program of the oven was isothermal for 5 min at 60 °C, followed by a warming of 5 °C/min up to 100 °C/min held by 2 min, and 10 °C/min up to 250 °C/min and held by 5 min. The interpretation of the mass spectra of the compounds was made using the NIST (National Institute of Standard and Technology) library.