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Phytochemical profile of methanolic extracts from Chilca (*Baccharis glutinosa*) roots and its activity against *Aspergillus ochraceus* and *Fusarium moniliforme*

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Abstract

Aim : The present study aimed to identify the phytochemicals of methanolic extract from *Baccharis glutinosa* (chilca) roots (MEBg) and to evaluate its antifungal activity on two major fungal pathogens of agricultural importance.

Methodology : The antifungal activity was evaluated by inhibition halo, minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and % sporulation against *A. ochraceus* and *F. moniliforme*. As a preliminary test, inhibition halo was tested using 1, 10, 100 and 270 mg ml⁻¹ of MEBg. Different concentrations of MEBg were applied for MIC and MFC tests. Ketoconazole was used as positive control. The treatments were applied in triplicate. The phytochemical compounds of MEBg were determined by GC-MS analysis.

Results : The MEBg produced an inhibition zone of 2 to 4 mm in the inhibition halo test, with concentrations of 100 and 270 mg ml⁻¹ for *A. ochraceus* and *F. moniliforme*, respectively. Reduction in % sporulation above 50 was shown in concentrations over 8 mg ml⁻¹. MEBg were reported to exhibit antifungal activities against *A. ochraceus* and *F. moniliforme* with the MIC values ranging from 2 to 5.6 mg·ml⁻¹ and the MFC from 12 to 15 mg ml⁻¹. GC-MS analysis of Chilca extracts revealed that the most abundant metabolites were furfural compounds and organic acids. The most abundant furfural compounds were 5-(hydroxymethyl) furan-2-carbaldehyde (38.59%), furan-2-carbaldehyde (4.103%) and 5-methylfuran-2-carbaldehyde (2.1%).

Interpretation : The MEBg revealed efficient antifungal activity, likely due to the presence of bioactive compounds, which could be used as an alternative for biological control of pathogenic fungi in maize and coffee crops.

Key words: Antifungal activity, *Aspergillus ochraceus*, *Baccharis glutinosa*, Furfural compounds, *Fusarium moniliforme*

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Introduction

Plants are a source of bioactive compounds. The most important of these compounds, are secondary metabolites, like polyphenols, alkaloids, saponin, flavonoids and tannins (Ertani *et al.*, 2015; O'Bryan *et al.*, 2015; Piasecka *et al.*, 2015; Savatin *et al.*, 2014). Recently, these phytochemicals obtained from different parts (roots, stems, leaves) of the plant have been used as bioinsecticides, antifungals, antibacterials, anti-protozoa and antioxidants (Kouame *et al.*, 2018; Bassiri-Jahromi *et al.*, 2015; Granados-Echegoyen *et al.*, 2015; Caballero-Gallardo *et al.*, 2014). It is known that these natural products are extensively used as they are less toxic to humans than most synthetic antifungals, (Pandey *et al.*, 2017; Avaz *et al.*, 2013; Bakkali *et al.*, 2008; Leeja and Thoppil, 2007). Common examples are sampangine, obtained from the root of African tree *Cleistopholis patens*, coruscanone A and B from *Piper coruscans*, several thiofenes from the Mediterranean plant *Echinops ritro* (Asteraceae) and a steroidal saponin from cayenne pepper (*Capcicum frutescens*) (Yoon *et al.*, 2013; Duke *et al.*, 2010).

Baccharis glutinosa, commonly known as chilca, has shown antimicrobial activity against *Microsporum canis*, *Microsporum gypseum*, *Trichophyton tonsurans*, *Epidermophyton floccosum*, *Sporotrix schenckii*, *Nocardia asteroides*, *Nocardia brasiliensis*, *Listeria monocytogenes*, *Clostridium perfringens* and *Proteus vulgaris* (Verástegui *et al.*, 1996). Extractions with organic solvents from aerial parts of *Baccharis glutinosa* have demonstrated the inhibition of β -1, 3-glucanase by acting as a competitive inhibitor (Buitimea *et al.*, 2013). However, to our knowledge there are few reports regarding phytochemical composition and antifungal activity by *B. glutinosa* against *A. ochraceus* and *F. moniliforme*.

These two fungi produce ochratoxin A and fumonisin mycotoxins (Bui-klimke and Wu, 2015; Adejumo and Langenkämper, 2012), and have been associated with pathologies such as nephrotoxicity, hepatotoxicity and renal tumors in a variety of animals (Buitimea *et al.*, 2013). In addition, according to recently reported data, the economic losses of food production due to mycotoxins are estimated between hundreds of millions and billions of pesos annually (Pinotti *et al.*, 2016). Therefore, the present investigation was carried out to study the *in vitro* effect of MEBg on mycelial growth and sporulation of *F. moniliforme* and *A. ochraceus*. Also, the phytochemical composition of *B. glutinosa* was analyzed in order to identify the probable presence of metabolites with antifungal potential.

Materials and Methods

Plant material: The roots of *B. glutinosa* were collected from Chiapas, México, in San Cristóbal de las Casas. Taxonomic identification of plants was done at 'Secretaría de Medio Ambiente e Historia Natural' of Chiapas State, México. Collected samples were filed in the scientific collection of the herbarium under registration number 49985 and scientific name *Baccharis glutinosa* Pers.

Methanolic extracts: The dried roots were ground to obtain a fine powder. Three grams of pulverized material were placed into a 250 ml erlenmeyer flask with 50 ml of methanol and macerated for 24 hr. Afterwards, the obtained mixture was then filtered and centrifuged at 3000x g for 5 min, the solvent was evaporated under vacuum at 55°C until the crude extract (MEBg) was obtained. The MEBg was dissolved in distilled water and prepared at defined concentrations for each bioassay.

Fungal species: *F. moniliforme* Wilhelm and *A. ochraceus* J. Sheld were obtained from 'Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias' (INIFAP), Mexico.

Spore suspension: Spores were harvested from a 15-day-old culture of *A. ochraceus* and *F. moniliforme* grown on potato dextrose agar medium (PDA) at 32°C. These were obtained by adding 10 ml sterile distilled water containing 0.01% (v/v) Tween® 80 (Merck KGaA, Darmstadt, Germany) and scraping the mycelial surface gently with an L-shaped glass spreader. The spores in the resulting suspension were counted using a Neubauer's Chamber (depth 0.1mm, 0.0025mm²) and expressed as spores per milliliter. The final spore concentration was adjusted by adding sterile distilled water and was prepared on the same day when the experiment was carried out.

Inhibition halo: Preliminary screening and susceptibility testing for antifungal activity the mycelial growth was determined by agar well diffusion method. Briefly, Petri dishes with a diameter of 90 mm diameter were spread with potato dextrose agar (PDA). The wells (6 mm diameter) were cut from the agar and filled with 100 μ l of methanolic extracts from *B. glutinosa* using the concentrations 1, 10, 100 and 270 mg ml⁻¹. Positive control wells containing ketoconazole were also tested. Then 1 ml of fungal spore suspension (1x10⁶ spores ml⁻¹) was spread over Petri dishes. The dishes were incubated at 32°C, and the mean value of two perpendicular diameter of inhibition zone was reported in millimeters after 48 h.

Minimum Inhibitory Concentration (MIC) and Minimal Fungicide (MFC): MIC was tested by use of a broth macrodilution method. Tubes containing 9 ml PDA broth with methanolic extracts from *B. glutinosa* in concentrations of 0.09, 0.16, 0.26, 0.43, 0.73, 1.21, 2, 3.4, 5.6, 8, 10, 12 and 15 mg ml⁻¹, were inoculated with 1 ml of fungal spore suspension (5x10⁴ spores ml⁻¹), *A. ochraceus* and *F. moniliforme*, respectively. MICs were read after 48 hr of incubation. The MIC was defined as the lowest concentration at which no growth was seen. MFC was determined using 1 ml aliquots from the tubes assayed in the MIC and were spread on PDA petri dishes and incubated at 32°C for 48 hr. The MFC was defined as the lowest concentration of methanolic extracts of *B. glutinosa* that showed no visible growth on the Petri dishes.

Sporulation: The % sporulation was assayed by collecting spores from Petri dishes (MFC) stored for 15 days at 32°C, with 10 ml sterile distilled water containing 0.01% (v/v) Tween® 80 by

scraping the mycelial surface gently with an L-shaped glass spreader. The obtained spore suspension was counted with a Neubauer's Chamber.

Phytochemical study: As an analysis strategy, the MEBg was partitioned into two fractions. MEBg was suspended in hexane and the soluble organic layer was separated and concentrated to dry under vacuum at 55°C. The insoluble residue was the methanolic fraction. Volatile components of hexanic and methanolic fractions were analyzed by an Agilent Technologies 7890A gas chromatograph, which was coupled to a VL MSD 5975C mass spectrometer. Analysis was based on retention rates and mass spectra. The procedure was performed on a 60 m DB-WAXetr column, with an internal diameter of 0.25 mm and 0.25 µm thickness. For the analysis of extract, the temperature of the injector was 70°C, whereas the temperature of the detector was 250°C. Helium was used as the gas for the mobile phase, with a volumetric flow of 1 ml min⁻¹. The GC-MS generated fragmentation patterns were analyzed according to ion rupture.

Statistical analysis: Statistical analysis was performed by Statgraphic Centurion version XV software. A simple ANOVA was performed with a comparison of means using Tukey test to determine significance different treatments at 95% confidence.

Results and Discussion

MEBg inhibited the growth of *A. ochraceus* and *F. moniliforme* at different concentrations (Fig. 1). For *A. ochraceus* strain, the presence of this extract at 1-10 mg ml⁻¹ did not inhibit the growth, but nevertheless occurred at 100 mg ml⁻¹ and 270 mg ml⁻¹ from 2 to 4 mm. Meanwhile for *F. moniliforme*, MEBg showed a significant inhibition halo ($P < 0.05$) at 270 mg ml⁻¹ with inhibition zone of 4 mm. Plant extracts with biological activity may have an

effect on organisms at different levels. The effect of these extracts depends on fungal species (Tej *et al.*, 2018). In the case of fungi, the effect may be inhibition of mycelial growth or spore germination (Juvaddi *et al.*, 2015). Once the susceptibility of extract was confirmed by well diffusion (inhibition halo), lower concentrations were tested for MIC and CFM. Antifungal activity was evaluated by determining MIC and CMF. The fungal species used were susceptible to MEBg, with MIC values ranging from 2 and 5.6 mg ml⁻¹, and MFC values are 15 and 12 mg ml⁻¹ for *A. ochraceus* and *F. moniliforme*, respectively. The inhibitory effect of MEBg on *A. ochraceus* revealed that the ability to sporulate was gradually lost as the concentration of extract increased up to 8 mg ml⁻¹ (Fig. 2), and spores were completely eliminated at 10 mg ml⁻¹. However, the MFC found to be 15 mg ml⁻¹. This means that although there were no spores, the fungus was still able to generate mycelial growth until reaching the MFC value.

These results suggest that MEBg have a fungicide effect as the positive control, Ketoconazole, used at bioassays. According to Parveen *et al.* (2004), when the fungal strain is under chemical stress on the membrane, the suppression of certain proteins occurs. It is speculated that proteins can be related to cell growth as well as its differentiation, which are regulated via activation of routes of transduction signals in response to external stimuli. Phosphorylation / dephosphorylation proteins have been shown to be important for the regulation of routes, as reported by Kanshin *et al.* (2015) and Brodhagen *et al.* (2006). They found that protein kinase (PKA) is involved in the morphogenesis of ascomycetes, noting that the ability to sporulate is greatly reduced when the pathway is affected by the induced absence of aforementioned protein.

The effect of different concentrations of MEBg on the growth and sporulation capacity of fungal strains, *A. ochraceus*

Fig. 1: Effect of MEBg concentration on the growth of *A. ochraceus* and *F. moniliforme*. Values were expressed as mean $\pm$ SD; * $p < 0.05$ indicated significant differences according to Tukey test.

Fig. 2: Effect of MEBg on the germination capacity of spores. K= ketoconazole. Values were expressed as mean $\pm$ SD; * $p < 0.05$ indicated significant differences according to Tukey test.

and *F. Moniliforme* (Fig. 2) showed that concentrations between 0.09 mg ml⁻¹ and 5.6 mg ml⁻¹ did inhibit sporulation. However, a concentration of 8.0 mg ml⁻¹ indicates substantial inhibition, which had an interval of inhibition between 95% and 100% at 10 mg ml⁻¹. For *F. moniliforme* the effect was similar. The inhibition increased at 8.0 mg ml⁻¹. In this case, 100% inhibition was achieved at 12 mg ml⁻¹ (Fig. 2). The difference in percent inhibition was statistically significant ($P < 0.05$). Although *A. ochraceus* and *F. moniliforme* belong to kingdom fungi, the nature of their genus have shown different characteristics, including their metabolic machinery, not yet clearly defined, as well as interaction with plants and life cycles. In addition, in spite of their cellular characteristics being similar in structure and function, they will present variations in chemical compositions and production stimuli (Pusztahelyi *et al.*, 2015). Different percentages of sporulation inhibition in fungus could be influenced by nature of each.

It was observed that, as the treatment doses increased, sporulation capacity was reduced to a limit concentration whereby both spore germination and mycelial growth were completely inhibited. Thus, it is possible that suppression of some essential proteins could have occurred (GSK-3 β , CK1, CDK1, MAPK) (Juvaddi *et al.*, 2015; Yang and Dickman, 1999). It has been suggested that there is a close association between proteins and the abundant production of mycelial growth. Also, an increase in the number of hyphae points has been correlated with an increase in protein production and its secretion in some cases, as well as synthesis of important phospholipids as signaling molecules, which seems to increase during mycelial elongation (Meyer *et al.*, 2009; Brodhagen and Keller, 2006).

In order to identify the main metabolites from *B. glutinosa* roots, MEBg were analyzed by GC-MS. As experimental strategy, the metabolites of crude extract were first physically separated into hexanic and methanolic fractions, according to polarity, to improve the chromatographic analysis. The more abundant components in the hexane fraction were polyketide structures (Table 1). These metabolites were (*E*)-13-ethoxytridec-12-en-1-yl acetate, (1*E*,6*E*,12*E*,14*E*)-1-(hydroxymethoxy) hexadeca-1,6,12,14-tetraen-4-ol, Tetradecyl-butyrate, 1-(2 oxopropoxy) tetradecan-2-one, Dodecyl-butyrate and (*E*)-12-(furan-3-yl)-4-oxododec-7-en-1-yl hydrogen carbonate. More than 1100 polyacetylene components have been identified in the Asteraceae family (Konovalov, 2014), wherein various types of activity have been demonstrated such as cytotoxic, antimicrobial, anti-inflammatory, among others.

The diversity of compounds of this class is long, including aliphatic and cyclic structures, which may contain oxygen, nitrogen and sulfides. The presence of furanopolyacetylenes has been found in some tribes of Asteraceae family, commonly in the genera *Leucanthemum* (Anthemideae) and *Atractylodes* (Cardueae) (Konovalov, 2014). Furanopolyacetylenes structures were reported for the first time in the roots of *B. thymifolia*, also furfural components has been reported for aerial parts, such as (2*E*, 6*E*)-2-(4-Methylpent-3-en-1-yl)-6-[3-(2,5-dihydro-2-oxofuran-3-yl)-propylidene] hept-2-enedioic acid, which was considered as an interference in larvae insect development (Hikawczuk *et al.*, 2008). Ramos *et al.* (2016) reported studies of hexane fractions, mainly finding anti-inflammatory activity in extracts of genus *Baccharis* (*B. latifolia*, *B. btusifolia*, *B. subulata*, *B. pentlandii*).

Table 1 : Principal components of *Chilca hexanic* extracts analyzed by CG-MS

Number	Retention time	Compound	Percentage (%)
1	12.09	2- hexanone	0.7
2	68.589	Dodecyl-butirate	4.32
3	70.34	Tetradecyl-butirate	6.89
4	72.087	(E) - 2 - hidroxi - 7 - metilhexadeca - 8,15 – dienoico acid	0.7
5	80.508	Heptadecyl acetate	0.74
7	82.099	(E)-1-(aminomethoxy)-12-(furan-3-yl)dodec-7-en-4-one	2.78
8	83.539	(E)-12-(furan-3-yl)-4-oxododec-7-en-1-yl hydrogen carbonate	3.64
9	94.155	(E)-6-((1-hydroxy-5-oxohex-2-en-1-yl)oxy)-5,6-dioxohexanoic acid	1.89
10	97.311	(E)-13-ethoxytridec-12-en-1-yl acetate	38.17
11	99.19	(4E,10E)-allyl dodeca-4,10-dienoate	2.18
12	104.79	(2E,6E,8E,10E)-5-oxohexyl 11-acetoxy-4-oxoundeca-2,6,8,10-tetraenoate	0.91
13	107.47	1-(2-oxopropoxy)tetradecan-2-one	5.16
14	113.236	(1E,6E,12E,14E)-1-(hydroxymethoxy)hexadeca-1,6,12,14-tetraen-4-ol	27.1

The analysis of MEBg separated by methanolic fraction (Table 2) detected that the most abundant compounds were 5-(hydroxymethyl) furan-2-carbaldehyde, (Z)-4-hydroxy-5-((6-hydroxyhexyl)oxy)pent-4-en-2-yn-1-yl acetate, furan-2-carbaldehyde, 3,5-dihydroxy-2-(2-hydroxyethyl)-6-(hydroxymethyl)-2H-pyran-4(3H)-one. Konovalov (2014) reported that the polyacetylenes present in plant species of Asteraceae family are classified into individual subgroups, such as thiophenes, dithacyclohexadienes (thiarubrin), thioesters, sulfoxides, sulfones, alquimides, chlorohydrins, enol esters spiroacetals, furans, pyrans, tetrahydropyrans, isocoumarols and aromatic acetylenes. This coincides with the furan and pyran structures reported in this study. Wei-Ming *et al.* (2013) reported that the functional group on the furan ring plays a crucial role in

inhibiting the enzyme tyrosinase, important for the development of fungi. They further confirmed the inhibition constants in the following order: furfural>furoic acid>furfuryl alcohol. Also, in this study it was observed that furan compounds inhibited the proliferation of *Salmonella bacteria* and *Bacillus subtilis* at different concentrations. Although it has not been defined, the mechanism of action of these compounds could be considered an inactivation of cellular enzymes. Lopes da Silva *et al.* (2017a) and (2017b) reported that the growth, physiology and ethanol production of *Saccharomyces carlsbergensis* was severely affected by furfural presence due to an increase in oxygen reactive species, membrane depolarization, increase in membrane permeability, and reduction in lipid synthesis. Liu *et al.* (2004) reported that yeast strains *Saccharomyces cerevisiae* and

Table 2 : Principal components of *Chilca* methanolic extracts analyzed by CG-MS

Number	Retention time	Compound	Percentage (%)
1	21.78	1-ethoxypropan-2-one	0.18
2	30.387	propan-1-ol	6.74
3	31.133	furan-2-carbaldehyde	4.1
4	36.76	5-methylfuran-2-carbaldehyde	2.1
5	40.59	furan-2-ylmethanol	0.9
6	41.056	butyric acid	2.22
7	48.073	2-hexylfuran	0.37
8	50.15	(E)-3-(E)-prop-1-en-1-yloxy)acrylic acid	1.22
9	70.408	tetradecyl butyrate	2.05
10	71.202	3,5-dihydroxy-2-(2-hydroxyethyl)-6-(hydroxymethyl)-2H-pyran-4(3H)-one	3.26
11	82.861	5-(hydroxymethyl)furan-2-carbaldehyde	38.6
12	83.652	(3E,6E,8Z)-1-ethoxy-11-(furan-2-yl)undeca-3,6,8-triene-1,2,9-triol	0.9
13	85.278	(E)-2-(hexadeca-7-en-12,14-diyn-1-yl)furan	0.85
14	94.29	3-hydroxy-N-((3E,5E)-2-hydroxy-6-(((E)-1-hydroxyhex-2-en-1-yl)oxy)hexa-3,5-dien-1-yl)hexanamide	1.72
15	97.488	(Z)-4-hydroxy-5-((6-hydroxyhexyl)oxy)pent-4-en-2-yn-1-yl acetate	22.17
16	99.553	(5E,14E)-3-hydroxy-8-oxohexadeca-5,14-dienoic acid	2.63

Pichia stipitis showed differences in inhibitor tolerance reflected by varied durations of lag phases for cultures exposed to furfural and/or hydroxymethylfurfural (HMF). Consequently, furfural and HMF have been shown to inhibit important metabolic enzymes *in vitro*, including alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase (Modig and Taherzadeh, 2002). Recent studies have shown that 5-hydroxymethylfurfural has therapeutic potential in the treatment of Alzheimer's disease (Aijing *et al.*, 2014).

In conclusion, the results obtained in this study show fungicidal capacity of MEBg on *A. ochraceus* and *F. moniliforme*. The most abundant component found in MEBg were polyketides, mainly furfurals. Overall, the antifungal activity of MEBg can be attributed to changes in reactions synthesis of certain proteins related to cell wall, which affect fungal growth and morphogenesis. To our knowledge, this is the first study reporting on phytochemical composition and biological activity of *Baccharis glutinosa* roots.

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