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Article

Antifungal effect and chemical study of *Eysenhardtia polystachya* (Fabaceae) on *Phanerochaete chrysosporium* and *Ganoderma lucidum*

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Abstract

Forest species are a source of timber and non-timber products. In addition to being important in obtaining botanical extracts, they contain in their tissues numerous secondary metabolites that have been recognized for their diversity and biological activity, considered as an alternative against fungi that cause rot and degradation in wood (xylophagous fungi), as well as fungi that cause diseases in agricultural crops. In this sense, it was proposed to study *Eysenhardtia polystachya* as a natural preservative source of wood due to its high resistance to stem rot. For this, extracts were obtained from leaf, sapwood, and heartwood with solvents of different polarities. Scrutiny and selection of the extracts with greater efficacy in *Phanerochaete chrysosporium* and *Ganoderma lucidum* were carried out; they were subjected to column chromatography. The content of phenols and flavonoids was determined. The leaf extracts showed selectivity regarding mycelial inhibition in *P. chrysosporium*, with greater sensitivity to the extracts than *G. lucidum*. The synergistic effect of the compounds present favors inhibition in *P. chrysosporium*. Therefore, *E. polystachya* presents chemical compounds that can preserve the wood and prolong its use time against attack by xylophagous fungi and generate control in phytopathogenic fungi.

Keywords:

mycelial inhibition, secondary metabolites, xylophagous fungi.

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Introduction

Wood, as a renewable natural resource, plays an important role in the global economy, particularly in the construction and manufacture of furniture (González-Laredo *et al*., 2015). Like any other organic material, wood is susceptible to deterioration by mechanical and biological factors, mainly by degrading insects and fungi (xylophages) (Goktas *et al*., 2007). To protect and prolong the useful life of products that use wood, different synthetic chemical compounds have been developed, which have not been widely accepted because they mostly contain toxic compounds that damage health and pollute the environment, such as soil and water.

Therefore, one of the priorities for the preservation of wood is to look for environmentally friendly alternatives that reduce the use of these chemical compounds and thus avoid negative effects as much as possible (Tascioglu *et al*., 2013). Considering that plants synthesize and store various secondary metabolites with biological activity, which can be extracted to evaluate their potential use as a sustainable alternative (Stevenson *et al*., 2017; Shakya *et al*., 2019).

For more than 100 years, several plant species have been considered as an option to evaluate their extracts or some of their fractions as biological control agents against organisms related to wood degradation, mainly termites and xylophagous fungi (Ramírez-López *et al*., 2016).

Thus; for example, Goktas *et al*. (2007) mentioned the antifungal effects of extracts obtained from leaves and flowers of *Nerium oleander* against the fungi *Postia placenta* and *Trametes versicolor*. As state Tascioglu *et al*. (2013), many tree species have been shown to have various functions in agronomic (pest control), industrial (wood for use in construction), and medicinal fields; the bark of mimosa (*Acacia mollissima*) and quebracho (*Schinopsis lorentzii*) proved to be efficient against wood-degrading fungi: *Trametes versicolor*, *Pleurotus ostreatus*, *Gloeophyllum trabeum,* and *Fomitopsis palustris*.

On the other hand, Brocco *et al*. (2017) in a study to assess the wood preservation potential, reported favorable effects of ethanolic extracts obtained from teak (*Tectona grandis*) heartwood against *Postia placenta*. In another more recent study, Lajnef *et al*. (2018) reported that extracts of pomegranate (*Punica granatum*) and *Melia azedarach* obtained from the bark were efficient as antifungal agents against *Coriolus versicolor* and *Coniophora puteana*, improving the durability of beech and maritime pine woods.

Despite existing studies on the effect of plant extracts on the growth of wood-degrading fungi, in Mexico, there is a diversity of plant species that have not been evaluated to know their potential as a source of natural compounds with antifungal activity, such as the legume known as 'palo dulce or 'palo azul' *Eysenhardtia polystachya* (Fabaceae).

Although this tree species is recognized in traditional medicine (Pablo-Pérez *et al*., 2016) and there are studies of extracts with pharmacological properties (Pablo-Pérez *et al*., 2018), reports of antimicrobial activity are still limited. Other reports consider that leaf extracts of *E. polystachya* demonstrated an effect on the growth of pathogenic microorganisms such as *Escherichia coli* (Alonso-Castro *et al*., 2018).

One of the studies carried out in the working group showed that extracts obtained from sapwood and heartwood of *E. polystachya* inhibited mycelial growth in the fungi *Rhizoctonia solani* and *Sclerotium cepivorum* (Bernabé-Antonio *et al*., 2017). The aim of this study was to evaluate the antifungal and chemical effects of different extracts obtained from 'palo dulce' *E. polystachya* against *Phanerochaete chrysosporium* and *Ganoderma lucidum*.

Materials and methods

Collecting *Eysenhardtia polystachya*

In July 2019, leaves and stems of the *E. polystachya* shrub were collected from wild populations located in the communities of San Luciano and Las Trojes, municipality of Jocotepec, Jalisco.

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Between the coordinates 20° 10' 00" north latitude and 103° 17' 30" west longitude at 1 840 masl. The specimen was transferred to the Natural Products Laboratory of the Department of Wood, Cellulose, and Paper (University of Guadalajara), where it was processed to obtain the biomass of its different organs (leaves, sapwood, and heartwood). The samples of the specimen collected from the shrub were classified according to what was reported by the Northwest Mexico Herbarium Network.

Obtaining extracts

Two hundred thirty-eight grams of leaves were obtained by drying the leaves at room temperature and grinding them; to obtain the samples of the woody tissue, we proceeded to the debarking of stems, splintering, and grinding of the splinters (mesh 1 mm) to obtain 300 g of sapwood sawdust and 285 g of heartwood. Each of the samples was macerated with solvents in ascending polarity (hexane, diclomethane, and methanol).

The maceration with hexane was carried out for three days, it was filtered with medium pore Whatman® paper (Sigma Aldrich, USA), concentrated in a rotary evaporator at 65 °C Rotava-RE-114 and vacuum pump B-169 (Büchi, Switzerland). Subsequently, the biomass was left to dry at room temperature to be subjected to maceration with diclomethane, concentrated in a rotary evaporator at 35 °C. Subsequently, the same samples were macerated with methanol and concentrated at 75 °C. The hexanic extract was defatted to obtain a fatty extract and a fat-free one.

From the dichloromethanic extracts, two fractions were obtained, that of the first wash with hexane and the second with ethyl acetate. Obtaining a total of 15 extracts, five extracts per type of plant biomass (sapwood, heartwood, and leaf), which were used for the evaluation against the xylophagous fungi *Phanerochaete chrysosporium* and *Ganoderma lucidum*.

Phanerochaete chrysosporium and *Ganoderma lucidum* strains

The fungi used for this study were obtained from pure strains sheltered in test tubes at 26 ° C, granted by the Bioengineering Laboratory of the Department of Wood, Cellulose, and Paper of the University Center of Exact Sciences and Engineering of the University of Guadalajara; they were reactivated in Petri dishes with potato dextrose agar to be subjected to evaluation in the presence of the extracts.

Evaluation of extracts against *P. chrysosporium* and *G. lucidum*.

Preparation of organic extracts

The extracts obtained were dissolved in ethanol at 96° (1 mg ml^{-1}). Cercobin® (methyl thiophanate) was used as a positive control, and ethanol was used as a negative control. The experimental design of the treatments that were evaluated in blocks is shown in Table 1, where a completely randomized model was performed, with an n= 3 per treatment.

Table 1. Experimental design of the antifungal activity vs. *P. chrysosporium* and *G. lucidum* of the organic extracts obtained (n= 3, per treatment).

Extract Organ	1	2	3	4	5
Leaf	T1	T2	T3	T4	T5
Sapwood	T6	T7	T8	T9	T10
Heartwood	T11	T12	T13	T14	15
Control (+) Cercobin®	T16	Control (-) Ethanol	T17		

1= fatty extract (FE); 2= defatted hexanic extract (DHE); 3= dichloromethane-ethyl acetate extract (DAE); 4= dichloromethane-hexane extract (DiHE); and 5= methanolic extract (ME). Each with sapwood, heartwood and leaf.

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Evaluation by the agar diffusion method

For each fungus, 15 Petri dishes (55 x 15 mm) were prepared with potato dextrose agar, and five discs of Whatman® filter paper (Sigma Aldrich, USA) of 0.5 mm in diameter were placed in each dish. Each disc was impregnated with 10 µl of each extract under study. The positive control (fungicide) and negative control (ethanol) were included in all dishes. Subsequently, 1 mm^3 of the mycelium of each fungus was placed on each disc; and it was incubated at 36 ±2 °C for 72 h. The data obtained were reported as the percentage of inhibition by measuring the growth halo of the control mycelium with respect to the treatments, as shown in the following formula: inhibition (%)= [(Gc - Gt)/(Gc + Gt)]*100. Where: Gc= control growth; and Gt= treated growth. An analysis of variance and a comparison of means with a Tukey test (α= 0.05) were performed, Stadistic version 7.

Fractionation of extracts with the highest antifungal activity against *P. chrysosporium* and *G. lucidum*

The leaf extracts that had the best antifungal effect were fractionated by column chromatography, and their activity on fungi was analyzed. Chromatography was performed using silica gel as the stationary phase and a mixture of hexane/ethyl acetate in ascending polarity as the mobile phase. Each fraction obtained constituted a treatment evaluated according to the methodology mentioned for the extracts (Table 1).

Determination of total phenols and flavonoids in the selected fractions

A determination of total phenols was performed, where it was extracted with 5 ml of methanol and centrifugation at 6 000 rpm for 20 min, then the concentration of phenols was determined by the Folin-Ciolcalteau method (Yim *et al*., 2012) using gallic acid as a standard. The results obtained were expressed as mg of gallic acid equivalents per gram of sample (mg GAE g^{-1} DW).

The total flavonoid content was determined according to the method used by Liu *et al*. (2009). For the standard curve, quercetin was used as an indicator, this was performed according to the protocol used by Bernabé-Antonio *et al*. (2017), and the results were reported as milligram of quercetin equivalents per gram of sample (mg QE g^{-1} DW).

Results and discussion

Effect of extracts on mycelial growth in *G. lucidum* and *P. chrysosporium*

It was found that extracts of *E. polystachya* showed fungicidal and fungistatic effect on the development of mycelium in both wood-degrading fungi, as has been reported in works showing the efficacy of other plant extracts with the same effect for xylophagous fungi that cause considerable damage to crops of agricultural importance (Bahraminejad *et al*., 2015).

The results show that there is selectivity of extracts of *E. polystachya*; *P. chrysosporium* (phytopathogen) was sensitive, while *G. lucidum* (fungus of medicinal importance) showed greater resistance. Some extracts proved to inhibit the growth of the fungus to its entirety (fungicide), while another group of extracts only stopped growth (fungistatic), like those reported by Bernabé-Antonio *et al*. (2017) for *R. solani* and *S. cepivorum*, as shown in Table 2.

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Table 2. Percentage of inhibition of the treatments evaluated in *P. chrysosporium* and *G. lucidum*.

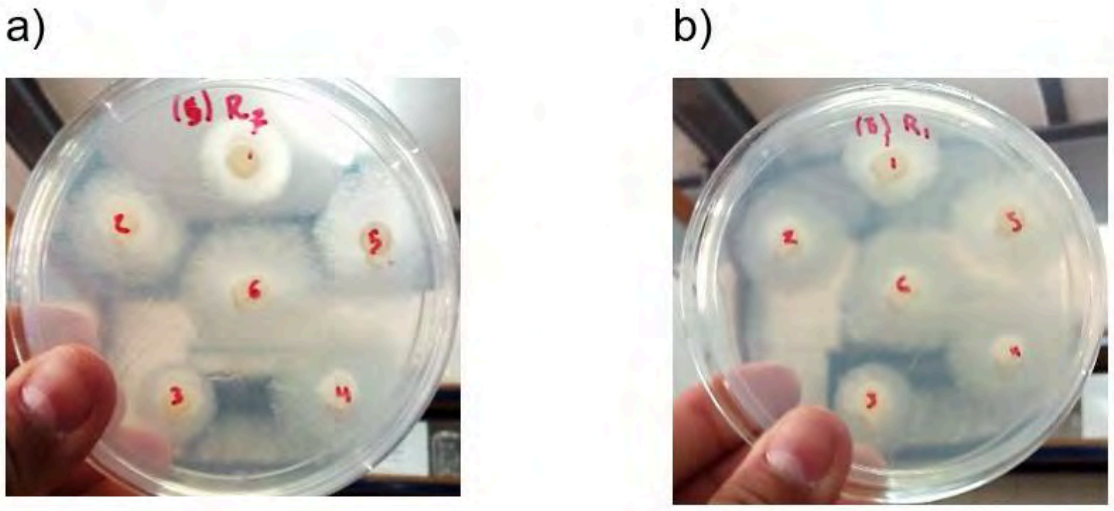
Type of biomass								
Leaf (%)			Heartwood (%)			Sapwood (%)		
Pc	*Gl*		*Pc*	*Gl*		*Pc*	*Gl*	
T1	14 ±1.9 d	0	T6	24 ±4.1 c	0	T11	45 ±4.1 a	0
T2	20 ±4.3 d	0	T7	78 ±7.1 a	0	T12	30 ±6.1 ab	42 ±2.1 a
T3	84 ±3.2 b	0	T8	70 ±6.3 a	0	T13	28 ±1.2 ab	0
T4	57 ±2.4 c	50 ±3.2	T9	60 ±1.8 ab	48 ±4.7	T14	25 ±4.2 b	28 ±2.6 b
T5	100 ±0 a	0	T10	57 ± .3 b	56 ±6.2	T15	6 ±0.9 c	18 ±1.5 c

The treatments are defined in Table 2. Pc= *P. chrysosporium*, Gl= *G. lucidum*. Values with the same letter in the same row did not show a significant difference according to Tukey's test with α= 0.5.

For the present work, it was considered that the extracts that inhibited the growth of the fungus in a percentage equal to or greater than 70% had a fungicidal effect, and those with inhibition of less than 70% had a fungistatic effect. The extracts with the greatest effect on *P. chrysosporium* were dichloromethane-hexane of leaves (E3H) with 84 ±3.2% and methanolic extract of leaves (E5H) with 100% inhibition on mycelial growth (Table 2).

The T4 (dichloromethane/hexane) treatment was selected to fractionate by column chromatography and determine the efficacy of secondary metabolites responsible for inhibiting mycelial growth in *P. chrysosporium*. The efficacy of the extracts is shown in Figure 1, where the inhibition of mycelial growth of the fungi, compared to the positive and negative controls with 70 and 100%, is observed (Figures 1a and 1b). The compounds present in the extracts can hinder the effectiveness that they can exert as synergism on the fungus. So, it is impossible to determine the chemical effectiveness and functionality in a complex mixture.

Figure 1. Effect of leaf dichloromethanolic/hexane extracts (T4 disc 3) on mycelial growth in *P. chrysosporium* at 72 h (a). Defatted hexanic extract (T2 disc 2) (b). Discs 6 (S/treatment), disc 1 (fungicide) for all experiments.



From the extracts that showed a fungicidal effect on *P. chrysosporium*, 25 fractions of the leaf dichloromethane/hexane extract (T4) were obtained, with a yield of 0.7 to 3.5%. Table 3 shows the effect of the fractions obtained from the selected extract on mycelial growth. Fraction 16 (T4-16) was the one that had the greatest fungicidal effect (88.2 ±9.8%) on *S. cepivorum*,

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coinciding with the highest concentrations of total phenols (17.61 ±1.31 mg GAE g^{-1} DW) and flavonoids (8.93 ±1.23 mg QE g^{-1} DW).

Table 3. Inhibitory effect of the fractions obtained from the leaf dichloromethane/hexane extract (T4) and the content of total phenols and flavonoids.

Treatment	Inhibition of mycelial growth (%)			Determination of aromatic compounds	
	P. chrysosporium	*R. solani*	*S. cepivorum*	Total phenols (mg GAE g^{-1} DW)	Flavonoids (mg QEg^{-1} DW)
T4-1	NA	NA	10.1 ±0.6	ND	ND
T4-4	7.1 ±0.9d	8.6 ±3d	ND	0.96 ±0.02b	0.32 ±0.01d
T4-6	ND	16.3 ±6.1c	ND	ND	ND
T4-7	ND	13.2 ±3.1cd	47.8 ±7.4b	0.75 ±0.01c	0.21 ±0.03d
T4-8	ND	8.4 ±2.1d	19.6 ±4.1e	ND	ND
T4-9	11 ±1.1c	3.8 ±0.7f	12.9 ±2.3ef	ND	ND
T4-10	13.9 ±1.3c	5.9 ±1.1e	10.1 ±0.9f	ND	ND
T4-11	2.6 ±0.5f	ND	41.3 ±6.1bc	0.71 ±0.11c	0.54 ±0.15c
T4-12	5.6 ±0.7e	ND	24.4 ±7.2d	ND	ND
T4-13	7.7 ±1.1d	8.9 ±2.3d	25.6 ±1.6cd	ND	ND
T4-14	26 ±4.3b	23.8 ±5.1ab	26.9 ±2.3c	ND	ND
T4-15	ND	ND	41.4 ±2.5dc	ND	ND
T4-16	6.7 ± 0.9de	7.9 ±0.8de	88.2 ±9.8a	17.61 ±1.31a	8.93 ±1.23a
T4-17	39.3 ±6.8a	29.4 ±7.3a	26.8 ±2.3c	1.64 ±0.79b	0.98 ±0.11b
T4-18	31 ±5.4ab	24.3 ±2.4ab	12.2 ±2.2ef	ND	ND

Only fractions that showed an inhibitory effect on mycelial growth are shown. No effect= they showed no inhibition. ND= not determined. Values with the same letter in the same column showed no statistically significant difference (α= 0.05) according to Tukey's test, Stadistic version 7. Nevertheless, Supradip reported similar results, attributing a synergy of other terpene-type compounds that favored fungal activity, where it is considered to act on the chemical composition of the fungal wall by stimulating degrading enzymes. Fraction 7 (T4-7) presented only a fungistatic effect (47.8 ±7.4%) and a phenol content of 0.75 ±0.01 mg GAE g^{-1} DW and flavonoids of 0.21 ±0.03 mg QE g^{-1} DW. The low content of phenols and flavonoids is due to the fact that hexanic extracts do not favor the obtaining of compounds of this polarity.

Table 4 shows the effect of the fractions obtained from the methanolic extracts of leaf (T5), 18 fractions of the extract were obtained. Fraction 1 (T5-1) presented a fungistatic effect on *P. chrysosporium* with 45.7 ±4.8, *R. solani* 36.9 ±7.5, and *S. cepivorum* 65.1 ±4.3, the content of total phenols was 8.91 ±0.94 mg GAE g^{-1} DW and total flavonoids of 5.75 ±1.21 mg QEg^{-1} DW.

Table 4. Inhibitory effect on mycelial growth in *P. chrysosporium*, *R. solani,* and *S. cepivorum* of the fractions obtained from the methanolic extract of leaf (T5) and the content of total phenols and flavonoids.

Treatment	Inhibition of mycelial growth (%)			Determination of aromatic compounds	
	P. chrysosporium	*R. solani*	*S. cepivorum*	Total phenols (mg GAE g^{-1} DW)	Flavonoids (mg QEg^{-1} DW)
T5-1	45.7 ±4.8a	36.9 ±7.5a	65.1 ±4.3b	8.91 ±0.94[a]	5.75 ±1.21b
T5-2	No effect	No effect	14.5 ±2.1e	ND	ND
T5-3	No effect	No effect	6.5 ±0.8f	ND	ND

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Treatment	Inhibition of mycelial growth (%)			Determination of aromatic compounds	
	P. chrysosporium	*R. solani*	*S. cepivorum*	Total phenols (mg GAE g^{-1} DW)	Flavonoids (mg QEg^{-1} DW)
T5-4	24.7 ±3.9bc	13.9 ±2.1c	No effect	ND	ND
T5-5	9.1 ±0.7d	No effect	No effect	ND	ND
T5-6	5.6 ±1.2e	No effect	No effect	ND	ND
T5-8	4.5 ±0.5e	13.9 ±6.1bc	46 ±9.3c	0.86 ±0.05b	0.59 ±0.03c
T5-12	28.4 ±1.9b	No effect	22.7 ±2.9d	ND	ND
T5-17	21.2 ±1.3c	19.7 ±2.1b	85.4 ±9.6a	9.78 ±1.23a	7.29 ±1.12a

Only fractions that showed an inhibitory effect on mycelial growth are shown. No effect= they show no inhibition. ND= not determined. The data presented in the table represent the means ± standard deviation of three repetitions. Values with the same letter in the same column showed no statistically significant difference (α= 0.05) according to Tukey's test.

The efficacy and selectivity of the molecules present at the extract level and fraction level demonstrate once again their fungicidal and fungistatic effect characteristic for each type of phytopathogen, so it is considered of great interest before the application of fungicides for commercial and botanical use, as well as the importance in their chemical structure and activity relationship.

The content of total phenolic compounds and flavonoids found in the fractions with inhibitory effect on mycelial growth of the three fungi evaluated was compared with those reported in leaf and cell culture of *E. polystachya* as well as with the values obtained with other species of the Fabaceae family (Bernabé-Antonio *et al*., 2017).

In other families of plant species, the content of total phenols and flavonoids has been reported to be lower than those presented in *E. polystachya*, as well as in the fractions; for example, in species such as *Artemisia absinthium* L., the phenol content was 3.57 mg GAE g^{-1} DW, lower than *E. polystachya* (Table 4), and flavonoids 1.89 mg QE g^{-1} DW, lower than the treatments present in Tables 3 and 4; *E. polystachya* is enriched by flavonoid-type compounds and chalcones in leaf and stem (Toroglu, 2007).

The treatments T5-1 and T5-17 were the only ones with a fungicidal effect on *S. cepivorum*, as shown in Tables 3 and 4. However, the T5-1 treatment demonstrated a fungistatic effect against *S. cepivorum*, so these three treatments were selected to determine their effective concentration 50 (EC_{50}) in *S. cepivorum*. To determine the effective concentration 50, 1:10 dilutions were made in an interval of 0.1 to 0.01 mg ml^{-1}, in which it was found that treatments T5-1 and T5-17 presented an EC_{50} on mycelial growth at the concentration of 12.1 and 14.5 µg ml^{-1}, respectively.

The T5-1 treatment presented an EC_{50} of 8.7 µg ml^{-1}. With these data, the efficacy of the fractions in terms of their EC_{50} is demonstrated, with a favorable effect compared to that reported by Marel-Abdelgaleil *et al*. (2018), in which an EC_{50} of 9.31 mg L^{-1} was determined in *Alternaria solani* and 24.69 mg L^{-1} in *Rhizoctonia solani* in extracts or fractions composed of monoterpenes.

Figure 2 shows the images of the considered effect of the fractions obtained from the extracts selected on *R. solani*, *S. cepivorum* (Bernabé-Antonio *et al*., 2017), and *P. chrysosporium*; *G. lucidum* did not present growth inhibition with the fractions evaluated. Nonetheless, the selectivity and efficiency of the extracts and fractions obtained from the same plant species are of interest for the inhibition of some phytopathogenic fungi; *G. lucidum* is considered a medicinal fungus; *R. solani* and *S. cepivorum* cause damage to products of agricultural importance, with the species of the genus *Sclerotio* being the ones with the greatest impact (Morales-Palacios, 2016).

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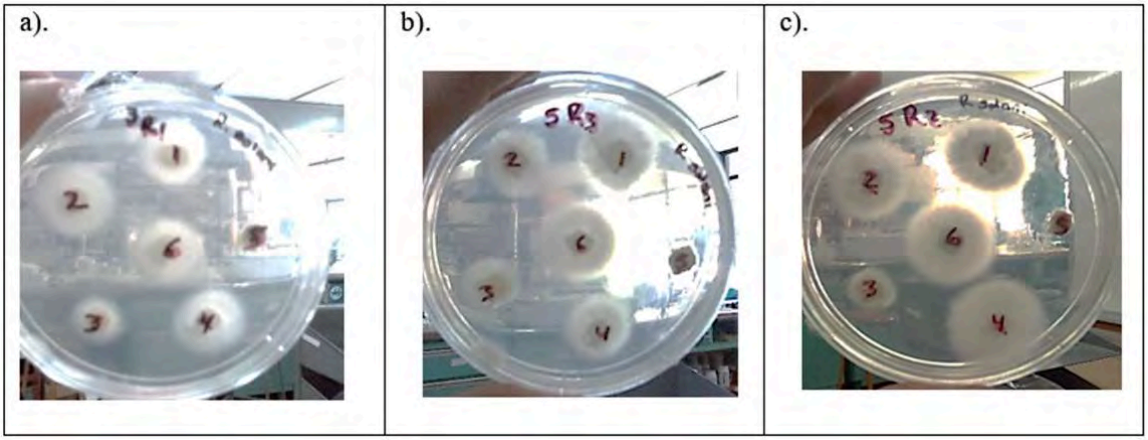
Figure 2. Effects of fractions obtained from selected extracts with antifungal activity on *R. solani*. Fracti ons 1 to 4 of the leaf hexanic/diclomethane extract (a). Fractions 1 to 4 of the leaf methanolic extract (b). Effect of fraction 7 obtained from the leaf dichloromethane-hexane extract (disc 3) (c).



Some reports consider that *E. polystachya* contains terpene-type compounds, most of them detectable in nonpolar extracts, such as hexane and petroleum ether, since they have as precursors fatty acid compounds, as well as phenolic compounds from simple structures; many of them give rise to chalcones and flavonoids (Rivas-Morales *et al*., 2009; García-Hernández *et al*., 2016).

Many of these compounds are considered secondary metabolites present in various organs of the plant and have been shown to have biological activity against phytopathogenic fungi. So, it was important to determine the content of total phenols and flavonoids in the selected fractions since a considerable amount of total phenols, of 155.17 mg GAE g^{-1} DW, was found in the leaf, compared to half the content reported in cell culture with 73.98 mg GAE g^{-1} DW (Bernabé-Antonio *et al*., 2017).

With this finding, new specific biofungicides for each fungus were demonstrated, *E. polystachya* can be considered an unexplored source of bioactive compounds against phytopathogens. Such effects may be related to chemical molecules present in this plant species and of which there are few studies on the possible secondary metabolites responsible for its activity.

Conclusions

The extracts obtained from *E. polystachya* (Palo dulce) showed an antifungal effect on xylophagous fungi, possibly due to the presence of flavonoids and some terpene-type compounds that have been reported in the species. Therefore, *E. polystachya* can be considered a biosource of secondary metabolites as a plant fungicide of interest in the agronomic and forestry fields.

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