Fungitoxic Potential of Extracts of Four *Pinus* spp. Bark to Inhibit Fungus *Trametes versicolor* (L. ex. Fr.) Pilát

María Guadalupe Lomelí-Ramírez,[a] Haydeé Dávila-Soto,[b] José Antonio Silva-Guzmán,[a] Héctor Guillermo Ochoa Ruíz,[a] and Salvador García-Enriquez [a]*

Antifungal potential of bark crude extracts of *Pinus strobus* L., *Pinus douglasiana* Martinez, *Pinus caribaea* Morelet [var. Hondurensis (scheclauze) W. H. G. Barrett & Golfari] and *Pinus leiophylla* Schltdl. & Cham, for inhibiting mycelial growth to fungus *Trametes versicolor* (L. ex Fr.) Pilát was investigated. *Pinus* bark was extracted through a mixture of acetone-hexane-water 54:44:2 V. Bioassays were performed according to the agar dilution method (petri dish) using concentrations of 0.1, 0.5, and 1.0 mg mL^{-1} for each of the obtained extracts. All extracts showed some inhibition degree. Crude extracts of *P. strobus* bark showed the greatest inhibition degree. Antifungal potential of all tested extracts at a concentration of 1.0 mg mL^{-1} was classified as toxic. Through simple regression models, concentrations were calculated in order to achieve a 50% inhibition (IC_{50}) and the minimal inhibition concentration (MIC). MIC values were 1.58, 1.72, 1.24, and 1.94 mg mL^{-1} for bark extracts of *P. strobus*, *P. douglasiana*, *P. caribaea*, and *P. leiophylla*, respectively.

Keywords: Antifungal potential; Bark extracts; Pine bark; Trametes versicolor

Contact information: a: Departamento de Madera, Celulosa y Papel del Centro Universitario de Ciencias Exactas e Ingeniarías, Universidad de Guadalajara, Km 15.5, Carretera Guadalajara-Nogales, Zapopan, Jalisco, México, CP. 45220; b: Tecnologías Químicas, Centro de Enseñanza Técnica Industrial, Circuito Loma Norte No. 8962 Col. Loma Dorada, Tonalá, Jalisco, México, C.P. 45418;
** Corresponding author: Salvador.garciae@cucei.udg.mx*

INTRODUCTION

Bark is the tree's main defense against phytophagous insect attacks, large animals, and other organisms that cause tree rot (Laks 1991). Conifers develop resin canals and parenchyma cells in the secondary phloem as a response to beetle bark and blue stain fungus joint invasion (Franceschi *et al.* 2000). These anatomic changes can be induced by organisms (Franceschi *et al.* 2005). At the same time, active chemical response mechanisms occur in order to repel such threats, and there are changes in quantity and constitution of secondary metabolites such as terpenes (Keeling and Bohlmann 2006; Goodsman *et al.* 2012) and phenolic compounds (Franchesi *et al.* 2005; Li *et al.* 2012). It has been suggested that monoterpenes and terpenoids are more effective against insects (Jost *et al.* 2008; Boulogne *et al.* 2012). On the other hand, the phenolic compounds show better antifungal bioactivity (Harun and Labosky 1985; Boulogne *et al.* 2012).

Plant bioactive metabolites and wood decay fungi interactions have been investigated widely and variously, such as: natural durability sapwood of 12 pine species (Eslyn and Highway 1976) and the effect of antifungal extracts of heartwood tropical timber on pine sapwood (Eslyn *et al.* 1981). Pinosylvins found on pine heartwood (Erdtman 1949), cones and bark (Celimene *et al.* 1999), as well as isolated flavonoids from branches and needles (Song *et al.* 2011) have been thoroughly studied. On the other hand, bioactive

fungi components that degrade wood have been studied as potential control agents for preserving wood (Teoh *et al.* 2011), and the fungi´s ability to biodegrade xenobiotics soil contaminants has been investigated (Baldrian 2008). Enzymes generated by fungi can be used as wood biopulped agents (Villalba *et al.* 2006), textile plant agents (Mohiuddin *et al.* 2005), cellulosic pulp bio-bleaching agents (Chandra *et al.* 2007), and also can be used on polymer synthesis, for antibiotics production and flavonoids functionalize (Mikolasch and Schauer 2009). Such enzymes have also been an object study on hydrolysis and oxidation-reduction reaction mechanisms (Hamel *et al.* 2002; Hofrichter 2002).

The major chemical content of extracted substances from pine bark, in general, is proanthocyanidines (Marham and Porter 1973; Harun and Labosky 1985). Other substances found are pinosylvins (Pan and Lundgren 1996; Celimene *et al.* 1999), phenolic acids (Hata *et al.* 1966), and lignans and flavonoids (Marham and Porter 1973). Also, it has been reported that bark from some pine species contain waxes, for instance, *Pinus caribaea* (Kolattukudy and Espelie 1989) and oleoresinous materials (Laks 1991). Antioxidant and antimicrobial properties have been attributed to waxes, resins, and tannins from bark (Singh and Singh 2012). Kokalis and Rodriguez (1994) evaluated the potential of pine bark extracts and bark powder from *Pinus taeda* L. and *Pinus elliottii* Engelm, as an inhibitor of growth of fungal plant pathogens. Mori *et al.* (1995) and Alessandrini and Vargas (2006) evaluated antifungal properties of *P. strobus* and *P. caribaea* bark acetone extracts, respectively. Alfredsen *et al.* (2008) evaluated the antifungal effect of bark extracts from some European tree species, including the wild *P. sylvestris* L. Regarding pine bark Mexican species, monoflavonoids including taxifolin, quercetin, and catechin have been identified (Weissmann and Meier 1989). Catechin was found in *P. leiophylla* bark, and its antioxidant capacity was evaluated (Rosales *et al.* 2009). Pietarinen *et al.* (2006) found a high percentage of pinosylvins with hexane in *P. strobus* barks. Cespedes *et al.* (2006) reported a 25.7% antifungal activity shown by methanol extracts of heartwood of *Araucaria araucana* toward *T. versicolor*.

The purpose of this paper is to evaluate fungal toxic potential of crude extracts obtained from the bark of four species of *Pinus*, to inhibit the growth of *Trametes versicolor* fungi through the agar dilution method (Petri dish). The minimum inhibitory concentration (MIC) and the concentration required to achieve a 50% inhibition (IC_{50}) were calculated. Likewise, the corresponding toxicity of obtained extracts was classified.

EXPERIMENTAL

Extracts Preparation

Bark from native pine trees were used from: *Pinus strobus* L., *Pinus douglasiana* Martínez, *Pinus caribaea* Morelet [var. Hondurensis (scheclauze) W. H. G. Barrett & Golfari], and *Pinus leiophylla* Schltdl. & Cham. The first two tree species are found in Oaxaca Mountains (Mexico) and the latter two in Tapalpa Mountains (Jalisco, Mexico). Barks were milled using the Wiley Retch GmbH, model 5657, followed by sieving, where particles that passed the mesh 40 and were retained on mesh 65 were selected. Subsequently, bark of each species was dried in an oven at 60 °C during 48 h. To obtain the extracts, a mixture of acetone (Sigma Aldrich 99.9%), hexane (Sigma Aldrich 97.5%), and water (bi-distilled) was employed, in a 54:44:2 V/V/V proportion (Steller and Labosky 1984; Harun and Labosky 1985). Bark extractions were carried out in a batch system, with continuous stirring during 10 h (without heating), with a solid to liquid relation of 1/8. The

solution extracts were concentrated on a rotary evaporator (BÜCHI RE -120) at 50 °C; residual solvent was evaporated outdoors in the laboratory during two weeks. Performance was determined gravimetrically as a function of the bark powder amount used and the amount of obtained extracts.

Bioassay Solutions

Organic solutions were prepared using ethanol (Sigma Aldrich 99.5%)-pyridine (Fermont 99.0 %)-water in a 84:3:13 V/V proportion for *P. strobus* extracts; while for the other three bark extracts from *P. douglasiana*, *P. caribaea*, and *P. leiophylla*, an ethanol-water solution 95:5 V/V was used. Solutions with the required amount to obtain final concentrations of 0.1, 0.5, and 1.0 mg mL^{-1} were prepared, and these mixtures were combined homogeneously with the culture medium malt-agar.

Fungal Strain

Bioassay was carried out using the *Trametes versicolor* (L. ex Fr.) Pilát white rot fungus (CFNL01760). This strain was provided by the Forest Department of the Autonomous University of Nuevo León, Mexico. The culture medium for fungal growth test was prepared using a 45 g L^{-1} malt-agar extract and was sterilized at 1.05 Kgf/cm^2 during 15 min.

Antifungal Assays

The agar dilution method (Rutiaga *et al.* 1995) for antifungal potential of pine bark extracts was used. To prevent alteration of physicochemical stability, the extracts were not sterilized. Concentration tests were obtained through incorporating into a Petri dish a liquid sterilized culture medium (23 mL) and a given amount of extract solution, to obtain the final concentration of interest. Petri dishes were uncovered, for evaporation of solvents, in sterile environment. Five assessments of those three concentrations and four extracts were made three times. Subsequently, Petri dishes were inoculated using 0.5 cm^2 of test fungus mycelium and were incubated during 10 days at 28 ºC ± 1 ºC, measuring radius of the mycelial growth every 48-h interval. Petri dishes containing only culture medium (blank) and other Petri dishes containing both culture medium and a solvent mixture (controls) were inoculated with fungi. Antifungal activity (AFA) was calculated according to the following formula (Rutiaga *et al.* 1995):

$$AFA\ (\%) = [(\text{Growth control} - \text{Growth treatment}) / \text{Growth control}] \times 100 \quad (1)$$

Toxicity was classified according to AFA value: 0-25% as Non-Toxic (NT), 26-50 as Little Toxic (LT), 51-75 as Moderately Toxic (MT), and >75% as Toxic (T) (Mori *et al.* 1995).

Determination of Inhibitory Concentration 50 (IC_{50}) and Minimum Inhibitory Concentration (MIC)

To calculate IC_{50} values (concentration in mg mL-1 that inhibited fungi mycelium growth at 50%) and minimum inhibitory concentration (MIC), AFA values were applied through simple regressions using the Statgraphics program (Centurion XVI, version 16.1.15)

RESULTS AND DISCUSSION

Extracts

Table 1 shows yields of bark extractions from the four *Pinus* species that were evaluated. The bark extract with the highest yield was obtained from *P. strobus*; the lowest one was from *P. caribaea*; whereas extracts from *P. douglasiana* and *P. leiophylla* were approximately 10%. The amount of obtained bark extract usually varies from one species to another; it also depends on the type of solvent used for extraction. Pietarinen *et al.* (2006) reported that 19% of bark extract was obtainable through a sequential bark extraction from *P. strobus*, firstly using pure hexane and then applying a ketone-water mixture 95:5% V/V. Over these types of bark extracts, for this work a mixture of the same three solvents was used, and the yield of bark extract obtained was 12.34%; the latter confirms that the solvent system used has a strong influence on the amount of extract obtainable. Pietarinen *et al.* (2006) also reported the following bark extract components: lignans (5%), lariciresinol (4%), flavonoids (8%), pinobanksin (4%), pinocembrin (2%), and pinosylvins (45%). In the case of bark extracts from *P. caribaea*, Alessandrini and Vargas (2006) reported a total yield of 13.05% of bark extractions that were obtained through a sequence of eight different solvents. Steller and Labosky (1984) used an acetone-hexane-water mixture to obtain bark extract, finding mostly essential oils, resins, tannins, and phlobaphenes.

Table 1. Yields of Bark Extract, Antifungal Potential, and Toxicity for Each Concentration Tested

Bark	Yield (%)	Concentration (mg mL^{-1})	Antifungal Potential (%)	Toxicity*
P. strobus	12.34	1.0	90.1 ±2.3	T
		0.5	72.9 ± 2.9	MT
		0.1	61.7 ± 3.2	MT
P. douglasiana	10.36	1.0	86.6 ± 3.2	T
		0.5	78.1 ± 3.2	T
		0.1	58.7 ± 2.6	MT
P. caribaea	8.92	1.0	84.9 ± 2.2	T
		0.5	70.6 ± 2.8	MT
		0.1	36.9 ± 2.9	LT
P. leiophylla	10.78	1.0	87.3 ± 2.2	T
		0.5	68.7 ± 2.8	MT
		0.1	57.0 ± 2.4	MT

*Nontoxic (NT) for <25% inhibitions, Little Toxic (LT) for inhibitions between 25 and 50%, Moderately Toxic (MT) for inhibitions between 50 and 75% and, Toxic (T) for >75% inhibitions (Mori *et al.*1995; García 2000]

Antifungal Potential

Antifungal activity (AFA) average values of each evaluated bark extract concentration are shown in Table 1. Crude extracts from *P. strobus* bark, obtained with hexane-acetone-water mixture (54:44:2% V/V/V), exhibit AFA of 61.7%, 72.9%, and 90.1% at concentrations of 0.1, 0.5, and 1.0 mg mL^{-1}, respectively. AFA average values were classified as Moderately Toxic (MT) at concentrations of 0.1 and 0.5 mg mL^{-1} and as Toxic (T) at a concentration of 1.0 mg mL^{-1}. Mori *et al.* (1995) reported that extracts from *P. strobus* bark obtained with acetone, showing an inhibition percent less than 25% at a concentration of 0.5 mg mL^{-1}, against *T. versicolor* fungus growth. Given the above results, most AFA comes from substances, such as waxes (Singh and Singh 2012; Kolattukudy and

Espelie 1989) and pinosylvins (Pietarinen *et al.* 2006), which are the main compounds extracted using hexane and acetone, respectively.

Crude bark extract from *P. douglasiana* exhibited AFA values of 58.7%, 78.1%, and 86.6% at concentrations of 0.1, 0.5, and 1.0 mg mL^{-1}, respectively; these extracts were classified as MT at the lowest concentration and as T at the highest two concentrations. In the case of crude bark extracts from *P. caribaea*, AFA values observed were 36.89%, 70.62%, and 84.87% at concentrations of 0.1, 0.5, and 1.0 mg mL^{-1}, respectively; these extracts were classified as LT, MT, and T, respectively. Alessandrini and Vargas (2006) reported inhibition percentages of 35.71%, 21.49%, 25.72%, 29.51%, and 6.04% against *T. versicolor*, applying extracts from benzene, methanol, ethanol, acetone, and toluene, respectively. Finally, crude bark extracts from *P. leiophylla* exhibited AFA values of 57.0%, 68.7%, and 87.3% at concentrations of 0.1, 0.5, and 1.0 mg mL^{-1}, respectively; the lower two concentrations were classified as MT and the highest as T. In general, all extracts that showed a fungal growth inhibition between 84.9% and 90.1% at a concentration of 1.0 g L^{-1}, were considered as T.

In Figure 1, images taken at the end of the test, show *T. versicolor* mycelial growth in Petri dishes, at concentrations of: a) 1.0 mg mL^{-1}, b) 0.5 mg mL^{-1}, c) 0.1 mg mL^{-1} and, d) control. It can be observed that by increasing the concentrations, fungus growth was lower, while the control Petri dish shows a normal fungus mycelium growth, covering the entire culture medium.

In Fig. 2, antifungal potential versus bark extract concentrations are evaluated for the four *Pinus* species. It is observed that crude bark extracts from *P. strobus* had an AFA increase of 11.2% and 17.2%, by increasing concentrations from 0.1 to 0.5 mg mL^{-1} and from 0.5 to 1.0 mg mL^{-1}, respectively. At the same concentration increases, bark extracts of the other species showed increases of: 19.4% and 8.5% for *P. douglasiana*, 33.7% and 14.4% for *P. caribaea* and, 11.7% and 18.6% for *P. leiophylla*. All evaluations showed that antifungal potential increases as extract concentration increases. In general, bark extracts from *P. strobus* exhibited a higher inhibitory potential than the other evaluated extracts, whereas bark extracts from *P. caribaea* had the lowest one, against *T. versicolor* fungi growth, at the concentrations tested experimentally.

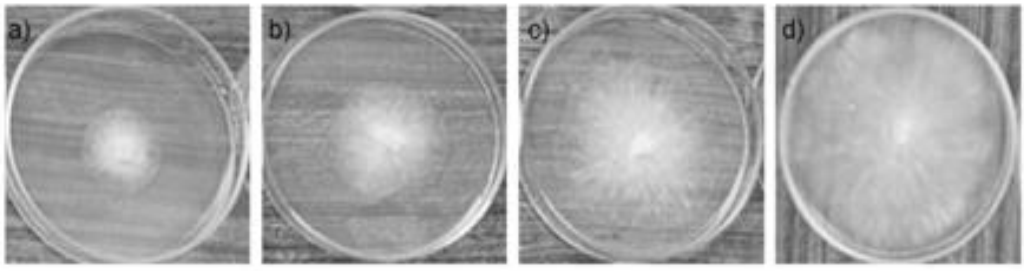

Fig. 1. *T. versicolor* growth exposed to culture media using *P. caribaea* extracts at concentrations of: a) 1.0 mg mL^{-1}, b) 0.5 mg mL^{-1}, c) 0.1 mg mL^{-1} d) Control

In previous studies, Rutiaga *et al.* (1995) reported that extracts from *Enterolobium cyclocarpum* obtained using ethanol and cyclohexane against *T. versicolor* fungus growth, at a concentration of 1.0 g L^{-1} show inhibition values of 80% and 25%, respectively; applying the same solvents and concentration to extracts from *Dalbergia granadillo*, inhibition values were 85% and 100%, respectively.

Cespedes *et al.* (2006) reported that extracts from *Araucaria araucana* (Mol.) obtained using methanol show an inhibition value of 25.7% against *T. versicolor* fungus growth, at concentrations of 0.1 g L^{-1}; on the other hand, pinoresinol shows a 15% inhibition against the same fungus and at the same concentration. Velásquez *et al.* (2006) evaluated inhibitory capacity of wood extracts obtained using acetone from *Hymenaea courbaril* L., *Centrolobium paraense* Tul. var. orinocense Benth, *Tabebuia serratifolia* (Vahl) Nicholson and, *Peltogyne porphyrocardia* Griseb; *T. versicolor* fungus growth showed inhibition values of 67%, 81%, 90% and 40%, respectively, at a concentration of 0.2% v/v. Martínez-Sotres *et al.* (2012) reported that heartwood extracts from *Dalbergia congestiflora* Pittier obtained using hexane, inhibited a 100% of *T. versicolor* growth at a concentration of 250 mg L^{-1}, whereas extracts obtained using acetone show a 0% inhibition at the same concentration. Conifer bark extracts in the present study showed better performance at the same concentrations than all the hardwood extracts cited above, except on *Dalbergia* extracts using hexane.

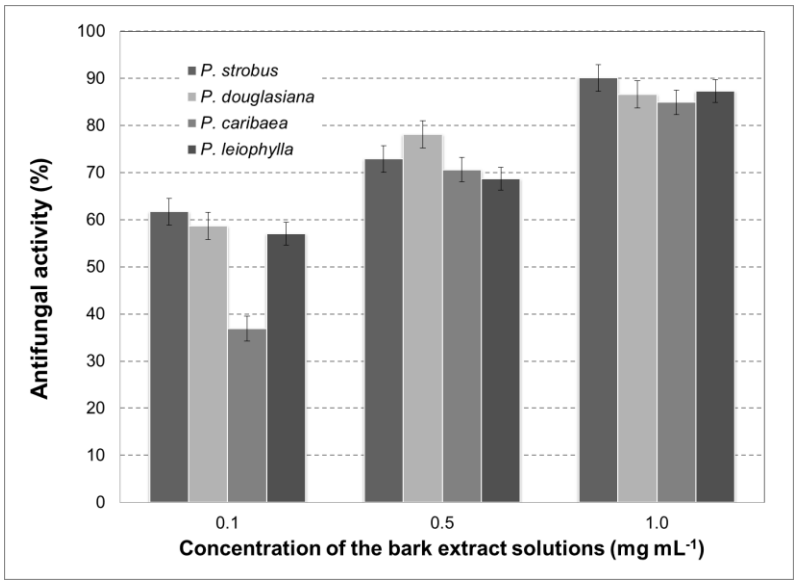

Fig. 2. Antifungal potential of the four bark extracts at different concentrations against *T. versicolor* fungus

Inhibitory Concentration 50 (IC_{50}) and Minimum Inhibitory Concentration (MIC)

At the end point of kinetics of fungal growth, inhibition percentages obtained were adjusted by simple regression models, and then IC_{50} and MIC were calculated (Table 2). Correlation coefficient (R^2) values obtained through statistical analysis show that the models are a good fit of the experimental data.

Figure 3 shows plotted models for each tested extract. It was found that pine bark extracts from *P. strobus*, *P. douglasiana*, *P. caribaea*, and *P. leiophylla* showed IC_{50} values of 0.071, 0.069, 0.279, and 0.096 mg mL^{-1}, respectively, against *T. versicolor* fungus. Theoretically the best extract to inhibit 50% of growth was the *P. douglasiana*, and the less effective the *P. caribaea*. MIC values were 1.58, 1.72, 1.24, and 1.94 mg mL^{-1} for bark extracts of *P. strobus*, *P. douglasiana*, *P. caribaea*, and *P. leiophylla*, respectively. Therefore, the bark extract of *P. caribaea* showed the strongest activity and that of *P. strobus* exhibited the second strongest activity.

Table 2. Adjusted Model by Simple Regression, IC_{50} and MIC Required for Inhibition of *T. versicolor* Growth

Extracts	Adjusted regression model	P- value model	R^2	IC_{50} (mg mL^{-1})	MIC (mg mL^{-1})
P. strobus	% AFA = sqrt (487.531 + 7558.47 X sqrt C)	0.020	0.96	0.071	1.584
P. douglasiana	% AFA = sqrt (636.621 + 7136.38 X sqtr C)	0.023	0.95	0.069	1.722
P. caribaea	% AFA = 4.964 + 85.27 X sqrt C	0.015	0.97	0.279	1.243
P. leiophylla	% AFA = sqrt (301.3 + 7111.1 X sqrt C)	0.017	0.97	0.096	1.861
C = concentration					

Cheng *et al.* (2005) reported that the heartwood, sapwood, and bark extracts from *Cryptomeria japonica* resulted in IC_{50} values of 91, 139, and greater than 500 mg mL^{-1}, respectively, in the inhibition of *T. versicolor* growth. This study showed that bark extracts have the least antifungal activity. Wang *et al.* (2011) reported that extracts from *Cunninghamia lanceolata* heartwood obtained using hexane, ethyl acetate, and methanol, required concentrations of 0.47, 0.64, and 0.84 g L^{-1}, respectively, in order to inhibit 50% *T. versicolor* growth. Finally, IC_{50} values of this work compared to those obtained by Cheng *et al.* (2005) and Wang *et al.* (2011) against fungus growth, were lower.

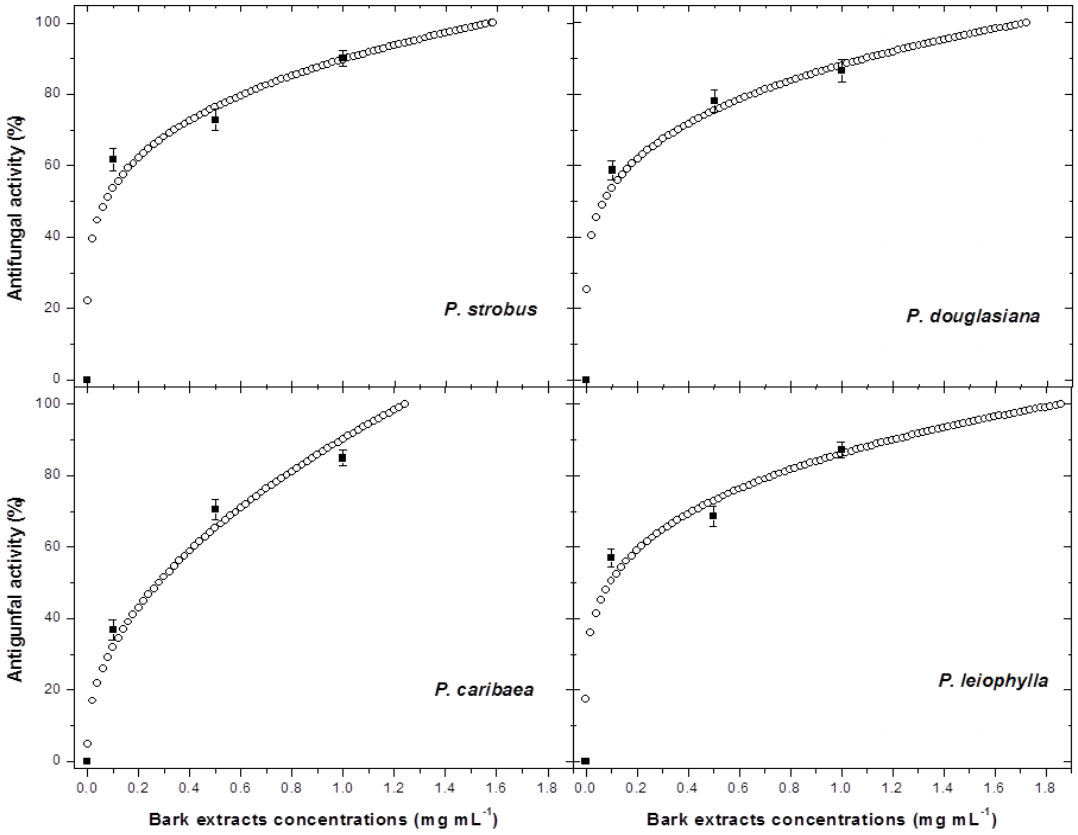

Fig. 3. ■ Experimental data; ○ model biological through simple regression for the extracts tested

CONCLUSIONS

1. Bark extracts from the four pine species that were evaluated showed a certain growth inhibition on the test fungus.
2. At a concentration of 1.0 mg mL^{-1}, *P. strobus* bark extract is slightly more toxic than *P. douglasiana* and *P. leiophylla* bark extracts, and, *P. caribaea* bark extract is the least toxic.
3. The antifungal potential probability of tested extracts can be strongly attributed to substances present in bark, such as waxes and pinosylvins, which are the main extracted compounds using hexane and acetone, respectively.
4. Antifungal potential of all tested extracts at the concentration of 1.0 mg mL^{-1} were classified as toxic.
5. MIC values of 1.58, 1.72, 1.24, and 1.94 mg mL^{-1} obtained for bark extracts from *P. strobus*, *P. douglasiana*, *P. caribaea*, and *P. leiophylla*, respectively, against fungus growth inhibition, were considered as good.

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