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In vitro antifungal activity of polyphenols-rich plant extracts against *Phytophthora cinnamomi* Rands

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Antifungal activity of water, ethanol, lanolin and cocoa butter plant extracts derived from seven Mexican Chihuahuan desert inhabiting plant species (*Larrea tridentata, Flourensia cernua, Agave lechuguilla, Opuntia ficus-indica, Lippia graveolens, Carya illinoensis* and *Yucca filifera*) were evaluated against *Phytophthora cinnamomi*. All plant extracts were active against *Phytophthora cinnamomi*. Two (*L. tridentata* and *F. cernua*) out of seven plant species tested had the optimal antifungal activity against this fungus specie, with minimum inhibitory concentration (MIC) values as low as 6.96 and 8.6 mg/L. Some of the plant extracts had moderate to low activity against *P. cinnamomi*, and the variations of active polyphenolic (condensed and hydrolysable tannins) compounds in the plant extracts estimated via colorimetric methods indicated that the inhibitory activity may not based on a general metabolic toxicity but perhaps the antifungal potency is conferred by group or groups of toxic metabolites. Based on the antifungal activity, crude plant extracts may be a cost effective way of protecting crops against *P. cinnamomi.* Because plant extracts contain several antifungal compounds, the development of resistant pathogens to these plant extracts may be delayed.

Key words: Antifungal activity, plant extracts, polyphenols, MIC_{50} *P. cinamomi*.

INTRODUCTION

The stramenopile *Phytophthora cinnamomi* Rands causes root rot of avocado and is one of the main limiting factors of this crop (Ceja et al., 2000; Messenger et al., 2000). In addition, this plant pathogen causes damages

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to others species as *Eucalyptus* and *Pinus* species (Linde et al., 1997), and pineapple (Allen et al., 1980). Their virulence is associated with temperature between 21 to 30°C, poorly drained soils and excessive moisture. This pathogen is diploid and heterothallic with two groups, A1 and A2 (Linde et al., 1997).

Its control is based on cultural practices, including management of soil moisture and improving ventilation by increasing drainage, and mineral nutrition care. The application of chemicals among which are the fungicides metalaxyl and fosetyl-aluminum to the soil, leaves or trunk injection (Whiley et al., 1986) and biological control agents including bacteria and fungi in soil, as *Pseudomonas* spp., *Streptomyces* spp. and *Trichoderma* spp., *Myrothecium roridum*, *Aspergillus* spp., or *Paecilomyces* spp., are other techniques useful to inhibit *P. cinnamomi* (Reeves, 1975; Gees and Coffey, 1989; Mass and Kotzé, 1990; Casale, 1990; Stirling et al., 1992; Duvenhage and Kotzé, 1993).

However, these management disease techniques present challenges and constraints in control of the disease, loss in efficiency, increased resistance to active ingredients and environmental hazards, so it is necessary to find new strategies for control, one of these strategies can be use of plant extracts as an alternative (Lira et al., 2007).

Several studies showed that secondary metabolites produced by plants have an effect on inhibiting the development of the mycelium of several pathogenic fungi (Hossein and Maldonado, 1982). Among the synthesis of secondary metabolites or phytochemicals are polyphenols which are a heterogeneous group of molecules having a structure of benzene substituted by various groups with hydroxyl functions, allowing them to be highly soluble is substances such as water.

These compounds are present in extracts of leaves, bark, wood, fruits and galls of certain ferns, gymnosperms and angiosperms (Swain, 1979). Polyphenols are important in plant physiology because they contribute to resistance to microorganisms, insects and herbivorous animals (Haslam, 1996).

Besides, these compounds help to preserve plant integrity during continuous exposure to environmental stressors, including ultraviolet radiation, high temperatures and dehydration (Lira et al., 2007). Polyphenol antioxidants are active in biological systems and probably the capacity or biological value explains its abundance in plant tissues (Meckes et al., 2004). Some plant species like *Larrea tridentata*, *Turnera diffusa*, *Flourensia cernua*, *Jatropha diocesan* among others are widely distributed in the Mexican Northern States, occupying an area of approximately 100 million hectares (González, 1975). These native plants have a high content of polyphenolic compounds (Lira et al., 2007). Plant extracts obtained with different solvents as methanol, acetone, chloroform, hexane, etc. have been reported with antimicrobial properties.

However, little attention has been given to obtaining polyphenols-rich extracts with unconventional solvents which have potential use in disease management of organic farming. The detected significant differences on the antifungal activity can be due to total polyphenols presents in the plant extracts. This is the first study on use polyphenols-rich plant extracts against *P. cinnamomi*, because there are some reports where plant extracts are used but to inhibit other *Phytophthora* species such as: *Phytophthora infestans* (Gamboa et al., 2003a, b), *Phytophthora capsici* (Galván, 2005) and *Phytophthora palmivora* (Mendoza et al., 2007) *in vitro.*

In addition, Nielsen et al. (2006), reported the effect of natural product derives from *Quillaja saponaria* which showed activity against root rot until 100% in disease control, this plant is native of desert regions and have high titers of saponins. Saponins have been reported to reduce surface tension in the nutrient solution of hydroponic systems in greenhouses and cause disintegration of the membrane of *Phytophthora* zoospores.

In this context this paper aims were to determine the *in vitro* antifungal activity of semi-desert plants extracts on inhibiting mycelial growth of *P. cinnamomi* and their MIC_{50}.

MATERIALS AND METHODS

Seven wild plant species (*L. tridentata* Sees and Moc. ex D.C. Coville, [Zygophyllaceae] *Flourensia cernua* DC [Asteraceae], *Agave lechuguilla* Torr [Agavaceae], *Opuntia ficus-indica* L. [Cactaceae], *Lippia graveolens* Kunth (Verbenaceae), *Carya illinoensis* K. Koch (Juglandaceae) and *Yucca filifera* Chabaud (Agavaceae)) were collected in the Southern region of Coahuila, (semi-desert region) during August and September, 2008. The collected plant material was transferred to the Microbiology Laboratory of The Food Research Department, School of Chemistry, Universidad Autonoma de Coahuila, for dehydration and milling. Dehydration was carried out at room temperature for 10 days and when required in an oven for two days to have moisture content between 5 to 10%, the milling process was carried out in a miller (Thomas Wiley) 1 mm mesh. The obtained fine powder was stored in amber bottles at room temperature until extraction of polyphenolic compounds was done.

The phytochemical compounds extraction was performed by a solid-liquid procedure, using four solvents (water, ethanol, lanolin and cocoa butter). For hydrophilic solvents group Soxhlet method was used and hydrophobic solvents group infusion method was used. In firth group distilled water and ethanol (70%) were used and second group mineral oil emulsions with 10% lanolin and cocoa butter were used. Each fine powder sample was mixed in a 1:4 (w /v) ratio with the corresponding extracting agent. Soxhlet method was performed in a rotary evaporator at 60°C for 7 h while infusion method was carried out heating the solvent at 60°C, once reached this temperature; the fine powder was added and remained under these conditions during 7 h. After this, extracts were filtered and stored at 5°C in container in ramber bottles until the extracted phytochemical compounds were identified and quantified.

In this case, only condensed and hydrolysable tannins were

determined which belong to polyphenols group. Concentration of hydrolysable tannins (HT) was determined by the Folin-Ciocalteu method (Makkar, 1999). Condensed tannins (CT) were spetrophotometrically determined using the method reported by Swain and Hillis (1959). For condensed tannins determination, an aliquot of 0.5 ml of plant extract was placed in a tube, with 3 ml of HCl/butanol (1:9) and 0.1 ml of ferric reagent.

On the other hand, it was added to a tube assay series catechin (standard) in distilled water at different concentrations (0, 200, 400, 600, 800 and 1000 ppm) to determine the reference curve. Tubes were plugged tightly and were heated for 1 h in water bath at 90°C. After that, tubes were leaved to cool and absorbance was read at 460 nm. For hydrolysable tannins determination, a reference curve was done by placing 400 µl of gallic acid at different concentrations (0, 200, 400, 600, 800, and 1000 ppm) in assay tubes. Gallic acid concentrations were prepared using distilled water. Each one of the plant extract were diluted in a test tube respectively, immediately to each tube were added 400 µl of commercial Folin-Ciocalteu reagent, samples were vortexed and held for 5 min. Then 400 µl of $NaCO_3$ (0.01 M) and 2.5 ml of distilled water were added.

Finally, absorbance was read at 725 nm in UV / visible spectrophotometer. Determination of polyphenolic compounds antifungal activity from 28 plant extracts on inhibition of mycelia growth was performed through the poisoned medium technique using different concentrations (ppm) of total polyphenols (hydrolysable plus condensed tannins). The response in inhibition mycelia growth was based in Minimum Inhibitory Concentration (MIC_{50}) defined as: the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism in 50% of radial growth in contrast to control (Kumar et al., 2011). Potato dextrose agar (PDA - Bioxon) as culture medium was used, in this case, volume of each extract according to the final concentration was determined and quantified; this volume was added to a flask with the water volume and PDA and sterilized at 120°C for 15 min, then flasks were left to cool and poured in Petri dishes. Subsequently, 0.5 cm plug *P. cinnamomi* mycelia 7 days old was add and incubated at 25 ± 2°C, until the untreated control (PDA only) completely covered the Petri dish. The response variable was radial growth (cm).

This data was transformed to percent of mycelia growth inhibition by the following equation P = (CT) / C x 100, where P is inhibition percentage, C is colony diameter of the control treatment and T is the colony diameter of a specific treatment. Treatments were established under a completely randomized design with four replications.

In addition, Probit analysis by maximum likelihood method (Finney, 1971) to determine the minimum inhibitory concentrations at 50% (MIC_{50}) of each extract was used. Data were analyzed using SAS V8.1 software. The MIC_{90} and MIC_{50} values were calculated as the 90th and 50th percentile of the minimum inhibitory concentration values and their fiducials limits respective.

RESULTS

The variance analysis detected significant differences on the antifungal activity by effect of polyphenols derives Mexican plants. We observed differences in percentage of mycelia growth inhibition of *P. cinnamomi*. These percentages in mycelia growth inhibition varied from 0% (control treatment) to 100% in the highest concentration treatment where plant extracts were used. In Figure 1, is shown as totals polyphenol concentration is increase, the algae mycelia growth inhibition also increases.

The antifungal effects of plant extracts on *P. cinnamomi* were variable. Figure 1a shows that the *L. tridentata* extracts promoted the high mycelium inhibition until 100% when those was obtained with ethanol and 80% when lanolin was used, while the lowest antifungal effect was observed with cocoa butter and water solvents. It also shows that as total polyphenols concentration increase *P. cinnamomi* mycelia growth inhibition also increases.

Results obtained with *Flourensia cernua* indicated that the highest fungal inhibition effect are reached using ethanol and water as solvents, while the lowest fungal inhibition effect was observed when lanolin was used during the extraction (Figure 1b). Although the fungal inhibition effect were equal (100%) with extracts obtained using water and ethanol, the concentrations required in the later case are lower (Figure 1b).

Pecan (*C. illinoensis*) nut husk extracts showed little or no effects on *P. cinnamomi* mycelium growth inhibition, the highest inhibition effect (16%) was observed when cocoa butter extracts was used as solvent (Figure 1f). In this case, it was so that the highest (3000 ppm) concentration inhibited only 50% the mycelium growth, extractions where ethanol, water and lanolin were used as solvents no inhibitory effect were observed.

This is the first study reporting the use *Opuntia ficus-indica, Agave lechuguilla, Lippia graveolens* and *Yucca filifera* extracts against *P. cinnamomi*. The highest fungal inhibition effects (100%) using *Opuntia* extracts were observed using water as solvents and a polyphenols concentration of 4000 ppm. While little or no mycelia growth inhibition was found with the other solvents (Figure 1c).

Agave extracts showed the best fungal inhibition effect (60%) observed when water was used during extraction at polyphenol concentration of 3000 ppm (Figure 1d). Not mycelium growth inhibition effects were observed with cocoa butter and lanolin emulsions, while inhibition effect (40%) was observed when ethanol was used in the extraction in polyphenols at 40 ppm concentration. The highest mycelium growth inhibition (80%) on *P. cinnamomi* by *Lippia* extracts was observed in lanolin at 40 ppm, while no fungal inhibitory effects were observed with aqueous extracts. In general it was observed less than 50% inhibition using ethanol and cocoa butter as solvents (Figure 1e). *Yucca* extracts showed little or no effect on *P. cinnamomi* mycelium growth inhibition at the evaluated concentration (Figure 1g).

The results obtained in the present study showed that the plant species has an effect on the level of *P. cinnamomi* mycelium growth inhibition. Figure 2 shows that the highest (75.3 and 68.1%) mycelium growth inhibition was reached when *Flourensia cernua* and *L. tridentata* were used as sources of extracts. On the other hand, all other plant species showed a maximum average effect on fungal inhibition of 30%. In general, it was observed plant and solvent interaction effects on mycelium

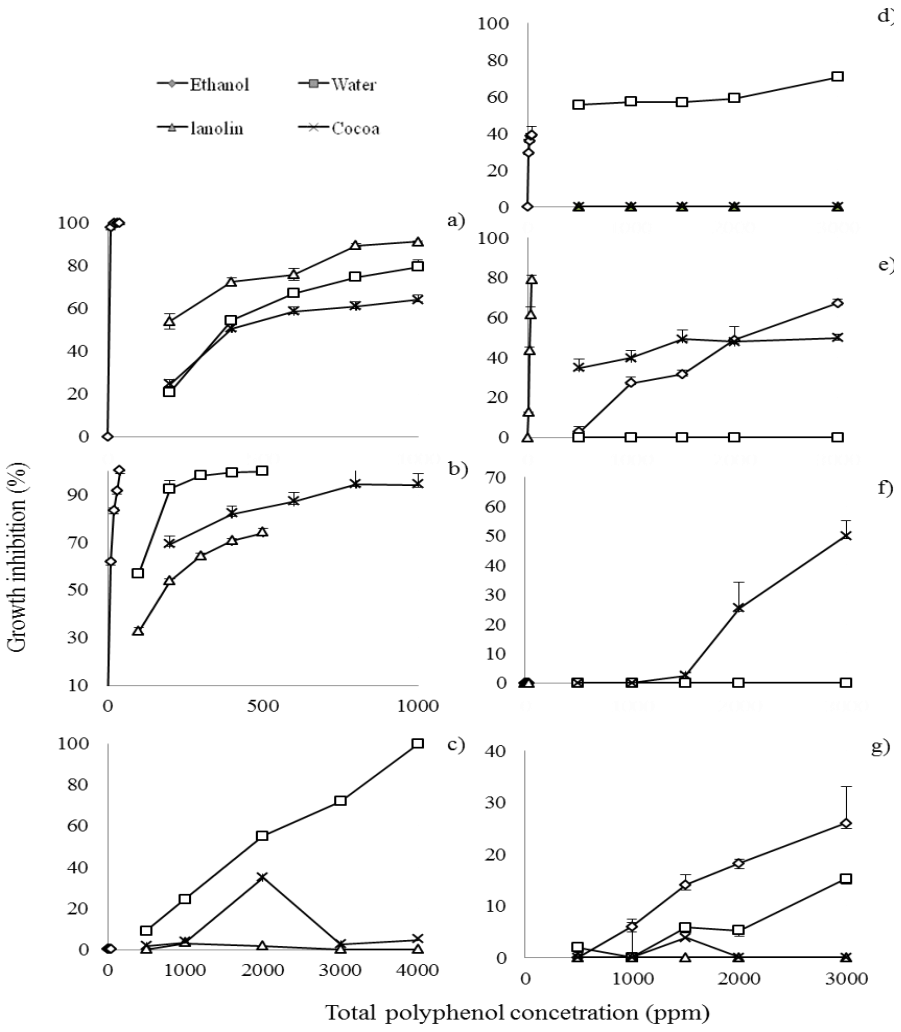

Figure 1. Inhibition response of *P. cinnamomi* at the total polyphenols concentration (PPM) obtained different solvents from (a) *L. tridentata*, (b) *F. cernua*, (c) *O. indica*, (d) *A. lechuguilla,* (e) *L. graveolens*, (f) *C. illinoensis* and (g) *Y. filifera*.

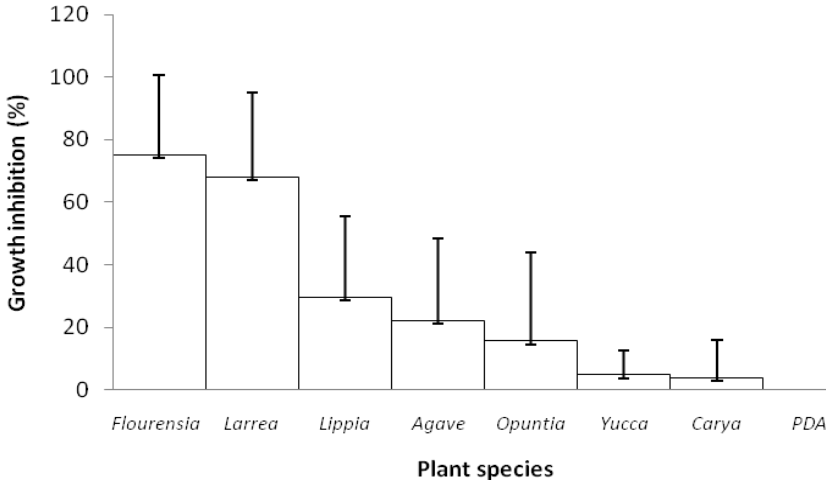

Figure 2. *In vitro* average effect on inhibition of *P. cinnamomi* with different plant extracts.

Table 1. Totals polyphenols minimum inhibitory concentrations (ppm) for inhibit mycelia of *P. cinnamomi.*

Species	Solvents	MIC_{50}	Fiducial limits 95% of MIC_{50}		MIC_{90}
			Inferior	Superior	
Larrea tridentata	Water	483.7	449.8	518.2	1431
	Lanolin	183.6	155.3	210.3	1008
	Cocoa	664	560.4	772.4	7213
	Ethanol	6.96	6.17	7.85	11.19
Flourensia cernua	Water	94.97	88.05	101.36	193.14
	Lanolin	230.12	212.83	247.65	1188
	Cocoa	112.19	62.78	157.25	619.14
	Ethanol	8.6	7.8	9.36	23.61
Opuntia ficus indica	Water	13039	5803	596284	68568
	Lanolin	5378	4524	6817	20636
	Cocoa	1867	1723	2013	3595
	Ethanol	341.95	80.82	576.41	409181
Agave lechuguilla	Water	28.87	22.39	38.05	121.7
	Lanolin	23.07	21.96	24.22	58.5
	Cocoa	252.7	209.07	298.87	326974
	Ethanol	2032	1908	2169	5952
Lippia graveolens	Water	2887	2704	3140	4825
	Lanolin	0	-	-	0
	Cocoa	0	-	-	0
	Ethanol	0	-	-	0
Yucca spp.	Water	0	-	-	0
	Lanolin	0	-	-	0
	Cocoa	0	-	-	0
	Ethanol	0	-	-	0
Carya illinoensis	Water	0	-	-	0
	Lanolin	0	-	-	0
	Cocoa	0	-	-	0
	Ethanol	0	-	-	0

Fiducial limit = confidence interval, MIC = Minimun inhibitory concetrantion in PPM.

growth inhibition of *P. cinnamomi*. The MIC_{50} of each plant extract on *P. cinnamomi*, was highly variable among solvents within each particular specie. The lowest MIC_{50} was obtained with *L. tridentata* in ethanol with 6.96 ppm, and the highest with *Opuntia* aqueous extract with 13039 ppm (Table 1). MIC_{50} analysis reveals that the lowest concentrations inhibiting 50% of mycelia growth of *P. cinnamomi* are: 6.96 of *L. tridentata* in ethanol, 8.60 of *F. cernua* in ethanol, 23.07 of *L. graveolens* in lanolin, 28.87 of *A. lechuguilla* in ethanol (Table 1).

The highest concentrations (ppm) to inhibit 50% of *P. cinnamomi* mycelia growth are: *Opuntia* aqueous extract at 13039.00, *Y. filifera* ethanol extracts with 5378.00, for *C. illinoensis* extracts using cocoa butter as solvent with 2887 and *L. graveolens* ethanolic extracts with 2032 (Table 1). The extracts that did not inhibit *P. cinnamomi* mycelia growth are: *Y. filifera* using both lanolin and cocoa butter as solvents, *O. ficus-indica* with lanolin, cocoa butter and ethanol as solvents, *A. lechuguilla* with lanolin and cocoa butter as solvent, *L. graveolens* with cocoa butter and *C. illinoensis* with water, lanolin and ethanol as solvents (Table 1).

DISCUSSION

The solvents used permitting the extraction of polyphenols from plants in this study. It was demonstrated the solvents chemical structure interaction in specific manner with the polyphenols type extracted from vegetal tissue. Because it was used two groups of solvents, one highly hydrophilic (water and ethanol) and other hydrophobic

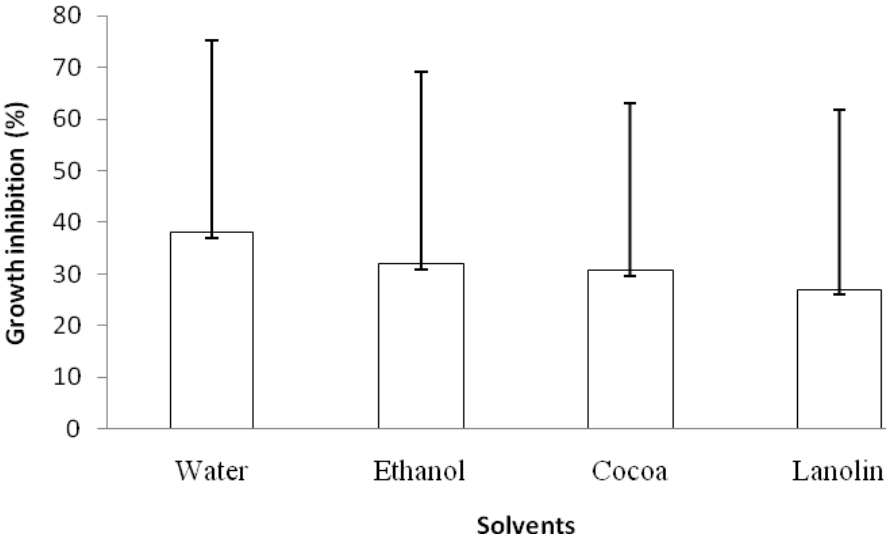

Figure 3. *In vitro* average effect on *P. cinnamomi* mycelia growth inhibition using different solvents.

(lanolin and cocoa butter) where polyphenols quantity differences obtained can be due to plant genera and solvent in this study (Figure 2). In addition, the polyphenols content in tissue is affected by season of plant tissue recollection, vegetative part, and plant growing conditions (Gamboa et al., 2003a; Hyder et al., 2005).

The differences shows on mycelia growth inhibition by polyphenols can be due to the chemical constitution of the polyphenols extracted associated with solvents (lanolin, cocoa butter, ethanol and water) may be due to the association formed between the hydrophobic region present in their structures, and the lipophilic region of the polyphenolic ester group, in comparison to the hydrophilic region of the water molecule. Lanolin is a complex mixture of esters of sterols, triterpene alcohols, esters of aliphatic alcohols and monohydroxyesters of sterols and triterpenes and aliphatic alcohols (Schlossman and McCarthy, 1978), while cocoa butter is composed by glycerides, mainly oleo-palmitostearin, oleo-distearin, oleodipalmitin, stearo-diolein, palmito-dioleintrisaturatedtriolein and triunsaturedtriolein (Beckett, 1994).

On the other hand, results of this study suggest that emulsions obtained with lanolin and ethanol inhibit better this pathogen than extracts using water or cocoa butter as solvents at low concentrations.

The antifungal effect of all extracts on *P. cinnamomi* inhibition contrast with studies shown by other authors, because research works using different plant species. Gamboa et al. (2003) reported the use *L. tridentata* extracts against *P. infestans* and shown an antifungal activity of 100% at concentrations of 4000 ppm. Our results indicated that *L. tridentata* ethanol-extracts has potential on *P. cinnamomi* control because it was observed 100% fungal growth inhibition with concentrations as low as 20 ppm (Table 1). Galván (2005) reported 100% inhibition effects on *P. capsici* using ethanolic resin at concentrations of 500 ppm derives from *F. cernua* and Gamboa et al. (2003) found mycelium growth inhibition of 67.28% to 20,000 ppm using methanolic extracts against *P.infestans*. Osorio et al. (2010) mentioned effects in inhibition (100%) on *Pythium* sp. using *C. illinoensis*.

In general, it was observed that the polyphenols obtained from plant extracts using different solvents have effects on mycelium growth inhibition of *P. cinnamomi*. Results obtained with these plant species are similar to those obtained by Gamboa et al. (2003a, b) against *Phytophthora* spp. and confirm the antifungal activity of polyphenols derives from *F. cernua* and *L. tridentata.*

From this study results, it can be inferred that solvent selection play important role on metabolites extraction. The present study showed that aqueous solvents present major antifungal response to Oomycetes (Figure 3). Ethanolic extracts is 20 times better than water and 5 times better than lanolin extracts for have higher effect on *P. cinnamomi* growth inhibition (Figure 2).

Also, lanolin can be the alternative solvent because it showed an interesting effect on polyphenol extraction from *L. graveolens* and excellent effect on antifungal response.

The MIC_{50} obtained with more effective plant extracts on mycelia growth inhibition was such as 20 ppm and derives from *L. tridentata* and *F. cernua.* These doses are lower than those needed to *in vitro* inhibit 100% of *P. cinnamomi* mycelia growth using a commercial fungicide

(Metalaxyl at 750 ppm) (Gamboa et al., 2003b).

Conclusions

It was possible *in vitro* mycelia growth total inhibition of *P. cinnamomi* using *L. tridentata* and *F. cernua* extracts obtained using ethanol and water, *L. graveolens* extracts obtained using lanolin. The best concentrations were lower than 20 ppm of total polyphenols.

Conflict of Interests

The authors have not declared any conflict of interests.

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