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Lippia graveolens and *Carya illinoensis* Organic Extracts and there *in vitro* Effect Against *Rhizoctonia Solani* Kuhn

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Abstract: Problem statement: Plant extracts with polyphenolic compounds obtained with different solvents have been evaluated against plant pathogens. However, most of these extract have been obtained using solvents no allowed under an organic production context. **Approach:** In the present research was to evaluate the inhibitory effect of polyphenolic extracts from *L. graveolens* and *C. illinoensis*, obtained with alternative organic solvents (lanolin and cocoa butter), water and ethanol against *R. solani* in order to determine the Inhibitory Concentration (IC_{50}) of each extract. **Results:** The results showed that extracts of both *L. graveolens* and *C. illinoensis* obtained with lanolin and ethanol (200 and 3000 ppm of total tannins, respectively) inhibited at 100% growth of *R. solani*. The IC_{50} for each extract was highly variable, low IC_{50} values were obtained with *L. graveolens* (4.50×10^{1}) and *C. illinoensis* (4.33×10^{2}) extract using lanolin and ethanol respectively. Tannins extraction was strongly dependent on plant species and in the solvent used. **Conclusion:** The alternative organic solvents lanolin and cocoa butter allowed the recovery of polyphenols compounds with antifungal activity against *R. solani*.

Key words: *Lippia graveolens*, *Carya illinoensis*, Lanolin, Cocoa butter, IC_{50}, *R. solani*, mycelia inhibition

INTRODUCTION

Pesticide applications for control of fungal pathogens, among other effects causes: environmental pollution, affectation to beneficial organisms (Anderson *et al*., 2003) and generation of populations of resistant pathogens to chemicals in response to selection pressure due to high dose and continuous applications (Leroux, 2003). Currently seeking alternative for disease control, consider using plant derived compounds in extract form, the potential use of these to control plant pathogens has been shown in laboratory studies (Qasem and Abu-Blam, 1996), in greenhouse (Bergeron *et al*., 1995; Lomeli and Ochoa, 1999) and at field level (Cruz *et al*., 1999). Plants possess a variety of secondary compounds in their tissues such as polyphenols, terpens and alkaloids. Among the polyphenolic polymers are tannins, these compounds have the ability to form complexes with proteins, polysaccharides, nucleic acids, steroids, alkaloids and saponins. Based on their chemical origin, tannins are classified into two main groups: hydrolysable and condensed tannins. The hydrolysable tannins are polymers of phenolic acids (gallic, hexahidroxidifenic acid), while condensed tannins are polymers of flavan-3-ols (Isaza *et al*., 2007). Among the plant families with presence of polyphenols are Leguminosae, Rosaceae, Polygonaceae, Fagaceae, Rhyzophoraceae, Myrtaceae and Melastomataceae (Isaza, 2007). In this sense, Mexico has native plants with high content of these compounds (Gamboa *et al*., 2003, Lira *et al*., 2007; Castillo *et al*., 2010), extracts obtained from these plants using methanol, acetone among others solvents, have proven effective antimicrobial activity, however there is a lack of knowledge about to obtaining these phytochemicals with unconventional solvents which can be used as a potential alternative disease management within a system of organic agriculture. In this context, this study aims determine antifungal *in vitro* effect of *L.*

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graveolens and *C. illinoensis* extracts obtained with lanolin, cocoa butter, water and ethanol on *R. solani* mycelia inhibition growth.

MATERIALS AND METHODS

Vegetal material: Whole plants (leaves, stems and roots) of Mexican oregano (*L. graveolens*) and pecan husk (*C. illinoensis*) were collected on August and September 2008 in the Southern region of Coahuila State, Mexico. The samples were transported to the Microbiology Laboratory, Department of Food Research, Faculty of Chemical Sciences, Universidad Autonoma de Coahuila, in plastic bags. Dehydration process was performed under environment temperature and in an oven (LABNET International, Inc.) for two days at a temperature of 60°C. Then, dry samples were ground in a mill (Thomas Wiley) and passed through a 1mm screen. The fine and dried powder was stored in amber bottles or plastic bags and at room temperature under darkness.

Extraction of polyphenols: Polyphenolic compounds extraction from each collected sample was done in a proportion of 1:4 (w/v) for infusion method using water and ethanol (70%). Lanolin and cocoa butter extractions were done using emulsions with mineral oil (10%) at 60°C for 7 h. After this, extracts were filtered. The obtained extracts were stored in containers covered with aluminum or amber bottles at 5°C.

Determination of hydrolysable and condensed tannins: The tannin concentrations were determined by spectrophotometry as hydrolysable tannins (Makkar, 1999), as condensed tannins (Swain and Hillis, 1959) of a sample extract diluted at 1:20 (extract: distilled water). For hydrolysable tannins determination, a reference curve was done by using 400 µL of gallic acid at different concentrations (0, 200, 400, 600, 800, 1000 ppm). Gallic acid concentrations were prepared using distilled water. Each one of the plant extracts was diluted in a test tube and was added 400 µL of commercial Folin-Ciocalteau reagent. Samples were vortexed and left for 5min at room temperature. Then 400 µL of NaCO3 (0.01M) and 2.5 mL of distilled water were added. Finally absorbance was read at 725 nm.

For condensed tannins determination, 0.5 mL of plant extract was used with 3mL of HCl/butanol (1:9) and 0.1 mL of ferric reagent. The standard was prepared using catechin dissolved in distilled water at different concentrations (0, 200, 400, 600, 800 and 1000 ppm). Samples were heated for 1 h in water bath at 90°C and absorbance was read at 460 nm.

Table 1: Plant extract concentrations (ppm) against *Rhizoctonia solani* used in this study

Extract /solvent	Concentration (ppm)
L. graveolens/water	500, 1000, 1500, 2000, 3000
L. graveolens/lanolin	200, 400, 600, 800, 1000
L. graveolens/cocoa butter	500, 1000, 1500, 2000, 3000
L. graveolens /Ethanol	500, 1000, 1500, 2000, 3000
C. illinoensis/water	500, 1000, 1500, 2000, 3000
C. illinoensis/lanolin	200, 400, 600, 800, 1000
C. illinoensis/cocoa butter	500, 1000, 1500, 2000, 3000
C. illinoensis/ Ethanol	500, 1000, 1500, 2000, 3000

Effect of the plant extracts on *R. solani* mycelia growth: This work step was carried out in the Laboratory of Microbiology, Agricultural Parasitology Department at Universidad Autonoma Agraria Antonio Narro, Saltillo Coahuila, Mexico. Tannin concentrations of the tested extracts are shown in Table 1. The poisoned culture medium technique was used to determine the effect of different extracts on inhibition of *R. solani* mycelia growth. In this case, four Petri dishes with one of the different extract concentration were used per treatment. First, the volume required for each extract and concentration was determined. This amount was added to an Erlenmeyer flask with the required water content and PDA medium. Then, flasks were sterilized at 120°C for 15 min.

Discs with fungal mycelia (0.4 cm in diameter) were placed on Petri dishes, which had poisoned culture media. This was done for each different extract and concentration. Petri dishes were incubated at 25±2°C. The efficacy of treatments was evaluated measuring fungal radial growth (cm). The percent of mycelia growth inhibition (P) was estimated using as reference the control treatment (Petri dishes only with PDA medium) as follows:

$$P = (C-T)/C\times100$$

where, C is the colony diameter under the control treatment and T is the colony diameter under the extract treatment. The experiment was established under a completely randomized design with four replications. The Probit analysis was made to determine the 50% Inhibitory Concentration (IC_{50}) of each extract. Data were analyzed using the software SAS V8.1.

RESULTS

Effect of solvents on total polyphenols content extraction: The extraction of polyphenols compounds varied among species according to the solvent used. In addition, it was observed an interaction between plant species and solvent for total tannins extraction. The highest concentration was obtained from *L. graveolens* with 2.327×10^5 ppm using ethanol, followed by *C. illinoensis* with 1.93×10^5 ppm using cocoa butter.

Table 2: Concentrations IC_{50} and IC_{90} (ppm) of the extracts of *Lippia graveolens* and *Carya illinoensis* obtained with different solvents against *Rhizoctonia solani*

			Fiducial limits			Fiducial limits	
Extract	Solvent	IC_{50}	Superior	Inferior	IC_{90}	Superior	Inferior
L. graveolens	Ethanol	1.93×10^{3}	1.72×10^{3}	2.21×10^{3}	2.25×10^{4}	1.45×10^{4}	4.19×10^{4}
L. graveolens	Lanolin	i*					
L. graveolens	Cocoa	1.11×10^{3}	8.61×10^{2}	1.36×10^{3}	4.03×10^{3}	2.87×10^{3}	7.69×10^{3}
L. graveolens	Water	0.00	0.00	0.00	0.00	0.00	0.00
C. illinoensis	Ethanol	4.34×10^{2}	2.39×10^{2}	5.88×10^{2}	1.45×10^{3}	1.15×10^{3}	2.11×10^{3}
C. illinoensis	Lanolin	2.14×10^{3}	1.69×10^{3}	3.10×10^{3}	1.25×10^{4}	6.61×10^{3}	5.11×10^{4}
C. illinoensis	Cocoa	0.00	0.00	0.00	0.00	0.00	0.00
C. illinoensis	Water	0.00	0.00	0.00	0.00	0.00	0.00

i*: Inhibition at 100% to 200 ppm

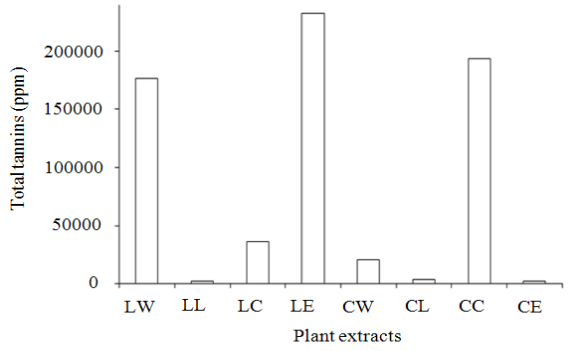

Fig. 1: Concentration of total tannins (hydrolysable tannins and condensed tannins) present in each plant extract of *Lippia graveolens* (LW in water, LL in lanolin, LC in cocoa butter and LE in ethanol) and *Carya illinoensis* (CW in water, CL in lanolin, CC in cocoa butter and CE in ethanol) in ppm (equivalent to gallic acid and catechin)

The solvent that yielded the highest tannin concentration was ethanol (1.17×10^{5} ppm) and with lanolin was obtained the lowest (2.79×10^{3} ppm) total polyphenols content (Fig. 1).

Effect of plant extracts on inhibition of *R. solani* mycelia growth: The effect of different extracts of Mexican oregano and pecan husk obtained using four different solvents on *R. solani* was highly significant at 72 h. Figure 2 shown that as extract increases, the mycelia growth of *R. solani* is significantly reduced, with exception of the water extract for both Mexican oregano and pecan husk and cocoa butter extract from pecan husk. According to the values of inhibition mean, lanolin and ethanol extracts were statistically ($p\leq0.01$) more efficient, with an inhibition mean of 69.1 and 63.5% respectively, water extract was the least efficient with an inhibition mean of 16.0%.

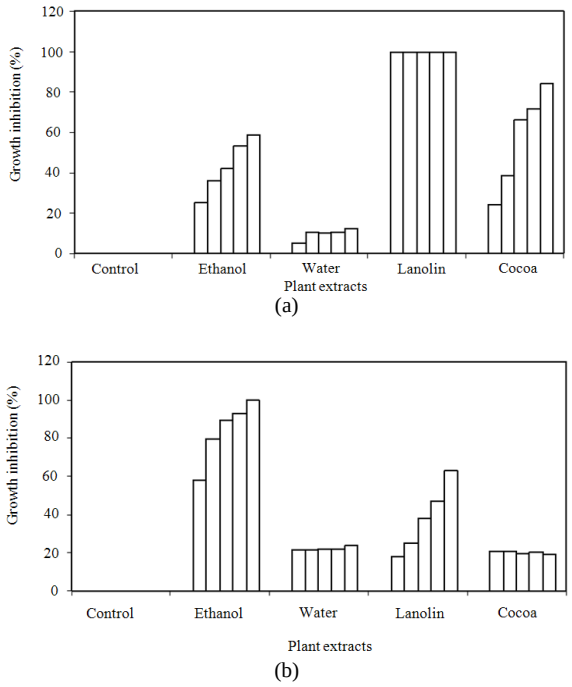

Fig. 2: Percentage of mycelia inhibition of *Rhizoctonia solani* with four plants extracts to five concentrations equivalent total tannins (ppm), A) *Lippia graveolens* extracts and B) *Carya illinoensis* extracts

The greater mycelia inhibition (100%) was observed with *L. graveolens* extract using lanolin as solvent at a concentration of 200 ppm of total tannins, followed by cocoa butter extract with 83% of mycelia inhibition to 3000 ppm, while that with ethanol extracts was obtained a 60% of mycelia inhibition at 3000 ppm, the water extract had little effect on *R. solani* mycelia inhibition (Fig. 2A).

In the case of *C. illinoensis* extracts, the greatest effect was found with ethanolic extracts with doses from 500-3000 ppm of totals tannins with a range of

mycelia inhibition of 60-100% (Fig. 2B), followed for the lanolin extracts with 62% of *R. solani* mycelia inhibition using 3000 ppm of total tannins and only 23% of mycelia inhibition was observed with water and cocoa butter extracts respectively.

Inhibitory concentrations of plant extracts to 50% (IC_{50}): The IC_{50} of each extract on fungus growth was highly variable among extracts with the different solvents within each particular species. The lowest IC_{50} was obtained with *L. graveolens* with 4.50×10^{1} ppm with lanolin as solvent and the highest with pecan husk to 2.13×10^{3} ppm using lanolin (Table 2).

DISCUSSION

These differences in total tannins production for ethanol, is correlated with the high polarity of ethanol and partition point to extract more polyphenolic compounds. These results are consistent with those reported by Lira *et al*. (2003) whom mentioned that absolute ethanol extracts less resin from *Larrea tridentata* (SEES and Moc. Ex DC) that its use at lower concentration. In this study, ethanol (70%) yielded higher concentration of polyphenol compounds from *L. graveolens* than the other solvents. There is only a reference about the use of lanolin and cocoa butter in extraction of polyphenolic molecules with antifungal effect, where it was found that the use of cocoa butter and lanolin as solvents allowed higher extraction of polyphenolic compound than water (Castillo *et al*., 2010).

This results corroborated the antifungal effect of *L. graveolens* against *R. solani* (Hernandez *et al*., 2008) and in contrast with those obtained by Garcia *et al*. (2006), whom found a fungicidal effect at 1000 ppm of oregano essential oil, although these differences are given by the different genus and specie (*Origanum vulgare*) used, as pointed out by Hernandez *et al*. (2008). Likewise, there is little information regarding the use of polyphenolic extracts from *C. illinoensis*, Osorio *et al*. (2009) found high sensitivity of *R. solani* to acetonic extracts obtained from *C. illinoensis* nuts shell to 0.20 mg L^{-1}, these results contrast with those obtained in this work to 3000 ppm of tannin required to inhibit in 100% this fungus, these differences could probably be explained by the different plant tissue used and the evaluation conditions, in our case, growth was assessed in a radial way as opposed to only qualitative presence or absence of growth on plaques of polystyrene employed by Osorio *et al*. (2009).

The use of lanolin and cocoa butter for the extraction of highly efficient phytochemicals to inhibit fungus growth *in vitro* was reported previously (Castillo *et al*., 2010), where they mentioned that efficiency differences for inhibit mycelia growth of extracts with these solvents are given because emulsions are best to extract more and different phytochemicals from plants, the type and concentration of phytochemicals recovered during the extraction process determines the efficiency of inhibitory capacity, due to differences in polarity and partition points of these solvents. Results with extracts using ethanol as solvent agree with those obtained with extracts from *Flourensia cernua* using ethanol and other solvents, where similar concentrations than those evaluated here, which inhibited the mycelia growth of *Alternaria alternata*, *Penicillium digitatum* and *Collectotrichum gloesporoides* from 80-100% respectively (Guerrero *et al*., 2007).

The obtained IC_{50} values are different to those obtained by Hernandez *et al*. (2008) whom found an IC_{50} of 10 ug mL^{-1} although these differences are probably given by the use of essential oil that may have other compounds with antifungal activity, so it is less than the required dose of methyl tolclofos for total inhibition of *R. solani in vitro* (Gamboa *et al*., 2003).

CONCLUSION

The use of different alternative organic solvents allowed the obtaining of extracts with antifungal activity against *R. solani*, in specific, the amount of phenolic compounds is a function of solvent and vegetal specie used. The highest activity against *R. solani* was obtained with extracts from *L. graveolens* with lanolin and C. illenoensis with ethanol. To the best of our knowledge, this is one of the first reports with nonconventional organic solvents (lanolin and cocoa butter) for extract phytochemicals with antifungal activity from *L. graveolens* and *C. illenoensis* husks. These solvents represent an attractive alternative for development of natural products to control plant pathogen fungi, which may avoid the use of synthetic fungicides.

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