PHENOLS AND FLAVONOIDS CONCENTRATION AND FUNGISTATIC ACTIVITY OF WOOD AND BARK OF FIVE COMMON TROPICAL SPECIES

[CONCENTRACIÓN DE FENOLES Y FLAVONIODES Y ACTIVIDAD ANTIFÚNGICA DE LA MADERA Y CORTEZA DE CINCO ESPECIES COMUNES TROPICALES]

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SUMMARY

This research determine the total phenol and flavonoids content as well as the fungistatic activity of hot water wood sawdust and bark extracts on *Coniophora puteana* and *Trametes versicolor*. Extracts tested were taken from *Condalia hookeri* M.C. Johnst., *Ebenopsis ebano* (Berl.) Britton et Rose, *Helietta parvifolia* (*Gray*) Benth, *Leucaena leucocephala* (Lam.) de Wit, and *Prosopis laevigata* (Humb. et Bonpl. ex Willd). The extraction was developed with soxhlet aparats, phenol concentration was determined with Folin-Ciocalteu method and flavonoide was determined by Heimler procedure. Phenol concentration ranged between 50±11 to 827±23 mg g^{-1} and flavonoids content between 15±2 to 708±30 mg g^{-1}. All extracts tested inhibited growth of the fungal species. The highest inhibition effect (88%±1) occurred on *C. puteana* with *L. leucocephala* wood sawdust extracts at 2 mg ml^{-1}, this species also reduced the growth on *T. versicolor* by 75%±12 when used at 10 mg ml^{-1}, wood sawdust extracts of *H. parvifolia* and *C. hookerii* at same concentration reduced the growth of *T. versicolor* in 43%±3 and 40%±4 respectively. Inhibition of bark extracts of *E. ebano* was 84±5 and 80±7 % for *H. parvifolia*. A negative relationship between growing inhibitory activity and the content of total phenolics in the extracts was obtained.

Key words: Fungistatic effect; growing inhibition; extracts; native species.

RESUMEN

La presente investigación determina el contenido de fenoles y flavonoides, así como y la actividad fungiestática en los hongos *Coniophora puteana* y *Trametes versicolor* de los extractos de madera y corteza de las especies comunes tropicales *Condalia hookeri* M.C. Johnst., *Ebenopsis ebano* (Berl.) Britton et Rose, *Helietta parvifolia* (Gray) Benth, *Leucaena leucocephala* (Lam.) de Wit y *Prosopis laevigata* (Humb. et Bonpl. ex Willd). La extracción se realizó con aparatos solxhlets, la contenido de fenoles se determinó con el procedimiento Folin-Ciocalteu y los flavonoides por el método de Heimler. La concentración de fenoles se presentó en un rango de 50±11 a 827±23 mg g^{-1} y el contenido de flavonoides fue de 15±2 a 708± 30 mg g^{-1}. Todos los extractos inhibieron el crecimiento de las especies de hongos. La mayor inhibición (88±1%) se presentó en *C. puteana* al ser expuesto a los extractos de madera de *L. leucocephala* a una concentración de 2 mg ml^{-1}. La inhibición producida por extractos de *E. ebano* fue 84±5 y 80±6% por *H. parviflora*. Los extractos de aserrín de madera de esta especie a una concentración de 10 mg ml^{-1} redujo el crecimiento de *T. versicolor* en 75±12%, *H. parvifolia* lo redujo en 43±3% y *Condalia hookerii* 40±4%. Se obtuvo una relación negativa entre la actividad inhibitoria y el contenido de fenoles totales en los extractos.

Palabras calve: Efecto fungiestático; inhibición en el crecimiento; extractos; especies nativas.

INTRODUCTION

Thornscrub Shrubland vegetation grows naturally under harsh environmental conditions. Temperature ranges from 20 to 45°C and annual precipitation is less than 500 mm (Návar *et al*., 1999). The vegetation growing in this area is known as common tropical species and covers approximately 125,000 – 200,000 Km^2. It is characterized by shrubs and tree species with short stems with diameters of 0.15 m – 0.20 m (Rzedowski, 2006). Low economic input is obtained by traditional agricultural, free cattle management, and forest harvesting (Reid *et al*., 1990; Villalón and Carrillo, 2010). Some shrubs and tree species have demonstrated high natural resistance against fungi and other microorganism present in soil (Wolf and Perales, 1985; Carrillo, 2010; Carrillo *et al*., 2011).
The natural durability of tree species is defined as the resistance to biological degradation and is attributed to several factors, including wood anatomic structure, certain physical properties and, above all, accumulation of extracts (Eaton, 1993; Schultz, 1995; Haupt *et al*., 2003; Eyles *et al*., 2003; Aloui *et al*., 2004). Wood extracts are present in heartwood, mostly in the parenchyma cells, but are also found in vessels, fibers, specialized cells, and substances with low molecular weight from 2 – 10 % of dry mass (Hillis, 1971; Mantanis, 1995). These substances are produced during the wood maturation process when cambium tissues form differentiated sapwood and heartwood cells. Trees growing in an environment with high UV or high temperatures as well as trees which have been injured mechanically by insects, or have suffered a microbial attack are rich in substances such as polyphenols, since these are part of defence mechanisms (Rowell, 2005; Kawamura *et al*., 2011).

Polyphenols are known to have strong antioxidant and antibacterial effects which act as radical scavengers, biocides, and metal chelators (Pietarinen *et al*., 2006). According to Schultz and Nicholas (2002) the combination of organic biocide with metal chelating and/or antioxidant additives improves protection against fungi. This enhances the development of environmentally benign wood preservative substances. Determining the total amount of extracts and flavonoids in trees and shrubs in there Thornscrub Shrubland areas can help to increase their economic importance.

Information with regard to the natural durability of wood, wood quality, chemical wood composition and the potential of wood extracts of Thornscrub Shrubland vegetation to inhibit the growth of decay fungi is lacking (Wolf and Perales, 1985; Carrillo, 2010). The aims of this study are: To determine the total phenol and flavonoids content of hot water extracted solutions from wood sawdust and bark of five tree species known to have high natural durability; and to establish how extracts inhibit the growth of *Coniophora puteana* and *Trametes versicolor* fungi under laboratory conditions.

MATERIAL AND METHODS

Species selection, extraction and determination of phenol and flavonoids content

Five tree species native to Northeast Mexico were selected according to importance, abundance, distribution and use. There were randomly selected three specimens from *Condalia hookeri* M.C. Johnst., *Ebenopsis ebano* (Berl.) Barneby & Grimes, *Helietta parvifolia* (Gray) Benth, *Leucaena leucocephala* (Lam.) DeWit and *Prosopis laevigata* (Humb. & Bonpl. ex. Willd.) M.C. Johnst. Bark and heartwood chips from the outer heartwood of them fresh cut tree were obtained at 1.3 m from the tree base, they were then air conditioned for two months and milled to a 40-mesh sieve size.

Soxhlet flasks were used for extraction procedures (Demirbaş, 1991; Schwanninger, 2002). Three portions of 100 g with 10% moisture content of saw wood and bark were separately grounded and sieved at 500 μm and then put in Soxhlet flasks. Each flask was filled with 200 ml of distillate water. The water solution and sawdust were boiled for four hours at a minimum boiling rate of 24 cycles. Extracts were then isolated from the solvent in a vacuum rotary evaporator (Rotavapor Type R-114 Büchi) at 40°C.

The total phenols were measured using the Folin-Ciocalteu index method according to Waterman (1994). Hot water extracts (10 mg) were diluted in 10 ml of acetone/water (1:1 v/v). Six ml of water and 0.5 ml of Folin-Ciocalteu reagent (Sigma-Aldrich) were successively added to 0.1 ml of this solution. In addition, 1.5 ml of a 20% sodium carbonate solution and water were added to obtain 10 ml. A reaction took place within 120 min. at room temperature. Absorbance was measured at 760 nm. Calibration was done using a gallic acid solution (0.5, 0.4, 0.3, 0.2 and 0.1 mg ml^{-1} in acetone/water 1:1 v/v, $y= 0.0011x + 0.016$, $R^2 = 0.9984$). The results were expressed as the equivalent to milligrams of gallic acid per gram of extract (mg GAE g^{-1}).

The total flavonoid content was determined according to Heimler *et al*. (2005). Ten mg of extracts were diluted in 10 ml of acetone/water (1:1 v/v). A solution of 0.25 ml of the suitably diluted sample was added to 0.75 μl of a $NaNO_2$ (5% w/v) solution, as well as 0.150 ml of a freshly prepared $AlCl_3$ (10% w/v) solution, and 0.5 ml of 1 M NaOH solution. The final volume was adjusted to 2.5 ml with deionized water.

The mixture was allowed to stand for 5 min and the absorption measured at 510 nm against the same mixture without the sample. Calibration was done using (+)-catechin as standard, for which a calibration curve was obtained with solutions of 0.25, 0.2, 0.15, 0.1 and 0.05 mg mL^{-1} ($y = 0.0032x + 0.0127$, $R^2 = 0.998$). The results were expressed as the equivalent to milligrams of catechin per gram of extract (mg CEQ g^{-1}).

Growing inhibition effect

The growing inhibition effect of extracts dissolved in a water/acetone solution (1:1) at 2 mg ml^{-1} and 10 mg ml^{-1} concentrations on brown rot fungus *C. puteana* and white rot fungus *T. versicolor* was determined. This solution was stirred with a mixer (Heidolph Type Mr 3003 Control G.) for 10 min. Subsequently, the extracts previously diluted with the water/acetone solution were transferred to 140 mm Petri dishes providing 2 mg ml^{-1} and 10 mg ml^{-1} concentrations. Fifty ml of malt-agar medium at 60°C were then added to the Petri dish. Plates were inoculated by placing a 10 mm diameter mycelium plug in the centre of each plate. The plugs were obtained from the edge of a growing colony of each fungus. Three replicates for each extractive were used; five from the acetone/water control (only acetone and water in the medium) and five with the medium as a control treatment. Cultures were kept in an incubator at 25°C until the mycelium of the control reached the edges of the plates. The inhibition effect was determined at the end of that period by calculating the average of diametric growth in the two perpendicular directions. The data were used in the following formula (Gérardin, 2004; Neya *et al*., 2004).

$$G_i = 100 \cdot (1 - \frac{d_i}{d_o})$$

Where:
Gi = Growing inhibition effect (%)
d_i= Average diameter of the culture in the presence of extracts (mm)
d_0= Average diameter of the control culture (mm)

Statistical analysis

The data obtained were statistically analyzed using the SAS program. Analysis of variance was carried out and statistically significant differences were set at a 95% confidence level. Split plot desing ANOVA was applied to determine whether the growing inhibition was significantly different between species and between extractive concentrations. Additionally, interactions of growing inhibition effect for each fungus were examined with respect to tree species extracts kind of extractive and concentration.

RESULTS AND DISCUSSION

Total phenols

A higher phenol concentration was found for *E. ebano*, *P. laevigata*, and *C. hookeri* wood sawdust with values of 827±23, 818±8 and 400±15 mg g^{-1} respectively (Figure 1). Concentration for *C. hookeri*, *H. parvifolia* and *L. leucocephala* in bark was 232±29, 165±0.2 and 115±9 mg g^{-1}, respectively. No data describing phenol concentration for these species is available; however, tannin concentration on hardwood was presented by Foroughbakhch *et al*. (2008) with values ranging from 56 mg g^{-1} for *P. glandulosa* to 172.5 mg g^{-1} in *C. hookeri*. Scalbert *et al*. (1989) reported lower total phenol values from 17 trees species with lower values than reported in this research: *Quercus rubur* (62.6 mg m^{-1}), *Castanea sativa* (53.4 mg m^{-1}) and *Q. petrea* (39.3 mg m^{-1}). Hărmănescu *et al*. (2008) found concentrations from 52 to 400 mg g^{-1} in medicinal plants from Romania. Sáenz-Esqueda *et al*. (2010) found phenols concentration ranging from 48.9 to 399.2 mg g^{-1} in catequin equivalents from needle extracts of *Pinus cooperi* and *Pinus teocote,* respectively. Rosales-Castro *et al*. (2009) reported values of phenols from 491 to 604 mg g^{-1} and flavonoid from 292 to 385 mg g^{-1} in *Pinus cooperi, Pinus engelmannii, Pinus leiophylla and Pinus teocote*.

Total flavonoids

The content of total flavonoids varied between wood sawdust and bark, with the wood sawdust showing higher values of 708±30, 572±17, and 41±4 mg g^{-1} for *P. laevigata*, *E. ebano* and *C. hookeri,* respectively. Bark extracts showed lower amounts 75±5, 47±4 and 32±6 mg g^{-1} for *H. parvifolia*, *C.* hookeri and *P. laevigata*, respectively (Figure 2).

Growing inhibition

ANOVA results (Table 1) show that the growing inhibition effect on *C. puteana* fungus was highly significant among tree species ($p<0.0001$, $F=26.5$) and significant among extracts ($p< 0.0157$, $F=6.28$). The growing inhibition effect is shown in Table 2. A higher inhibition for *C. puteana* growth was shown by *L. leucocephala* wood sawdust and bark extracts at both concentrations: levels from 68±0.6 to 88±0.1% for bark (2 mg ml^{-1}) and wood sawdust (2 mg ml^{-1}). Biocide activity for *L. leucocephala* was also demonstrated on seedlings (Aderibigbe *et al*., 2011). The activity was attributed to the high concentration of chitinases. These enzymes break down glycosidic bonds in chitin which composes the cell walls of fungi and exoskeletal elements of some worms and arthropods (Kaomek *et al*., 2003). Chitosan was also used on wood modification under experimental

conditions (Larnøy *et al*., 2006). *E. ebano* extracts from bark at 10 mg ml^{-1} also reduced the growth of *C. puetana* (84±5%).

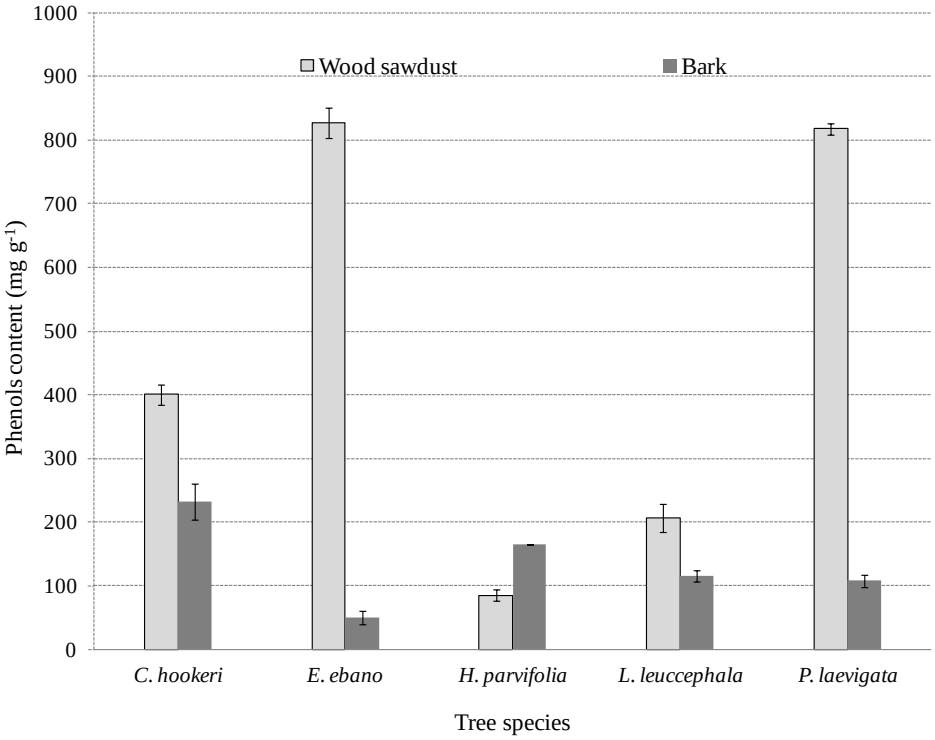

Figure 1. Total phenol concentration (mg g^{-1}) of sawdust wood and bark form five tree species.

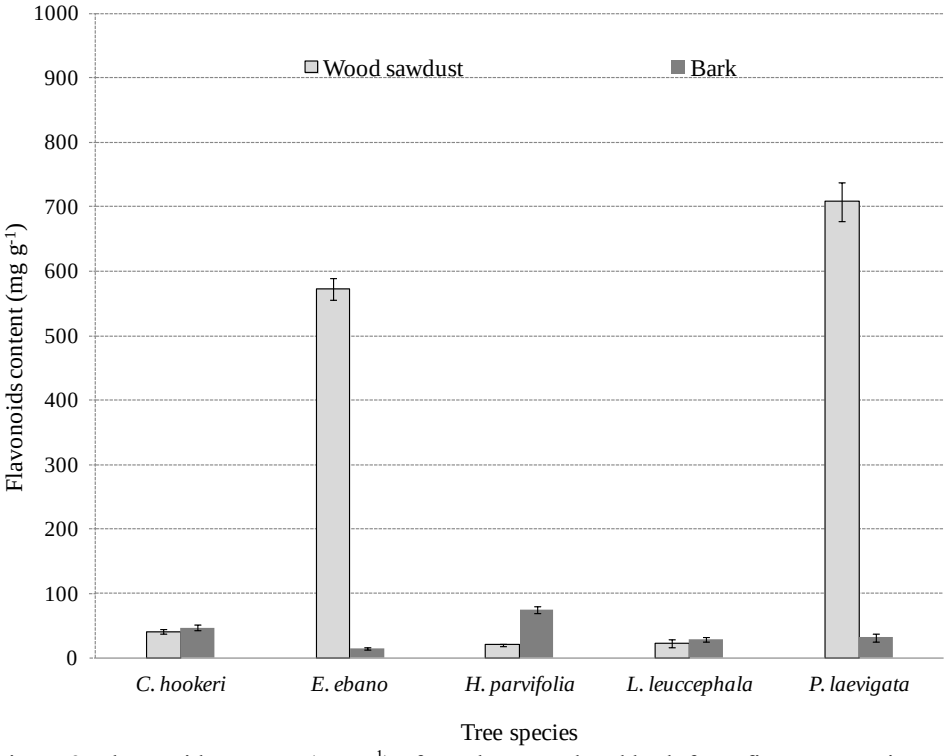

Figure 2. Flavonoids content (mg g^{-1}) of sawdust wood and bark form five tree species.

Table 1. Analysis of variance for growing inhibition of *C. puteana* fungi produced by wood and bark extracts from five tree species.

Source of variation	df	Sum of Squares	Mean Square	*F* value	*p* value
Wood species (WS)	4	33588.76	8397.19	26.50***	0.0001
Extracts (E)	1	1989.35	1989.35	6.28**	0.0157
WS*E	4	10246.63	2561.66	8.08***	0.0001
Concentration (C)	1	379.53	379.53	1.20NS	0.2792
E*C	1	12.57	12.57	0.04NS	0.843
Error	48	15209.96	316.87		

Table 2. Growing inhibition effect (%) on *C. puteana and T. versicolor* fungi by wood sawdust and bark extracts.

Tree species	*C. puteana*				*T. versicolor*			
	Wood sawdust mg ml^{-1}		Bark mg ml^{-1}		Wood sawdust mg ml^{-1}		Bark mg ml^{-1}	
	2	10	2	10	2	10	2	10
C. hookeri	55±1.3	9±5.1	3±0.3	1±0.2	3±0.0	40±4.4	4±2.0	2±2.1
E. ebano	16±0.4	45±13.8	56±2.4	84±5.1	2±2.3	15±5.7	2±0.4	30±1.5
H. parvifolia	5±1.4	47±5.8	44±3.7	80±6.5	22±1.7	43±12.8	21±0.8	49±3.3
L. leucocephala	88±0.1	80±5.9	68±0.6	87±0.2	37±1.6	76±11.8	36±1.0	45±0.5
P. laevigata	7±0.8	10±1.7	52±10.9	1±0.6	3±1.0	16±0.4	3±1.0	2±0.8

(±) standard deviation

Table 3 shows that the growing inhibition effect on *T. versicolor* was highly significant between species and concentration ($p<0.0001$, $F=59.24$), and significant between extracts ($p< 0.0027$, $F=10.03$). *T. versicolor* growth was reduced 76±12% by *L. leucocephala* wood sawdust extracts at 10 mg ml^{-1} concentration; it was followed by bark extracts at 10 mg ml^{-1} of *Helietta parvifolia* (49±3%). The wood of *H. parvifolia* has been classified with a high natural durability by Wolf and Perales (1985). In this research *P. laevigata* hot water extracts showed a low level of inhibition compared to the 82 and 90% values for *C. puteana* and *T. versicolor* presented by Carrillo *et al*. (2011). Juglone bark extract from *Juglans mandshurica* at concentrations ≥ 0.5 mg ml^{-1} displayed similar inhibition for *Aspergillus niger*, *Paecilomyces variotii*, *Trametes versicolor,* and *Gloeophyllum trabeum* (Dongmei *et al*., 2009). A similar inhibition effect of up to 50% was found for water extracts and acetone extracts from *P. africana* heartwood diluted to concentrations of 1 mg ml^{-1}. The use of diethylether extracts as an inhibition agent affected the growth up to a level of 80% at 1 mg ml^{-1} (Gérardin, 2004.). Results from this research are somewhat similar to those studied by Huang *et al*. (2009). Here too no relationship was found between the amount of phenols and flavonoids and a growing inhibition effect on fungi.

CONCLUSION

Sawdust of *Prosopis laevigata* and *Ebenopsis ebano* has high concentrations of phenols and flavonoids. The fungistatic effect produced by sawdust and bark from species investigated is not related to high phenol and flavonoids yields. Wood sawdust and bark extracts of species such as *Helietta parvifolia* and *Condalia hookeri*, whose concentration of phenol and flavonoids were relatively low, showed high growth inhibition rates. Wood extracts from high natural durability tree species have a potential for use in wood protection. Still, further research is needed with regard to the identification of compounds present in wood sawdust and bark extracts and to their use in impregnating wood from a variety of tree species.

Table 3. Analysis of variance for growing inhibition on *T. versicolor* fungi produced by wood and bark extracts from five tree species.

Source of variation	Df	Sum of Squares	Mean Square	*F* value	*p* value
Wood species (WS)	4	15298.78	3824.69	59.24***	0.0001
Extracts (E)	1	647.33	647.33	10.03**	0.0027
WS*E	4	1544.52	386.13	5.98***	0.0005
Concentration (C)	1	4988.24	4988.24	77.26***	0.0001
E*C	1	580.88	580.88	9.00**	0.0043
Error	48	3099.24			

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