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Antifungal activity of naphtoquinone from *Tabebuia serratifolia* (Vahl. Nicholson)

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Abstract

The presence of naphtoquinones has been reported in several species of *Tabebuia.* It is known that naphtoquinones are biologically active against pathogenic fungi. An ethanolic extract of the wood of *Tabebuia serratifolia* showed activity against the wood rotting fungi *Gloeophyllum trabeum* and *Trametes versicolor*. Chromatography of the ethanolic extract yielded lapachol, [2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphtoquinone], which was proved to be the active principle and its identity was established by comparison of its spectroscopic properties with published data. The antifungal activity of this naphtoquinone was investigated using the agar dilution method with superficial inoculation, at concentrations of 30, 40, 50, 60, and 70 μg/mL. Lapachol showed fungicidal activity against *G. trabeum* and *T. versicolor* at 60 μg/mL and fungistatic between 30 and 50 μg/mL. Correlation analysis showed that as much as 96% of the variation in growth inhibition could be accounted for increased lapachol concentration.

Key words: Antifungal activity; *Gloeophyllum trabeum;* lapachol; naphtoquinone; *Tabebuia serratifolia*; *Trametes versicolor*.

Actividad antifúngica de una naftoquinona obtenida de *Tabebuia serratifolia* (Vahl. Nicholson)

Resumen

La presencia de naftoquinonas ha sido reportada en muchas especies de *Tabebuia* y *s*e conoce que las naftoquinonas son activas biológicamente frente a hongos patógenos. Un extracto etanólico de la madera de *Tabebuia serratifolia* mostró actividad frente a *Gloeophyllum trabeum* y *Trametes versicolor*, dos hongos responsables de la pudrición de la madera. Una separación cromatográfica del extracto etanólico permitió el aislamiento de lapachol [2-hidroxi-3-(3-metil-2-butenil)-1,4-naftoquinona], que demostró ser el principio activo y cuya identidad fue establecida mediante comparación de sus propiedades espectroscópicas con datos publicados en la literatura. Se investigó la actividad antifúngica de esta naftoquinona utilizando el método de la dilución de agar con inoculación superficial a concentraciones de 30, 40, 50, 60 y 70 μg/mL. El lapachol demostró actividad funguicida ante *Gloeophyllum trabeum* y *Trametes versicolor* a 60 ìg/mL y fungistática entre 30 y 50 μg/mL. Un análisis por correlación demostró que hasta un 96% en la variación de la inhibición del crecimiento podía ser atribuida al aumento de la concentración de lapachol.

Palabras clave: Actividad antifúngica; *Gloeophyllum trabeum;* lapachol; naftoquinona; *Tabebuia serratifolia*; *Trametes versicolor*.

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Introduction

Tropical and subtropical trees are an unexplored reservoir of new substances with potentially useful biological properties which may play a role in warning off predators and repelling fouling organisms (1, 2). The natural durability of Angiosperms' heartwood to Basidiomycetes is usually attributed to the presence of chemical compounds with fungicidal properties in the cell-wall of the wood (3-6). Several species of *Tabebuia* (Bignonaceae) have proven to be a rich source of many of such organic compounds, especially of phenolic and poliphenolic nature (5, 7). Such substances ha been classified as cytotoxic, antimicrobial and antifungal (2,8-12). The presence of anthraquinone and naphtoquinone compounds such as lapachol has been reported in several species of *Tabebuia*, (7, 13-16). The biological activity of naphtoquinones isolated from natural sources against pathogenic fungi of medical importance has been reported (17-19), as well as their anti-inflammatory action (13), analgesic (20), anti-tumor (21, 22), and anti-ulcerogenic effects (23). Their activity as anti-cancer (16), and as anti-malaria agents (12) have also been reported. However their toxic properties against fungi that cause biodegradation of wood have not been studied in detail. In the present study the antifungal activity of lapachol, a naphtoquinone isolated from the heartwood of *Tabebuia serratifolia* (Vahl. Nicholson) is reported. The antifungal activity of the crude ethanolic extract was confirmed by Velásquez et al. (24). Subsequently, the crude extract was fractionated and only those fractions which tested positive for antifungal activity were evaluated.

Materials and Methods

General Methods

Melting points were determined on Fisher-Johns melting point apparatus and are uncorrected. IR spectra were measured on a Perkin Elmer FT-1710 instrument, as KBr disks. NMR spectra were recorded with a Bruker Avance 400 MHz instrument for solutions in $CDCl_3$. Lapachol was characterized by acquisition of ^{1}H, ^{13}C, DEPT, ^{1}H-^{1}H-COSY, HMQC, and HMBC experiments. Analytical thin-layer chromatography was performed on E. Merck aluminum-backed silica gel foils (F254). Chromatography was performed on silica gel, by gradient elution with hexane-EtOAc, or hexane-diethyl ether.

Plant Material

The heartwood of *Tabebuia serratifolia* (Vahl. Nicholson) was collected during the rainy season (June, 1999) in the tropical rainforest of the Venezuelan Guayana region (latitude 08°23'00'', longitude 62°15'00") A voucher specimen (4898) was deposited at the Herbarium of Jardin Botánico del Orinoco, Venezuela.

Microorganisms and culture medium

Two wood rotting fungi were used. The brown rot fungi *Gloeophyllum trabeum* (Fr.) Murr.(mad.-617-R) and the white rot fungi *Trametes versicolor* (L:Fr) Pilát (FP-133255-R). A 3% malt extract agar was used in the antifungal assay.

Extraction and Isolation

Dried and ground heartwood (3000 g) was exhaustively extracted with 95% ethanol in a Soxhlet apparatus. The extract was concentrated under vacuum at 30°C to give a brownish viscous residue which was subjected to Si gel column chromatography with vacuum application (25-26) and eluted with n-hexane-ethyl acetate mixtures of increasing polaritiy. Fractions were combined according to TLC performance.

The ethanolic extract (300 g) obtained from the heartwood of *Tabebuia serratifolia* was subjected to open column chromatography on silica gel to produce 17 fractions. On a first screening fraction 9, which was eluted using a 70:30 n-hexane-AcOEt mixture, showed strong activity against both rot wood fungi. This fraction was further concentrated under vacuum to give 51 mg of yellow colored

crystals mp128°C. The IR spectrum of this substance showed a band at 1660 cm^{-1} which is characteristic of the quinone carbonyl. The ^{1}H-NMR spectrum in $CDCl_3$ showed two vynilic methyls at δ 1.62 and δ 1.76, one allylic methylene at δ 3.12, one olefinic hydrogen at δ 5.16, one exchangeable hydrogen at δ 7.3 (1H, s) and four aromatic hydrogens at δ 7.5. The values of the ^{13}C-NMR signals are shown on Figure 1.

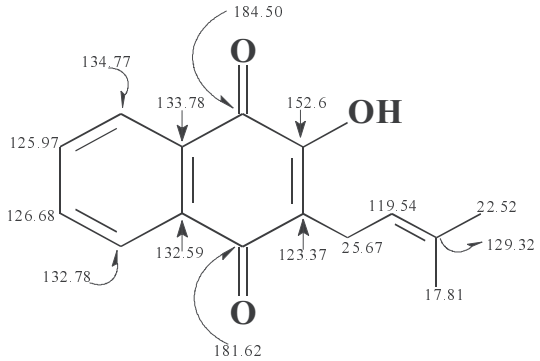

Figure 1. Structure and ^{13}C-NMR signals of lapachol.

Antifungal assay

To test for antifungal activity the agar dilution method with superficial inoculation was used (27-28-29-30). The compounds diluted in ethanol were incorporated into molten medium (ca 40°C) in aseptic conditions at concentrations of 30, 40, 50, 60 or 70 g/mL. Into each Petri dish (90 cm in diameter) 20 mL of medium was uniformLy distributed. Circular blocks of mycelia from a stock culture were punched and centrally placed onto solidified agar which incorporated the compound to be tested. Each test was carried out in triplicate for each concentration and fungus. The growth inhibition of each fungus was observed for a period of 7 days at 26 ± 2°C under constant humidity. The fungitoxicity, in terms of percentage inhibition of mycelial growth, was calculated over control on which only ethanol was used according to Singh and Tripathi (6).

Results and Discussion

The yellow crystals isolated from fraction 9 were tentatively identified as lapachol [2-hidroxy-3-(3-methyl-2-butenyl)-1,4-naphtoquinone]. The structure of lapachol was confirmed by comparison of its carbon-13 signals with the values reported by Goel et al. (23). Figure 1 shows the structure of lapachol and the ^{13}C-NMR signals obtained in $CDCl_3$.

The inhibitory effect of lapachol isolated from *T. serratifolia* on the growth of fungi is shown on Table 1. *Gloeophyllum trabeum* and *Trametes versicolor* constitute the major agents of damage on service wood (31). Data analyzed using the one-way ANOVA (SPSS 10.0 for Windows) showed that *G. trabeum* and *T. versicolor* showed a different response, with regards to their capacity to use this new substrate as a source of carbon. The susceptibility of *G. trabeum* is higher towards lapachol. This may be attributed to enzymatic and metabolic differences between both microorganisms (cellulase and phenol-oxidase activity respectably).

At the end of a growing period of 7 days, statistical analysis of data using the Multiple Comparisons Tukey test, showed a very significant difference (P<0,01), relative to the control group on both tested microorganisms, which was observed even at the lower concentration evaluated (e.g. 30μg/mL), These results indicated that a limited amount of lapachol (30-70 μg/mL), would be required to protect the wood against rotting. Therefore, the higher natural durability observed in the wood of this species in Venezuela (32-33) could be attributed to the presence of lapachol in its heartwood.

The statistical analysis showed with 99% confidence that on both fungi the five concentration levels evaluated differed significantly in relation to growth inhibition. At concentrations higher than 60 μg/mL, lapachol caused 100% inhibition on growth on both fungi. Whereas, at lower concentrations (e.g. 30-50 μg/mL), the performance of

Table 1
Antifungal activity of lapachol against *Trametes versicolor* and *Gloeophyllum trabeum*

Fungus test	Percentage mycelial inhibition at different concentrations (μg/mL)					
	Control	30	40	50	60	70
T. versicolor	0	33	52	75	100	100
G. trabeum	0	54	71	87	100	100

lapachol was different, showing only a regulatory effect on growth on both microorganisms. These results indicated that between 30-50 g/mL, both microorganisms were tolerant to lapachol.

A simple determination coefficient was obtained for the correlation between the growth inhibition (%) against the independent variable lapachol concentration (μg/mL) on both fungi tested. This relationship is shown on Figure 2. The results indicated that inhibition of growth rate on both fungi tested increased linearly with lapachol concentration, showing a strong positive correlation between variables. In other words, the antifungal activity of lapachol is evident when 96% of growth inhibition on *T. versicolor* and *G. Trabeum* is obtained at higher concentration levels (R^2 0,9638 and R^2 0,9665 respectively).

Fungitoxic compounds may inhibit the growth of a fungus temporally acting as fungistatic substances or permanently, that is fungicidal (6). In order to elucidate if the observed antifungal effect was fungistatic or fungicidal, agar plugs from the test plates were transferred to fresh malt agar and incubated in the same conditions for an additional 2 week period. The culture medium inoculated with agar plugs from 30, 40 and 50 μg/mL showed a normal growth, but those inoculated with agar plugs from 60 and 70 μg/mL did not show any growth after incubation for an additional period. This experiment suggested that lapachol acts as a fungicidal substance against *G. trabeum* and *T. versicolor* at concentrations higher

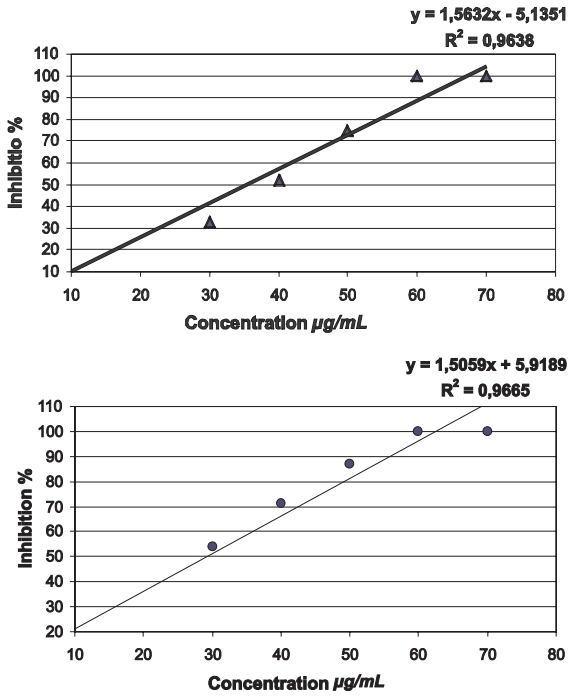

Figura 2. Growth inhibition (%) vs lapachol concentration (μg/mL) *T. versicolor* (A) and *G. Trabeum (B).*

than 60 μg/mL, but it is only fungistatic between 30 and 50 μg/mL.

Some woody plants produce secondary metabolites that undoubtedly play a key role on the natural durability of their timber (1, 34). Extraction, isolation and identification of those bioactive compounds (e.g. lapachol) from the hardwood of species of higher naturally durability *as T. serratifolia*, might be a potential route to develop new fungicides or wood preservative active ingredients with low toxicity to mammals (4, 10, 30, 34).

However, it would be advisable to investigate further their mechanism of action, possible synergistic effects, and the economic potential of their production.

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