IRG/WP 09-10696

THE INTERNATIONAL RESEARCH GROUP ON WOOD PROTECTION

Section 1 Biology

Antinomic natural self-protection mechanism in long-lasting woods: a case study with three tropical species from French Guiana

Alice Rodrigues[1,6], Mariana Royer[2], Nadine Amusant[3,*], Jacques Beauchêne[4], Gaëtan Herbette[5], Véronique Eparvier[2], Anne Thibaut[4], Laila Salmen Espíndola[6], Bernard Thibaut[2], Didier Stien[2]

[1] Université des Antilles et de la Guyane, UMR Ecofog, BP 792, 97337 Cayenne, France
[2] CNRS, UMR EcoFoG, BP 792, 97337 Cayenne Cedex, France
[3] CIRAD, UR 40, 73 rue JF Breton, 34398 Montpellier Cedex 5, France
[4] CIRAD, UMR EcoFoG, BP 709, 97387 Kourou, France
[5] Spectropole, Université d'Aix-Marseille, Faculté de Saint-Jérôme, service 511, Avenue Escadrille Normandie Niémen, 13397 Marseille cedex 20, France
[6] Laboratório de Farmacognosia, Universidade de Brasília, Brasília, DF, Brazil

* Corresponding author: Nadine Amusant, 73 rue JF Breton, TA 10/16, 34398 Montpellier Cedex 5, France. Tel.: +33 - 4 67 61 44 97. Fax +33 - 4 67 61 57 25. E-mail: nadine.amusant@cirad.fr.

Paper prepared for the 40th Annual Meeting
Beijing, China
24-28 May 2009

IRG SECRETARIAT
Box 5609
SE-114 86 Stockholm
Sweden
www.irg-wp.com

Antinomic natural self-protection mechanism in long-lasting woods: a case study with three tropical species from French Guiana

Alice Rodrigues[1,6], Mariana Royer[2], Nadine Amusant[3,*], Jacques Beauchêne[4], Gaëtan Herbette[5], Véronique Eparvier[2], Anne Thibaut[4], Laila Salmen Espíndola[6], Bernard Thibaut[2], Didier Stien[2]

[1] Université des Antilles et de la Guyane, UMR Ecofog, BP 792, 97337 Cayenne, France
[2] CNRS, UMR EcoFoG, BP 792, 97337 Cayenne Cedex, France
[3] CIRAD, UR 40, 73 rue JF Breton, 34398 Montpellier Cedex 5, France
[4] CIRAD, UMR EcoFoG, BP 709, 97387 Kourou, France
[5] Spectropole, Université d'Aix-Marseille, Faculté de Saint-Jérôme, service 511, Avenue Escadrille Normandie Niémen, 13397 Marseille cedex 20, France
[6] Laboratório de Farmacognosia, Universidade de Brasília, Brasília, DF, Brazil

* Corresponding author: Nadine Amusant, 73 rue JF Breton, TA 10/16, 34398 Montpellier Cedex 5, France. Tel.: +33 - 4 67 61 44 97. Fax +33 - 4 67 61 57 25. E-mail: nadine.amusant@cirad.fr.

ABSTRACT

We demonstrate in this work through 3 examples that Amazonian trees may specialize long-lasting woods by means of at least to different approaches. Wallaba impregnates its wood with large amounts of weakly antifungal compounds acting in synergy, while tatajuba and louro vermelho woods are naturally impregnated with antifungal agents. Comparison of biological activities *in vitro* and concentrations in the woods indicate that these compounds alone may account for the natural durability of the two woods.

Keywords: wood, natural durability, wood degradation, antifungal extractives

1. INTRODUCTION

Wood and wood products can be degraded by many different organisms especially when the wood is placed in an aggressive outdoor environment. In Amazonian rain forest, trees grow under biotic stress and long-living trees have adapted to reach the canopy and live well in this environment. Some lean on hollow trunk to stand while many have evolved long-lasting heartwood in this purpose. It is generally admitted that trees capable of specializing long-lasting heartwood do so through natural chemical impregnation with compounds decreasing fungi activity. Indeed, several weakly antifungal agents have been isolated in various woods in the literature (see Hsu *et al.* 2007 and references cited therein). In many cases, the biological activity of the antifungal agent alone does not seem high enough relatively to its concentration in the wood to account for the natural durability of the wood. However, Schultz has demonstrated that extractives can protect heartwood with several mechanisms affecting processes associated with fungal growth and wood digestion (Schultz and Nicholas 2000, Binbuga *et al.* 2007, Schultz and Nicholas 2002). These extractives are only poorly active when isolated, but act in synergy though different modes of action. We hypothesized that in rain forests, trees may have developed specific defense mechanism for their woods. We therefore embarked upon searching for antifungal agents in three Amazonian species able to specialize long-lasting wood: wallaba (*Eperua falcata* Aubl., Caesalpiniaceae), tatajuba (*Bagassa guianensis* Aubl., Moraceae) and louro vermelho (*Sextonia rubra* (Mez.) van der Werff formerly *Ocotoea rubra* Mez., Lauraceae).

Wallaba is the most abundant species in French Guiana. Its chemical composition had been studied by us in preliminary experiments but we did not find at this time any compound that might account for its high durability (Amusant *et al.* 2007). Tatajuba is the only member of its gender within the large Moraceae family. Its wood is renowned for its exceptionally high durability even under very aggressive outdoor conditions, and the chemical composition of the wood had never been regarded before our work. As regards louro vermelho, it is durable and it is the most exploited species in French Guiana. Initially, we thought that this species had never been investigated in the literature. However, we discovered after identification of the compounds isolated in the wood that it had been regarded but under an inappropriate scientific name: *Nectandra rubra* (Franca *et al*. 1977).

Overall, understanding why some heartwoods display considerable resistance against insects attack or fungi colonization and degradation may inspire the discovery of new environmentally-benign wood preservatives.

2. EXPERIMENTAL METHODS

2.1 General remarks

The white-rot *Coriolus versicolor* (L.ex Fr Quélet (CTB 863 A) was used in this study. Fungus was grown on an autoclaved (121°C) malt-agar medium at 25°C. Malt extract and agar-agar were purchased from Sigma-Aldrich. This medium were prepared with 40g/l (+/- 0,5g) malt extract and 20g/l (+/- 0,5g) agar-agar dissolved in distilled water. Manipulation of fungal cultures was done in a laminar flow hood. The fungus strain was maintained on maltose-agar at 2°C.

Solvents used for extraction were extraction grade ethyl acetate or methanol. Column chromatographies were conducted under silicagel with distilled reagent grade solvents.
NMR structural analysis were performed on a Bruker Avance DRX500 spectrometer (^{1}H-500.13 MHz) equipped with a 5mm triple resonance inverse Cryoprobe TXI (^{1}H-^{13}C-^{15}N), with z gradient. Spectra were recorded with 1.7mm NMR capillary tube in 40µL deuterated solvent at 300K. The ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data are reported in ppm. Hydrogen connectivity (C, CH, CH_2, CH_3) information was obtained from edited HSQC and/or DEPTQ-135 experiments. Proton and carbon peak assignments were based on 2D NMR analysis (COSY, NOESY, HSQC, and HMBC).

2.2 Bioassays

2.2.1 Antifungal screening test

Qualitative antifungal activity tests were carried out against *Coriolus versicolor* using a modified agar-well diffusion method described by Okeke *et al.* (2001). A suspension that was just turbid (~ 0.5 Mc Farland standard) by visual inspection was prepared by suspending the *C. versicolor* conidia. 80 µL aliquots of this suspension were spread on malt-agar sterile plate (diameter 14 cm). Excess liquid was air-dried under a sterile hood. 5 millimeters cavities were bored on dried spread test plates. After inoculation, each fraction dissolved in 50 µL of DMSO (dimethyl sulfoxide) was poured into the cavities. All test plates were incubated in the darkness at room temperature (30°C). 50 µL of DMSO were poured into cavities as negative controls. The activity was recorded by measuring the clear zone of growth inhibition on agar surface around the cavities after 5 days. Each experiment was performed three times

2.2.2 Biological activity of pure compounds

Antifungal indexes were measured based on Archer *et al.* (1995). Extracts and fractions activities are reported as the capacity to reduce the mycelium radial growth on impregnated maltose/agar medium. Extracts were dissolved in DMSO (1% w/w) and the DMSO solution was diluted in the warm maltose/agar medium (6 mL) in order to reach the initial test concentration of 1 mg/mL. Half of the medium (3 mL) was poured into a 35 mm diameter Petri dish and the other half was diluted with more warm culture medium (3 mL) in order to reach the second test concentration. Eight Petri dishes with concentrations ranging from 1 mg/mL to 7.8 µg/mL were prepared this way by successive dilutions. A fungus contaminated culture medium square of 6 mm of side was transferred in the middle of the dishes which were then incubated at 27°C until the mycelium of fungi reached the edges in the control dishes (approximately 96 h). At this time, the antifungal index (AI) was calculated as followed:

$AI\ (\%) = [1 - (D_a\text{-}6)/(D_b\text{-}6)] \times 100$

where, D_a is the diameter of growth zone in the test dish (in mm) and D_b is the diameter of growth zone in the control dish (35 mm). If AI equals 0, the extractives have no fungicidal activity. If AI equals 100, the extractives have a total fungicidal activity. Each experiment was performed three times. The correction of 6 mm on the diameter values represents the size of the initial sowing square.

2.3 *E. falcata*: plant material, extraction and fractionation

Eperua falcata Aubl. (wallaba, 2 trees, approximately 40 cm diameter at breast height) were collected in Régina, French Guiana. Botanical identification was done at the French Guiana Herbarium (CAY) where a voucher specimen was conserved.

Dried and powdered outer heartwood of wallaba external heartwood was prepared in order to be submitted to a successive extraction with five increasing polarity solvents: hexane, methylene chloride, ethyl acetate, methanol and water. 30 g of sawdust was placed in Erlenmeyer and submerged in 200 ml solvent. The flask was shaken at room temperature for 1 week. Extract solutions were collected by filtration in order to perform antifungal screening tests. None of the extracts displayed antifungal activities in the qualitative cavity bioassay. Quantitative tests performed on the crude successive extracts demonstrated that the most active extract was the EtOAC one, which inhibited 14% of the fungi growth at 0.5% w/w. HPLC analyses and separations were conducted on this extract using a Waters system equipped with a W600 pump and a W2996 photodiode array absorbance detector. HPLC separations were performed on a Discovery® C18 column (250 x 21.2 mm, 5 µm, Supelco®) with a linear gradient of H_2O/CH_3CN starting with a relative proportion of 80:20 and changing over 10 min to pure CH_3CN. The flow rate was 15 $mL.min^{-1}$ and the detection of compounds was operated at 300 nm. 8 known compounds were isolated and identified as eperuic acid (0.31 %) (Amusant *et al.* 2007), engeletin (0.28%) (Yinrong and Yeap 1999), isoengeletin (0.12 %) (Xu *et al.* 2005), a mixture of the epimers, neoengeletin and neoisoengeletin (0.22%), a mixture of the isomers, astilbin and neoastilbin (0.15%), *para*-hydroxybenzoic acid (0.09%), 3,4-dihydroxy-5-methoxy-benzoic acid (0.16%) (Saito and Kawabata 2006), (+)-catechin (0.44%) (Nay *et al.* 2001), (-)-epicatechin (0.65%) (Mendoza-Wilson and Glossman-Mitnik 2006), (-)-epicatechin 3-*O*-para-hydroxybenzoate (1.02%) (Watanabe 1998) and (+)-katuranin (0.41%) (Yinrong and Yeap 1999). None of these compounds displayed any antifungal activity.

2.4 *B. guianensis*: plant material, extraction and fractionation

Bagassa guianensis (tatajuba, 2 trees, approximately 40 cm diameter at breast height) was collected at Régina, French Guiana.

Dried and powdered outer heartwood (100 g) was extracted successively with AcOEt and MeOH. Antifungal activity was detected in the AcOEt fraction, which was further purified by column chromatography on silicagel (hexane/EtOAc 80/20, 50/50, 20/80 and 0/100). 9 fractions were obtained and biological activity concentrated in a fraction which proved after HPLC and NMR analysis to be a pure compound identified as *trans*-oxyresveratrol (300 mg, 0.30%) (Schultz *et al.* 1995).
Biological activities of *trans*-oxyresveratrol were:

IC_{50} = 0.34 mg/mL (approximately 0.034% w/w)

IC_{100} = 2.0 mg/mL (approximately 0.20% w/w)

2.5 *S. rubra*: plant material, extraction and fractionation

Sextonia rubra was collected in a commercially exploited forest in July 2007 at Régina, French Guiana. Botanical identification was done at the French Guiana Herbarium (CAY) where a voucher specimen was conserved.

Dried and powdered outer heartwood and 70 g were powdered and extracted by cold maceration in ethyl acetate for 24 h. The macerate was filtered and concentrated to dryness under reduce pressure below 30°C to yield 2 g of a brown gum. The wood was further extract with MeOH to yield a brown gum again (0.80 g). Antifungal activity was retained in the AcOEt extract. This crude extract was therefore chromatographed over silicagel (AcOEt, then MeOH). The ethyl acetate fraction was evaporated and residual fat was removed by washing the product with hexane to yield a white powder and liquid grease in the hexane fraction. The white powder, the hexane fraction and the MeOH-eluted compounds where all tested again using the fungi cavity method. The biological activity was retained in the white powder only, which proved to be an inseparable mixture of lactones rubrenolide and rubrynolide (1:1, 0.70 g, 1.0 %) (Franca *et al*. 1977, Thijs and Zwanenburg 2004, Taylor *et al.* 1991).

Biological activities of the mixture of lactones were:

IC_{50} = 90.2 µg/mL (approximately 0.009% w/w)

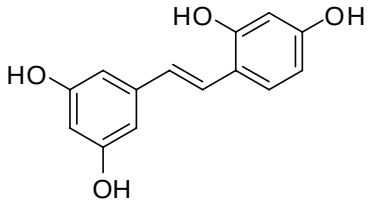
IC_{100} = 500 µg/mL (approximately 0.050% w/w)

3. RESULTS AND DISCUSSION

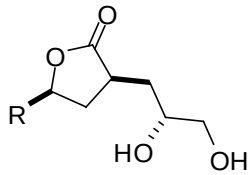
Extensive investigation of wallaba extracts did not allow us to isolate any antifungal agent. The crude successive extracts are very moderately active for inhibition of fungi growth. The most active extract is the crude ethyl acetate extract, which is only able to inhibit 14% of *Coriolus versicolor* growth at 0.5% w/w in the culture medium. We further ensured that this fraction did not contain any antifungal agent by fractionation and extensive analysis of its components. We isolated therein known eperuic acid, epicatechin, catechin and 6 rhamnosylated flavanones (astilbin and neoastilbine, engeletin and 3 stereoisomers). All these compounds had been described before in the literature and none of them was active in our antifungal assay. It was clear from these experiments that wallaba wood does not contain any antifungal agent strictly speaking. However, since wallaba is a long-lasting wood, it can be hypothesized that wallaba protects its wood in the same way as species studied and described by Schutlz and Nicholas

(2000), where extractives are present in the wood at very high concentration (up to 15% total extract in the external heartwood) and capable of acting in synergy against fungal wood deterioration.

Bioguided fractionation of *B. guianensis* heartwood allowed us to isolate one bioactive compound in the test against *C. versicolor*. This compound was identified as *trans*-oxyresveratrol (Figure 1). The compound had been found before in the literature in Osage orange wood (Schultz *et al.* 1995). Osage orange also belongs to the Moraceae family. In this case, its concentration in the wood is 0.3% based on isolated yield and we have measured that 0.2% of *trans*-oxyresveratrol inhibits 100% of the growth of *C. versicolor* in vitro. Therefore, *trans*-oxyresveratrol alone is active enough to account for the exceptional natural durability of this wood. In conclusion, it seems that tatajuba protects its wood by impregnation with one active compound at the appropriate concentration allowing for complete inhibition of fungi growth.



trans-oxyresveratrol



Rubrenolide: R = $-(CH_2)_8-CH=CH_2$
Rubrynolide: R = $-(CH_2)_8-C\equiv CH$

Figure 1: antifungal agents isolated from tatajuba and louro vermelho heartwoods

Bioguided fractionation of *S. rubra* heartwood allowed us to isolate and identify a 1 to 1 mixture of two lactones, rubrenolide and rubrynolide, which proved to inhibit *C. versicolor* growth *in vitro* (Figure 1). These two lactones have been described before in the literature, isolated from *Nectandra rubra* (Franca *et al*, 1977). It seems that *Nectandra rubra* is either an old name or an inappropriate name given to *Sextonia rubra*. Therefore, these compounds have probably been isolated before in this same wood. Rubrenolide and rubrynolide could not be separated by chromatographic methods. The mixture of the two inhibits 100% of fungi growth *in vitro* at the concentration of 0.05% w/w. Since its concentration in the wood can be estimated as 1% w/w based on isolated yield, it can be concluded that the relative concentration of the lactones is largely high enough in the wood to account for its natural durability. Therefore, we believe that louro vermelho also protects its wood by natural impregnation with a large amount of one antifungal agent.

4. CONCLUSIONS

In conclusion, while the 3 Amazonian species studied in this article are all capable of specializing long-lasting woods, we have identified two mechanisms for wood self-protection. Wallaba specializes a highly durable wood by impregnation with a very large relative proportion of extractives which do not display any antifungal property when they are isolated. Its seems that these extractives are able to reduce wood degradation when they are together in the wood and we hypothesize here that they act in synergy and inhibit fungi growth by affecting processes involved in fungi development and wood digestion by fungi. On the other hand, we have clearly demonstrated that tatajuba and louro vermelho synthesize antifungal agents above the concentration required for total inhibition of fungi growth. Other metal-chelating agents or antiradical compounds present in these woods may enhance the biological activities of these components, but we have demonstrated that the natural durability may be explained by the sole presence of these antifungal biomolecules.

5. REFERENCES

Amusant N, Moretti C, Richard B, Prost E, Nuzillard JM, Thévenon MF (2007): Chemical compounds from *Eperua falcata* and *Eperua grandiflora* heartwood and their biological activities against wood destroying fungus (*Coriolus versicolor*). *Holz Roh Werkst,* **65**, 23-28.

Archer, K, Nicholas D D, Schultz T P (1995): Screening of wood preservatives: comparison of the soilblock, agar-block, and agar-plate tests. *Forest Products Journal*, **45**, 86-89.

Binbuga, N, Ruhs, C, Hasty, J K, Henry, W P, Schultz, T P (2007): Developing environmentally benign and effective organic wood preservatives by understanding the biocidal and non-biocidal properties of extractives in naturally durable heartwood. *Holzforschung*, **62**, 263-269.

Franca, N C, Gottlieb, O R, Coxon, D T (1977): The chemistry of Brazilian Lauraceae. Part 42. Rubrenolide and rubrynolide: an alkene-alkyne pair from *Nectandra rubra*. *Phytochemistry*, **16**(2), 257-262.

Hsu, F-L, Chang, H-T, Chang, S-T (2007): Evaluation of antifungal properties of octyl gallate and its synergy with cinnamaldehyde. *Bioresource Technology*, **98**, 734-738.

Mendoza-Wilson A M, Glossman-Mitnik D (2006): Theoretical study of the molecular properties and chemical reactivity of (+)-catechin and (-)-epicatechin related to their antioxidant ability. *Journal of Molecular Structure. Theochem*, **761**(1-3), 97-106.

Nay B, Arnaudinaud V, Vercauteren J (2001): Gram-scale production and applications of optically pure ^{13}C-labelled (1)-Catechin and (2)-Epicatechin. *European Journal Organic Chemistry*, (12), 2379-2384.

Okeke, M I, Iroegbu, C U, Eze, E N, Okoli, A S, Esimone, C O (2001): Evaluation of extracts of the root of Landolphia owerrience for antibacterial activity. *Journal of Ethnopharmacology,* **78**, 57-60.

Saito S, Kawabata J (2006): DPPH (= 2,2-diphenyl-1-picrylhydrazyl) radical-scavenging of protocatechuic acid (= 3,4-dihydroxybenzoic acid): difference in reactivity between acids and their esters. *Helvetica Chimica Acta,* **89**, 1395-1407.

Schultz, T P, Nicholas, D D (2002): Development of environmentally-benign wood preservatives based on the combination of organic biocides with antioxydants and metal chelators. *Phytochemistry*, **61**, 555-560.

Schultz, T P, Nicholas, D D (2000): Naturally durable heartwood: evidence for a proposed dual defensive function of the extractives. *Phytochemistry*, **54**, 47-52.

Schultz, T P, Harms, W B, Fisher, T H, McMurtrey, K D, Minn, J, Nicholas, D D (1995): Durability of angisperm heartwood: The importance of extractives. *Holzforschung*, **49**, 29-34.

Taylor, S K, Hopkins, J A, Spangenberg, K A, McMillen, D W, Grutzner, J B (1991): Synthesis of (±)-rubrynolide and a revision of its reported stereochemistry. *Journal of Organic Chemistry*, **56**(20), 5951-5955.

Thijs, L, Zwanenburg, B. (2004): Rubrenolide, total synthesis and revision of its reported stereochemical structure. *Tetrahedron*, **60**(24), 5237-5252.

Watanabe M (1998): Catechins as Antioxidants from Buckwheat (Fagopyrum esculentum Moench) Groats. *Journal of Agricultural and Food Chemistry*, **46**, 839-845.

Xu J, Li X, Zhang P, Li Z L, Wang Y (2005): Antiinflammatory Constituents from the Roots of *Smilax bockii* warb. *Archives Pharmacal Research*, **28**(4), 395-399.

Yinrong L, Yeap F L (1999): The polyphenol constituents of grape pomace. *Food Chemitry*, **65**, 1-8.