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Daniela T. SILVA, Nadia H. BIANCHINI, Marlove F. B. MUNIZ, Berta M. HEINZMANN, Jalel LABIDI

CHEMICAL COMPOSITION AND INHIBITORY EFFECTS OF *NECTANDRA GRANDIFLORA* LEAVES ESSENTIAL OIL AGAINST WOOD DECAY FUNGI

The environmental toxicity and potential human health problems that can be caused by most common wood preservatives are pushing forward the search of safe preservatives from renewable resources. Consequently, several studies have been realized to assess the potential of plant extracts in terms of fungal resistance for wood products. In this context, the present work aims to evaluate the inhibitory effects of essential oil obtained from the leaves of Nectandra grandiflora against wood decay fungi. Chemical characterization was carried out by gas chromatography and the antifungal activity was performed by the radial growth technique. Firstly, the potato sucrose agar medium was supplemented with essential oil at concentrations ranging from 0 (control) to 5.0 $\mu L \cdot mL^{-1}$. Afterwards, mycelial discs of Pycnoporus sanguineus and Gloeophyllum trabeum were transferred to the plates and the results were evaluated by the probit method. Chemical analysis revealed a complex mixture of sesquiterpenoids in the essential oil, which presented dehydrofukinone as a major compound. The essential oil and dehydrofukinone proved to be effective in the mycelial growth control of G. trabeum and P. sanguineus. These preliminary reports demonstrated the suitability of the N. grandiflora essential oil as a component of preservative solutions.

Keywords: antifungal property, wood preservatives, Lauraceae, volatile components

Introduction

Some fungal species can be pathogenic to forest and urban trees and consequently, they deteriorate wood products [Bento et al. 2014]. Wood decay fungi are mainly responsible for the destruction of structural elements of the cell wall that can result in economic and material losses and subsequently, reduces

Daniela T. SILVA✉ (*dthomasdasilva@gmail.com*), Nadia H. BIANCHINI (*nadia_bianchini@hotmail.com*), Marlove F. B. MUNIZ (*marlovemuniz@yahoo.com.br*), Berta M. HEINZMANN (*berta.heinzmann@gmail.com*), Federal University of Santa Maria, Santa Maria, Brazil; Jalel LABIDI (*jalel.labidi@ehu.es*), University of the Basque Country, San Sebastian, Spain

the wood quality [Stangerlin et al. 2013]. Among the most important saprophytic wood-rot basidiomycetes, are *Pycnoporus sanguineus* (white-rot fungi) and *Gloeophyllum trabeum* (brown-rot fungi) which are able to degrade wood components. The first species attacks the lignocellulosic materials, while the second destroys the polysaccharide constituents.

Although traditional synthetic fungicides such as arsenate-based wood preservatives are very effective, its continued employment has led to environmental pollution, resistance development and human health toxicity [Yoon et al. 2013]. Plants produce large amounts of secondary metabolites for protection against adverse environmental conditions and biological pests. Extractives and isolated components, therefore, from individuals of diverse botanical families have been studied and have demonstrated promissory potential to fungal control [Cowan 1999; Schultz and Nicholas 2002; Wang et al. 2005; Sen et al. 2009].

In addition to the low toxicity, the application of substances from vegetal resources could improve the efficacy of antifungal products through its synergistic effects including a positive interaction between the components. Hwang et al. [2007], Schultz and Nicholas [2002] reported the usefulness of the combined use of tannins, heartwood extractives and synthetic biocides. In this background, the study aims to investigate the influence of essential oil extracted from *Nectandra grandiflora* leaves, on the mycelial growth of two species of wood-rot fungi. Additionally, a comparison of the antifungal property of essential oil and its major constituent, dehydrofukinone, was performed.

Materials and methods

Essential oil obtainment

Leaves of the *Nectandra grandiflora* Nees were collected on a native population located in the Jaguari city, South of Brazil (at 29º 26’ S and 54º 40’ W). The fresh leaves were fragmented and afterwards the essential oil was extracted by a hydrodistillation process using a Clevenger-type apparatus for three hours. The essential oil yield was quantified based on the mass weight (0.7 g per 100 g of dried leaves). An aliquot of the obtained extractive was chemically analysed by gas chromatography.

Chemical characterization and quantification

The chemical composition of the essential oil was determined by an Agilent 7890A gas chromatograph connected to a mass spectrometer 5075C (GC-MS) using a non-polar HP5-MS fused silica (5% phenyl, 95% methylsiloxane) capillary column (30 m × 0.25 mm i.d. × 0.25 mm film thickness), and an electron ionization mode at 70 eV. The carrier gas was helium at a flow rate of 1.0 mL·min^{-1}, injector and detector temperatures of 150°C and 280°C,

respectively, the split inlet injection mode (ratio 1:100), oven temperature at 40°C for four minutes, and up to 320°C at 4°C·min^{-1} were the employed parameters. Quantitative evaluation was performed in triplicate using a flame ionization detector (GC-FID), according to Silva et al. [2015]. The constituents were then identified by comparison of the retention indices and mass spectra with libraries [NIST-EPA-NIH 2009; Adams 2009].

Following the chemical characterization, three chromatography columns (CC) were performed to fractionate the leaves essential oil and to isolate the main constituent. In the first CC, 11 g of essential oil were added to 630 g of silica gel 60 (7.2 × 30.5 cm) and eluted with hexane-acetone (95:5 v/v). Fractions of 45 mL were gathered in nine main fractions based on the thin layer chromatography (TLC) profile and concentrated under reduced pressure at 40°C. The TLC was carried out on silica gel 60 F_{254} chromathoplates and the spots were detected by vanillin sulfuric acid-UV 365 nm.

The fractions 4 and 5 were grouped (2.8 g) and submitted to another CC (4.1 × 43.2 cm, 260 g silica gel 60, hexane-ethyl ether 95:5 at 1.25 mL/min). From the resulting ten main fractions, 6 and 7 were grouped (1.4 g) and submitted to the third CC (2.4 × 66 cm, 90 g of silica gel sixty impregnated with 10% $AgNO_3$, hexane-acetone 95:5 at 1.0 mL/min) [Williams and Mander 2001]. Among the four main fractions obtained, the fraction 1 (1 g) was identified as dehydrofukinone (100% purity), according to Schenato et al. [2001], Alkhathlan et al. [2005], Bolzan [2007] and Silva et al. [2015].

Antifungal activity

The antifungal assay was evaluated by the radial growth technique [Wang et al. 2005]. All assays were carried out in quadruplicate. Primarily, potato sucrose agar (200 g, 20 and 18 g in 1 L of distillated water) medium was supplemented with essential oil at concentrations of 0 (control), 0.25, 0.50, 1.0, 2.0 and 5.0 µL/mL, which were dissolved in ethanol (1:1), and placed into the Petri dishes. Mycelial discs (1.5 cm diameter) were then transferred aseptically to the center of the plates and incubated at 25 ±4°C in 12 h-photoperiod for seven days.

In another experiment, the inhibition effect of dehydrofukinone was analysed at equivalent concentration to that detected in 5 µL/mL (4.63 µg/mL) of essential oil. Thereby, considering the density of essential oil (0.926 g/mL), the substance purity (100%) and the content of dehydrofukinone in essential oil (26.85%), dehydrofukinone was tested at 1.25 µg/mL. This assay was performed as mentioned above.

The growth of the fungal colony was estimated by an average of two perpendicular measurements and mycelial-growth rate was calculated using the following equation (1).

$$MGR\ (\text{mm}) = \frac{dt}{n_1} + \frac{dt}{n_2} + \frac{dt}{n_5} + \frac{dt}{n_7} \quad (1)$$

where: n – days of the beginning of the experiment,
dt – average diameters of fungal colony (mm).

Mycelial-growth inhibition (%) was calculated through the equation (2).

$$\%\ \text{inhibition} = \frac{MGR_c - MGR_t}{MGR_t} \times 100 \quad (2)$$

where: MGR_c – mycelial-growth rate of control,
MGR_t – mycelial-growth rate of treatment.

The concentration-response data of the first experiment were analysed by probit analysis [Finney 1971] to obtain the 50% lethal concentration (LC_{50}) values and 95% confidence interval (CI). Mycelial-growth rate (MGR) results of dehydrofukinone in comparison with the essential oil were evaluated by t-test. A significant difference was considered at a level of $P < 0.05$.

Results and discussion

Chemical characterization

GC-MS and GC-FID analysis led to the identification of twenty-four components, representing 73.16% of the total essential oil obtained from *N. grandiflora* leaves. The chemical composition revealed a complex mixture of sesquiterpenoids in the extractive (tab. 1) and the chromatogram of GC-MS peaks is shown in figure 1A. The major volatile components were dehydrofukinone (26.85%, fig. 1B), valencene (6.89%), kaurene (6.03%), aristolochene<4,5-di-epi> (5.41%), selin-11-en-4-α-ol (5.34%) and bicyclogermacrene (5.06%).

In comparison with other studies about the chemical compositions of the essential oils of the *Nectandra* species, differences in the major components could be observed. According to Amaral et al. [2015], *N. megapotamica* presented the highest quantities of monoterpenes such as α- and β-pinene, similar to those detected for the essential oil of *N. membranacea* leaves [Wu et al. 2006]. The sesquiterpenoid atractylone was extracted from the leaves of *N. salicina* amounting to 14.6% of essential oil [Cicció et al. 2009]. Conversely, previous reports showed dehydrofukinone as the main component of the leaf extracts from the *Senecio* species [Bohlmann et al. 1981; Nachman 1983]. To date, this compound was only described in the Lauraceae target by Garlet et al. [2016].

Table 1. Chemical composition of the essential oil obtained from *N. grandiflora* leaves

RT[a]	Component	RI[b] calc	RI lib[c]	Content (%)[d]	Identification
10.4	α-Pinene	931	930[N]	0.37	RI, MS
12.0	β-Pinene	973	975[N]	0.25	RI, MS
14.7	*Z*-β-Ocimene	1039	1038[N]	0.30	RI, MS
15.0	*E*-β-Ocimene	1049	1047[N]	1.38	RI, MS
17.1	Linalool	1100	1100[N]	0.41	RI, MS
20.3	*Z*-3-Hexenyl butyrate	1188	1186[N]	0.95	RI, MS
27.3	β-Elemene	1393	1391[N]	3.29	RI, MS
28.1	β-Caryophyllene	1420	1418[N]	0.80	RI, MS
28.8	α-Guaiene	1442	1441[N]	1.38	RI, MS
29.2	α-Caryophyllene	1455	1454[N]	0.49	RI, MS
29.7	Aristolochene<4,5-di-epi>	1471	1473[A]	5.41	RI, MS
30.1	α-Amorphene	1482	1485[A]	0.71	RI, MS
30.3	Valencene	1489	1488[A]	6.89	RI, MS
30.4	*Z*-β-Guaiene	1495	1493[A]	1.84	RI, MS
30.5	Bicyclogermacrene	1498	1500[A]	5.06	RI, MS
30.8	Germacrene A	1506	1509[A]	0.24	RI, MS
31.8	Kessane	1541	1539[A]	2.68	RI, MS
32.9	Spathulenol	1579	1578[N]	1.49	RI, MS
34.2	Humulane-1,6-dien-3-ol	1624	1619[N]	0.31	RI, MS
34.4	Eremoligenol	1632	1631[A]	0.36	RI, MS
35.1	Selin-11-en-4-α-ol	1657	1660[A]	5.34	RI, MS
37.4	Isobicyclogermacrenal	1741	1734[A]	0.33	RI, MS
39.0	Dehydrofukinone	1807		26.85	MS, NMR
44.8	Kaurene	2039	2043[N]	6.03	RI, MS
Components identified				73.16	

[a]Retention time; [b] Retention indices relative to n-alkanes (C8-C31) on a HP5-MS capillary column; [c]retention index from libraries: [A]Adams [2009]; [N]National Institute of Standards and Technology – U.S. Environmental Protection Agency – National Institutes of Health [NIST-EPA-NIH 2009]; [d]Content obtained by GC-FID.

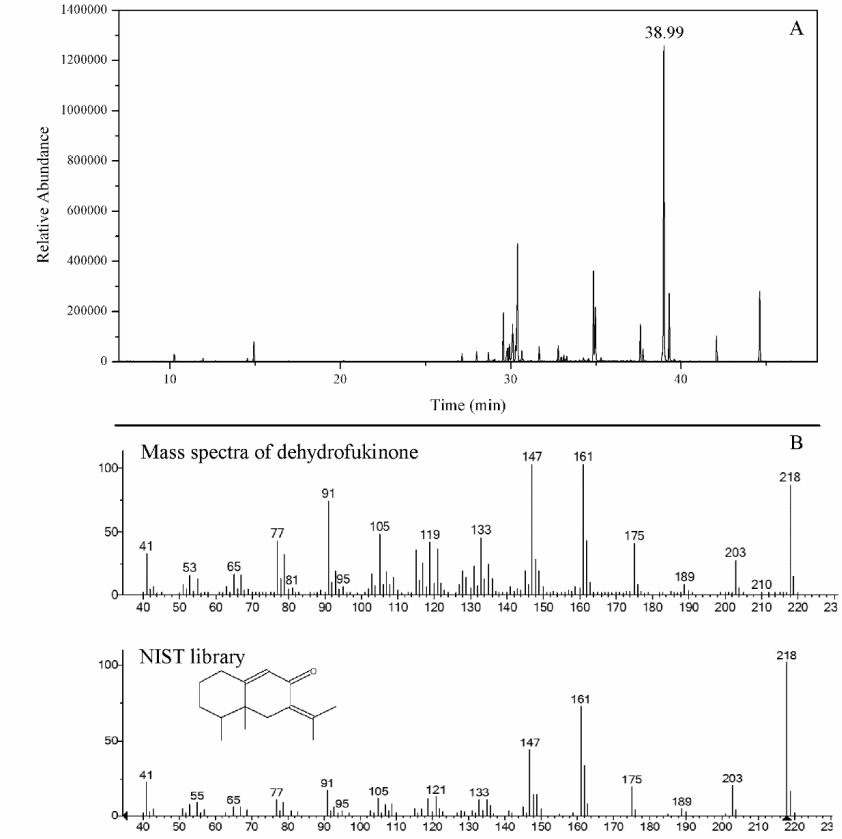

Fig. 1. GC-MS chromatogram of the essential oil from *N. grandiflora* leaves (A) and mass spectra of dehydrofukinone (B) in comparison to NIST library [NIST-EPA-NIH 2009]

Moreover, *N. grandiflora* is an endemic tree from Brazil and its leaves were employed in local medicine as a diuretic and digestive [Correa 1984]. There are a few studies about the biological activities of this plant [Moreno et al. 1993], however, little is known about its essential oil.

Antifungal activity

Regarding the antifungal assay, the essential oil of the *N. grandiflora* proved to be effective in the inhibiting of *P. sanguineus* and *G. trabeum* and the detected activity was in a concentration-dependent manner (tab. 2).

Table 2. Mycelial-growth inhibition (%) of essential oil o from *N. grandiflora* leaves against *P. sanguineus* and *G. trabeum*

Fungi species	Concentration (µL/mL)	Inhibition mean ± SEM (%)	LC_{50} ± SEM (µL/mL) 95% confidence limits	Intercept	Slope	χ^2
P. sanguineus	0.25 0.50 1.0 2.0 5.0	26.75 ± 0.99 33.34 ± 0.46 46.57 ± 1.08 52.20 ± 0.93 79.03 ± 0.41	1.22 ± 0.16 (0.96-1.61)	4.91	1.06	1.50
G. trabeum	0.25 0.50 1.0 2.0 5.0	47.82 ± 0.46 55.24 ± 0.75 61.70 ± 1.40 69.53 ± 3.69 94.76 ± 0.63	0.39 ± 0.26 (0.015-0.84)	5.42	1.02	2.27

χ^2 values were calculated by probit method [Finney 1971].

The calculated χ^2 values were obtained and compared with tabulated χ^2 to verify the adequacy of results for the probit model. All values showed appropriate fit, with calculated χ^2 lower than tabulated χ^2 (7.81). The CI values obtained after seven days of treatment did not overlap, indicating the significant differences between the effects of essential oil on both fungi species. *G. trabeum,* however, seems to be more vulnerable to essential oil, because it presented the lowest LC_{50} (0.39 ±0.26 µL/mL) and CI values (0.015--0.84 µL/mL).Our results agree with Yen and Chang [2008], who proposed that the antifungal activity is strongly related to the fungal species.

Furthermore, figure 2 shows a comparative analysis of antifungal effects among essential oil and the isolated substance against *P. sanguineus* and *G. trabeum*.

The t-test showed no significant difference between the MGR results of essential oil and dehydrofukinone with respect to *P. sanguineus* (8.03 ±1.05 mm and 9.89 ±0.74 mm, respectively; $P = 0.171$). This similarity, however, was not found in the *G. trabeum* assay, which showed more susceptibility to the essential oil. Such behavior could be explained by the synergistic or additive effects of substances present in *N. grandiflora* essential oil [Schultz and Nicholas 2002; Yen and Chang 2008]. Mycelial-growth inhibition results observed in this work, ranged from 76.06% to 79.45% for dehydrofukinone and it was greater than 80.56% for essential oil. Secondary metabolites such as essential oils are a promising source of active substances that can provide the fungal protection of wood surfaces. Some studies reported the potential use of essential oil from *Cinnamomum osmophloeum* [Wang et al. 2005] and *Eucalyptus camaldulensis* leaves [Salem et al. 2016], as well as pure substances [Marei et al. 2012] with this purpose.

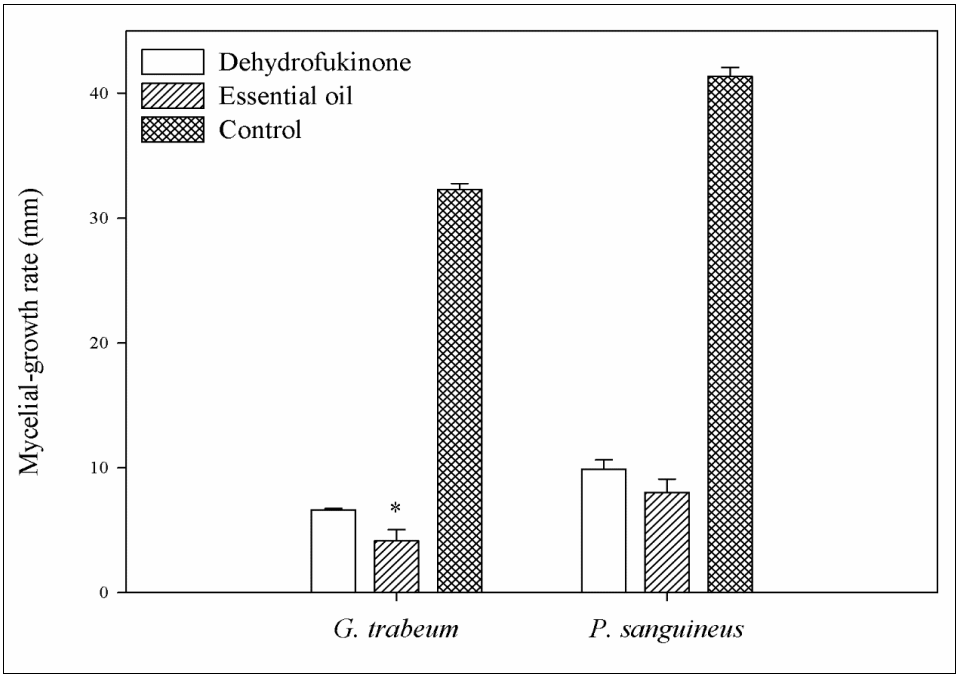

Fig. 2. Comparative analysis of mycelial-growth rate (MGR) concerning essential oil from *N. grandiflora* leaves (4.63 µg/mL) and dehydrofukinone (1.25 µg/mL) against *G. trabeum* and *P. sanguineus*
*Indicates significant differences among essential oil and dehydrofukinone by t-test ($P < 0.05$).

Conclusions

The essential oil of *Nectandra grandiflora* leaves was mainly composed of sesquiterpenoids and among them dehydrofukinone was the major one. Preliminary findings display the *in vitro* efficacy of leaves extractives from *N. grandiflora* against two wood-rot fungi. Moreover, the results suggested that the detected effect is partially due to dehydrofukinone. The essential oil and its major component can then be applied as a natural fungicide in wood treatments.

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