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Pharmacognosy

Duroia saccifera: *in vitro* germination, friable calli and identification of β-sitosterol and stigmasterol from the active extract against *Mycobacterium tuberculosis*

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Abstract

Duroia saccifera (Rubiaceae) occurs in the Amazon rainforest and their extracts showed antibacterial properties. To obtain greater quantities of active substances, leaf segments from *in vitro D. saccifera* seedlings were used as explants for calli induction; calli were multiplied via multiple subcultures, dried and extracted with hexane followed by ethyl acetate (EtOAc) and methanol (MeOH). As *D. macrophylla* had been reported to produce antimycobacterial substances, we assayed calli extracts against *Mycobacterium tuberculosis* (H37Rv strain). Calli EtOAc extract was active, with a minimal inhibitory concentration (MIC) of ≤ 25 μg mL^{-1}, IC_{90} of 19.5 μg mL^{-1} and minimal bactericidal concentration (MBC) of 200 μg mL^{-1}. EtOAc extract was analyzed by Thin Layer Chromatography (TLC) and Nuclear Magnetic Resonance (NMR) to determine its chemical profile, and was found to be rich in terpenes. Chromatographic fractionation of the EtOAc extract yielded a mixture of two sterols, β-sitosterol and stigmasterol (in proportion of 2:1), which were identified by ^{1}H and ^{13}C NMR analysis. As far as we know, this is the first report of *Duroia saccifera in vitro* cell culture, antituberculosis activity of calli extract and β-sitosterol and stigmasterol isolation from *in vitro* plant cell culture.

Key words: antituberculosis, phytochemistry, plant tissue culture, Rubiaceae, sterols.

Resumo

Duroia saccifera (Rubiaceae) ocorre na floresta Amazônica. Seus extratos apresentaram atividade antibacteriana. Para obter maiores quantidades de substâncias ativas, foram utilizados segmentos de folhas de plântulas *in vitro* de *D. saccifera* como explantes para indução de calos; os calos foram multiplicados através de múltiplos subcultivos, depois secos e extraídos com hexano, seguido por acetato de etila (AcOEt) e metanol (MeOH). Como *D. macrophylla* foi relatada como produtora de substâncias antimicobacterianas, os extratos de calos foram testados contra *Mycobacterium tuberculosis* (cepa H37Rv). O extrato AcOEt dos calos foi ativo, com uma concentração inibitória mínima (CIM) de ≤ 25 μg mL^{-1}, IC_{90} de 19,5 μg mL^{-1} e concentração bactericida mínima (MBC) de 200 μg mL^{-1}. O extrato AcOEt foi analisado por cromatografia em camada delgada comparativa (CCDC) e Ressonância Magnética Nuclear (RMN) para determinar o seu perfil químico e mostrou ser rico em terpenos. O fracionamento cromatográfico do extrato AcOEt conduziu ao isolamento de uma mistura de dois esteroides, β-sitosterol e estigmasterol (na proporção de 2:1), os quais foram identificados por análises de RMN de ^{1}H e bidimensionais. Pelo levantamento bibliográfico realizado, este é o primeiro relato da cultura de células *in vitro* de *Duroia saccifera*, da atividade antituberculose do extrato de calos e do isolamento de β-sitosterol e estigmasterol da cultura de células vegetais *in vitro* da espécie.

Palavras-chave: antituberculose, fitoquímica, cultura de tecidos vegetais, Rubiaceae, esterois.

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Introduction

Tuberculosis (TB) is a chronic contagious disease caused by *Mycobacterium tuberculosis* (WHO 2017). TB is one of the 10 leading causes of mortality worldwide and more than 95% of TB deaths occur in low- and middle-income countries. In recent decades, the drugs clinically available for TB treatment (rifampin and isoniazid) have become ineffective, as the causative microorganism has developed resistance, so causing an increase in the number of patients who have the disease in a resistant form (WHO 2017). This serious public health problem has led to a renewed initiative to search for novel effective drugs, including new natural plant products, which might potentially fight TB.

As part of the search for new antituberculosis substances conducted by our bioprospection research group, we had previously isolated two triterpenes and eight indole alkaloids from *Duroia macrophylla* that were active against *M. tuberculosis* (H37Rv strain) (Martins *et al.* 2013; Nunez *et al*. 2014).

In order to discover other plant sources, we studied the congener, *Duroia saccifera* (Mart. *ex* Roem. & Schult.) K. Schum. (Rubiaceae). This rainforest tree occurs in Brazil, Colombia, Venezuela, and Peru (Taylor *et al.* 2007). In Brazil, it is popularly known as "cabeça-de-urubu", "puruí-da-mata" or "puruí-grande" (Cavalcante 1974), and is present in the states of Acre, Amazonas, Pará, and Rondônia, where it occurs in unflooded ("terra firme") and adjacent seasonally-inundated forests, on both white-water ("várzea") and black-water ("igapó") rivers (Taylor *et al.* 2007).

The main activities reported for extracts obtained from wild individuals of *D. saccifera* are antioxidant activity (Mesquita *et al.* 2015), antifungal activity against *Candida glabrata*, *C. albicans* (Reis *et al.* 2016), antibacterial activity against *Salmonella enterica*, *Propionibacterium acnes* and *Enterobacter cloacae* (Contreras-Mejia 2017), and also antimycobacterial activity against *Mycobacterium tuberculosis* (Reis *et al.* 2016; Carrion *et al.* 2013).

To obtain a renewable source of *D. saccifera* material, we carried out an *in vitro* callus establishment protocol and phytochemical study of the ethyl acetate calli extract to isolate and identify calli-derived substances biologically-active against *Mycobacterium tuberculosis*.

Material and Methods

Plant material

To establish an *in vitro* cell culture fruits and seeds of *Duroia saccifera* were collected from wild plants growing in the Reserva Adolfo Ducke (2°55'46.25"S, 59°58'29.69"W), Manaus, Amazonas state, Brazil, under IBAMA's collection permit number 16970-1. A voucher (number 259229) was deposited in Herbarium of Instituto Nacional de Pesquisas da Amazônia-INPA, Manaus.

Germination

A pre-disinfection of the fruit was made using a disinfectant solution of Mancozeb® (2 g L^{-1}) plus streptomycin (100 mg L^{-1}) for 30 minutes, followed by ethanol (70% v/v) immersion for 5 minutes. Seeds disinfection followed an ethanol (70% v/v) immersion for 1 minute in a laminar flow chamber, followed by an immersion in NaClO (2% v/v) for 5 minutes, with three washes in autoclaved distilled water between the disinfecting agents.

After disinfection, seeds were inoculated onto MS medium (Murashige & Skoog 1962) supplemented with sucrose (30 g L^{-1}), agar (7 g L^{-1}) and 21.54 µmol L^{-1} of gibberellic acid (GA_3), and cultivated for 90 days, without changing medium or subcultures. The seeds were maintained in a growth room at 26 ± 2 °C and 60% average humidity, under a 16:8 h photoperiod (light:dark), with a light intensity of 50 μmol^{-2} m^{-2} s^{-1} given by white fluorescent lamps. After germination, seedlings were subjected to successive subcultures every 30 days.

Callus induction

Complete leaves from *in vitro* seedlings of *D. saccifera* were used as explants for callogenesis induction. They were cut at the base of the leaf blade and inoculated into sterile test tubes containing 5 mL MS medium (Murashige & Skoog 1962), supplemented with sucrose (30 g L^{-1}) and agar (7 g L^{-1}) in a laminar flow chamber. We added 2,4-dichlorophenoxyacetic (2,4-D) (17.24 µmol L^{-1}) and kinetin (8.62 µmol L^{-1}) as growth regulators. Cultures were then maintained in a growth room at 26 ± 2 °C and 60% average humidity, under a 16:8 h photoperiod (light:dark), with a light intensity of 50 μmol^{-2} m^{-2} s^{-1} given by white fluorescent lamps.

After 60 days in culture, induced calli were subcultured in new tubes. After 30 days they were transferred from the test tubes to glass jars with 25 mL of medium, and subsequently subjected to successive subcultures every 30 days in order to increase mass.

Extraction, fractionation and isolation

Duroia saccifera calli were manually removed from the culture medium and combined achieving a fresh calli total weight of 1.238 kg. They were lyophilized (model ALPHA 1-2 LDplus, Christ) and powdered yielding 54.49 g (4.4% of dry material). The lyophilized powder was then extracted first with hexane (1 g:10 mL), using ultrasound for 20 min, filtered and re-extracted with hexane, repeating 4 times. Then the calli was extracted with ethyl acetate (EtOAc) by the same procedure 5 times and finally with methanol (MeOH), also 5 times by the same procedure. All extracts (hexane, EtOAc and MeOH) were concentrated in a rotary evaporator (model 802, Fisatom). Extracts and all fractions were analyzed by Thin Layer Chromatography (TLC) (Merck) of silica with UV_{254} on aluminum support, eluted with suitable systems, and revealed under UV light (λ = 254 and 365 nm), sulfuric *p*-anisaldehyde, $Ce(SO_4)_2$, $FeCl_3$, and Dragendorff's reagent.

The crude EtOAc extract (1.7 g) was subjected to chromatographic column CC (33.5 × 3 cm) fractionation with silica gel (SiO_2) (86.8 g), eluted with a gradient solvent system of DCM/MeOH (95:5, 97:3, 9:1, 85:15, 8:2, 7:3, 6:4, 1:1) and MeOH 100%, yielding 98 fractions. Fractions (25 mL) were collected and grouped by TLC analysis. Fractions 5–11 (126 mg) were again fractionated in SiO_2 (12.6 mg) CC (25 × 1.5 cm) and eluted with a gradient of DCM/Acetone (98:2, 95:5, 9:1, 85:15, 8:2, 7:3, 1:1) and 100% Acetone, yielding 57 fractions. A white amorphous powder was formed in the fraction 7–9 and was named compound 1 (7 mg).

Structural characterization

In order to determine the chemical structure, the fraction was dissolved in $CDCl_3$ and analyzed by Nuclear Magnetic Resonance (NMR) obtained with a Fourier 300 spectrometer (Bruker), operating at 300 for ^1H and 75 MHz for ^{13}C nuclei, respectively. All NMR shifts were expressed in ppm related to TMS signal at 0.00 ppm as internal reference.

Antituberculosis activity

The resazurin microtiter assay (REMA) was performed to test extract activity against *Mycobacterium tuberculosis*, at the Medical Microbiology Research Nucleus (NUPEMM), Federal University of Rio Grande (FURG). Extracts were tested at the initial concentration of 400 μg mL^{-1}. In each experiment, 100 μL of extracts, serially diluted in Middlebrook 7H9 medium supplemented with 10% OADC (oleic acid, albumin, dextrose and catalase) and 100 μL of the inoculum, were incubated at 37 °C for 7 days in a 96 well plate. Following incubation, 30 μL of resazurin (0.02%) was added in each well and further incubated for two days at 37 °C. Biological activity was assayed based on the change of color from blue to pink, when oxidation of the reagent occurs due to bacterial growth. Rifampicin and isoniazid drugs were used as positive controls (Palomino *et al*. 2002).

Results

Germination

Of the 108 initial seeds, 24% developed fungus contamination and 25% did not germinate. After 50 days of cultivation, the emergence of the radicle was observed in the remainder. The hypocotyl and radicle elongation occurred after 60 days. Secondary root formation occurred after 72 days and fully developed cotyledons after 82 days. After 90 days, 50% of the seedlings were fully formed (Fig. 1a-f).

Callus induction

Callus formation began after 15 days, when cell growth occurred in the central part of the leaf and at the end of leaf segments. After 60 days, the explants were completely transformed into callus and were then subcultured in new tubes, even though they still had zones of green coloration, which indicated cell masses with some degree of differentiation. After 90 days, they had a friable appearance, when the surface of the calli took on a smoothly nodulated appearance, and coloration ranged between whitish and yellowish (Fig. 2a-f).

Extraction, fractionation and isolation

TLC analysis of EtOAc extract obtained from *D. saccifera* calli indicated a high concentration of terpenes when the plates were sprayed with $Ce(SO_4)_2$ and sulfuric anisaldehyde. When revealed with UV light (λ = 365 nm) fluorescence,

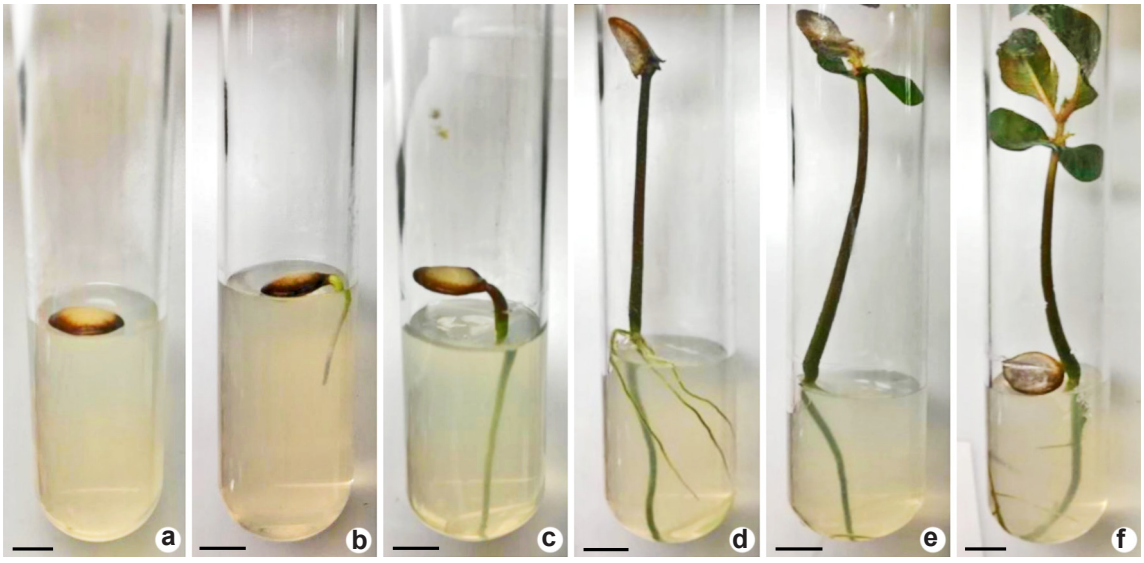

Figure 1 – a-f. *In vitro* germination of *D. saccifera* – a. seed; b. emerging radicle; c. hypocotyl and radicle elongation; d. secondary root formation; e. emergence of cotyledons; f. extension of leaf primordium. Scale bar: 0.5 cm.

the plates indicated the presence of molecules with conjugated double bonds. When the plates were sprayed with $FeCl_3$ and Dragendorff's reagent no spots were visible, which indicated the absence or very low concentrations of phenolic substances and alkaloids.

Structural characterization

^{1}H-NMR data for fraction 7–9 showed the presence of several signals in the shielded region between δ 0.6 (s) and 1.25 (s), characteristic of methyl hydrogens; and two signals at δ 5.35 (4H, d, J = 5.22 Hz) and 3.53 (4H, sept, J = 5 Hz) suggesting the presence of C-6 and C-3 of skeletal hydrogens of a stigmastane steroid. Two resonances at δ 5.15 (1H, dd, J = 15.18, 8.47 Hz) and δ 5.01 (1H, dd, J = 15.18, 8.47 Hz) suggested the presence of stigmasterol, but the integrals of these signals showed values half of the previous signals, indicating a mixture of two steroids, with β-sitosterol present in greater quantity (2:1).

The ^{13}C-RMN data showed signals at δ 140.75 and 121.74, common for both steroids, with signals in δ 138.24 and 129.25 being less

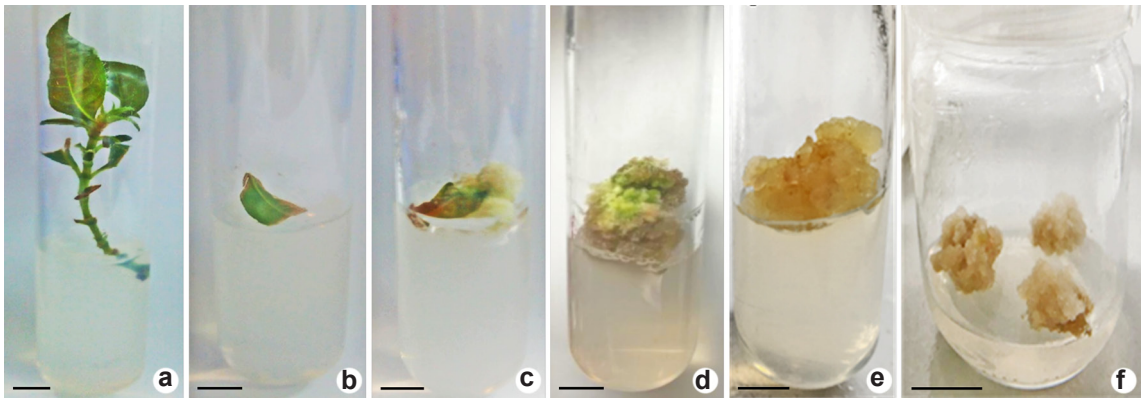

Figure 2 – a-f. Callogenesis induction of *D. saccifera* – a. *in vitro* seedlings; b. leaf segments used as explants; c. cell growth in the central part of the leaf and at the end of leaf segments; d. complete transformation of the explant into callus; e. friable appearance; f. calli subcultured in jars. Scale bar: a-e. 0.5 cm; f. 2 cm.

intense, these being a characteristic signals of stigmasterol. The HSQC spectrum showed the following correlations for the observed signals that made it possible to distinguish steroids: δ 5.35 with 121.74, δ 5.15 with 138.34, δ 5.01 with 129.25, and δ 3.53 with 71.81.

It was also possible to observe steroid methyl singlets in the ^{1}H-RMN spectrum, which could be correlated with each carbon atom by HSQC experiment analysis. For β-sitosterol, the correlations of these methyl groups were: δ 0.68 with 11.85, δ 0.80 with 18.93, δ 0.82 with 19.73,

Table 1 – ^{1}H and ^{13}C NMR chemical displacements observed for β-sitosterol and stigmasterol (300 MHz, $CDCl_3$).

Position	β-Sitosterol			Stigmasterol		
	^{13}C	^{1}H	^{13}C Literature [1]	^{13}C	^{1}H	^{13}C Literature [2]
1	37.24	1.80, 1.05	37.3	37.24		37.31
2	31.89	1.82, 1.52	31.6	32.41		31.72
3	71.81	3.53	71.8	71.81	3.53	71.85
4	42.29	2.28, 2.22	42.3	42.29		42.37
5	140.75	-	140.8	140.75		141.01
6	121.74	5.35	121.7	121.74	5.35	121.73
7	31.89	1.95, 1.52	32.1	31.65		31.72
8	31.89	1.53	32.1	31.65		31.79
9	50.1	0.91	50.2	50.1		50.19
10	36.49	-	36.5	36.15		36.17
11	21.08	1.46	21.1	21.22		21.11
12	39.76	1.99, 1.14	39.8	39.76		40.10
13	42.3	-	42.3	42.3		42.41
14	56.85	0.87	56.8	56.75		56.12
15	24.30	1.55, 1.03	24.3	24.30		23.12
16	28.94	1.82, 1.24	28.3	29.35		29.72
17	56.02	1.08	56.1	55.96		56.12
18	11.85	0.68	12.0	11.97		12.01
19	19.40	1.01	19.1	19.86		19.42
20	35.89	1.34	36.2	39.74		41.10
21	18.77	0.94	18.8	20.53		21.10
22	33.92	1.29, 0.99	34.0	138.34	5.15	138.00
23	26.00	1.15	26.2	129.25	5.01	128.95
24	45.80	0.90	45.2	51.23		50.15
25	29.1	1.64	29.2	29.70		31.72
26	19.01	0.80	18.9	21.86		21.30
27	19.84	0.82	19.1	18.70		19.06
28	23.03	1.23	23.1	25.4		25.38
29	11.85	0.85	11.9	11.97		12.28

Assignments made on the basis of COSY, HMQC and HMBC correlations; chemical displacements are in δ (ppm). [1] Kovganko *et al*. 1999 (360 MHz, CDCl3); [2] Khatun *et al*. 2012 (400 MHz, CDCl3).

δ 0.83 with 18.32, δ 0.85 with 11.86, δ 1.01 with 19.13, while the correlations for stigmasterol were: δ 0.70 with 11.85, δ 0.80 with 18.93, δ 0.83 with 20.62, δ 0.85 with 20.60, δ 1.03 with 19.13, plus δ 1.04 with 21.55 (Tab. 1).

Anti-tuberculosis activity

The EtOAc extract showed activity against *M. tuberculosis* (H37Rv strain), exhibiting MIC ≤ 25 μg mL^{-1} and Minimum Bactericidal Concentration (MBC) of 200 μg mL^{-1}. The MeOH extract showed no determined activity (Tab. 2).

Discussion

Previous studies on wild collected plants of *D. macrophylla* and *D. saccifera* have found biological activity against three *M. tuberculosis* strains: one pan-susceptible H37Rv (ATCC 27294) and two mono resistant strains, INH (ATCC 35822) and RIF (ATCC 35338) where leaves and branches crude extracts showed MIC values varied from 50 to 200 μg mL^{-1} (Reis *et al*. 2016; Carrion *et al*. 2013). A second study reported the isolation of the triterpenes oleanoic and ursolic acid active against *M. tuberculosis* strain (MIC 200 μg mL^{-1}) from *D. macrophylla* extracts (Martins *et al.* 2013).

Several other studies have shown terpenes to be responsible for antimycobacterial activity (Seidel & Taylor 2004; Aguiar *et al.* 2005; Higuchi *et al*. 2008; Subramaniam *et al*. 2014; Evina *et al.* 2017; Suja *et al.* 2017). Their high lipophilicity is probably the main factor that allows their penetration through the mycobacterial cell wall (Higuchi *et al.* 2008).

Reviews of the numerous plant species and active compounds showing antimycobacterial activity indicate β-sitosterol and stigmasterol to be important phytochemical components in this process (Chinsembu 2016; Tiwari *et al.* 2019). Suja (2017) reported growth inhibition activity of *M. tuberculosis* with a MIC of 25 and 100 μg mL^{-1} for β-sitosterol and stigmasterol, respectively. A study conducted by Saludes *et al.* (2002) reported the β-sitosterol and stigmasterol MICs to be 128 and 32 μg mL^{-1}, respectively. In the current study, we found a mixture of both substances which have similar structures, differing only in the position of an extra double bound (present in stigmasterol), which complicates their separation (Xu *et al.* 2005; Pierre & Moses 2015). This, and the small quantity of isolated mixture, meant it was not possible try to purify them further.

Conclusions

In this study, for the first time, we report *in vitro* germination, callus induction, and inhibitory activity, of EtOAc extract of *D. saccifera* calli against *M. tuberculosis*. β-sitosterol and stigmasterol were obtained for the first time from *in vitro* callus culture of this species. *In vitro D. saccifera* cultures have a broad potential for supply of active secondary metabolites, mainly terpenes. Fractionation of the EtOAc extract should be continued in order to isolate, identify and assay other possible active substances that could lead to the discovery of new anti-tuberculosis drugs.

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Table 2 – Minimum inhibitory concentration (MIC), Inhibitory concentration of 90% of bacterial growth (IC_{90}) and Minimum Bactericidal Concentration (MBC) of callus extracts of *Duroia saccifera* against *Mycobacterium tuberculosis* (H37Rv).

Extract / control	MIC (μg mL^{-1})	IC_{90} (μg mL^{-1})	MBC (μg mL^{-1})
EtOAc	≤ 25	19.5	200
MeOH	> 400	> 400	> 400
RMP	≤ 0.01	nd	nd
INH	0.03	nd	nd

EtOAc = ethyl acetate; MeOH = methanol; RMP = rifampicin; INH = isoniazid; nd = not determined. Extract with MIC > 400 μg mL^{-1} were considered inactive.

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