ARTÍCULO ORIGINAL

In vitro antimalarial activity of fractions and constituents isolated from *Tabebuia billbergii*

Actividad antimalárica *in vitro* de fracciones y constituyentes aislados de *Tabebuia billbergii*

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ABSTRACT

Introduction: *in vitro* antimalarial activity of naphthoquinones (1-5), isolated from *Tabebuia billbergii* (Bureau & K. Schum.) Standl., was investigated. *Tabebuia billbergii,* commonly known as *guayacán*, is a plant traditionally used in the Amazon in numerous conditions like bacterial and fungal infections, fever, syphilis, malaria, trypanosomiasis, as well as stomach and bladder disorders, and tumours.
Objective: to study the dichloromethane extracts of both the trunk and the inner bark of *Tabebuia billbergii* and to demonstrate the antimalarial activity of some of its bioactive components.
Methods: some bioactive components were evaluated for the antimalarial activity against *Plasmodium berghei*, by using the inhibition of the differentiation cycle of the parasite measure by the 3h-hipoxanthine incorporation and compared to that obtained for chloroquine.
Results: conventional chromatographic techniques and bioassay-guided fractionation (*Artemia salina*) allowed isolating from the active fractions one naphthoquinone (lapachol) and four naphtho-furan-4,9-diones. These compounds proved to have an important antiplasmodial effect, with very encouraging IC_{50}'s, especially when compared to the results shown by Chloroquine in the same experiment. In addition, two triterpenes, b-sitosterol and stigmasterol, were obtained from the bark.
Conclusions: the activity-guided fractionation (*A. salina*) of dichloromethane extracts of the trunk and the inner bark of *Tabebuia billbergii* led to the isolation and the identification of five quinonoid compounds with antiplasmodial effect. The

significant inhibitory activity *in vitro* against *Plasmodium berghei* observed for compound 2-acetyl-naphtho-[2,3*b*]-furan-4,9-dione allow us to present them as a potential antimalarial compound.

Key words: Tabebuia billbergii, naphthoquinones, Plasmodium berghei.

RESUMEN

Introducción: se evaluó la actividad antimalárica *in vitro* de una serie de naftoquinonas (1-5), aisladas de *Tabebuia billbergii* (Bureau & K. Schum.) Standl., que es conocida comúnmente como guayacán, una planta utilizada tradicionalmente en la Amazonía en numerosos problemas de salud como infecciones bacterianas y fúngicas, fiebre, sífilis, paludismo, tripanosomiasis, así como en problemas estomacales, tumores y trastornos de la vejiga.
Objetivo: estudiar los extractos en diclorometano tanto del tronco como la corteza interna de *Tabebuia billbergii* y evaluar la actividad antimalárica de algunos de sus componentes bioactivos.
Métodos: la actividad antimalárica contra *Plasmodium berghei* se evaluó en algunos componentes bioactivos, por la inhibición del ciclo de la diferenciación de la medida de los parásitos mediante la incorporación de 3H-hipoxantina y se comparó con la obtenida para la cloroquina.
Resultados: a través de técnicas cromatográficas convencionales y el fraccionamiento guiado por bioensayo (*Artemia salina*) se aislaron de las fracciones activas, una naftoquinona (lapachol) y 4 nafto-furan-4,9-dionas. Estos compuestos presentaron un efecto antiplasmodial importante, con buenos valores de IC_{50}, especialmente cuando se compara con los resultados mostrados por la cloroquina en el mismo experimento. Además, se obtuvieron de la corteza 2 triterpenos, b-sitosterol y estigmasterol.
Conclusiones: el fraccionamiento guiado por *Artemia salina* de los extractos en diclorometano del tronco y la corteza interna de *Tabebuia billbergii*, condujo al aislamiento y la identificación de 5 compuestos de naturaleza quinoidal con efecto antiplasmódicol. La actividad *in vitro* contra *Plasmodium berghei* observada para el compuesto 2-acetil-nafto-[2,3 *b*]-furan-4,9-diona, permite proponerlo como un potencial compuesto antimalárico.

Palabras clave: Tabebuia billbergii, naftoquinonas, Plasmodium berghei.

INTRODUCTION

Malaria is a serious infectious disease caused by protozoan parasites in tropical and subtropical regions. Global spread of multiple drug-resistant malaria has become a major health problem and efforts to search for new antimalarial are needed. The inner bark of *Tabebuia* spp*.* has been used for many years by the Tukuna Indians of the Colombian Amazon as antimalarial.[1-3] The genus *Tabebuia* belongs to the Bignoniaceae family. It comprises about 100 species that are distributed from Mexico to northern Argentina. Many of these species have interesting biological activities against tumours, viruses, bacteria and the plasmodia.[4] Previous studies on the chemistry of some Tabebuia spp have resulted in the isolation of naphthoquinone and anthraquinone derivatives,[5,6] which were shown active against

malaria,[7-9] bacteria[10] as well as cancer cells.[11,12] A particularly interesting species is *Tabebuia chrysantha*, from which lapachol and â-lapachone were isolated, together with several other naphthoquinones and antraquinones, which have shown antitumoral activity.[13-15] From *T. cassinoides* were isolated three naphtho-furan-diones, which were shown to have *in vitro* cytotoxic activity against KB cells.[16] Natural and synthetic naphthoquinones have shown significant antimalarial activity against chloroquine-resistant *Plasmodium* parasites.[17,18]

These important findings justified our studies on the flora of the Atlantic coast of Colombia. As a result, this paper deals with the isolation of lapachol and several naphtho-furan-4,9-diones from the trunk and inner bark of *Tabebuia billbergii* (Bureau & K. Schum.) Standl. (Bignoniaceae) and their effects on the differentiation cycle of *Plasmodium berghei*, measured by the 3H-hypoxanthine incorporation.

METHODS

General

Column chromatography (CC): silica gel 60 Merck (70-230 mesh). Preparative TLC: pre-coated TLC plates, silica gel 60 F_{254} (2 mm, Merck). UV: spectrophotometer Milton Roy Spectronic 3000 Array; 1mg/mL MeOH. FT-IR spectra: (KBr) spectrophotometer Nicolet 5DX. ^{1}H-NMR spectra: Bruker AM 300 (300 MHz) in $CDCl_3$, TMS as internal standard. EIMS: Kratos MS25RFA, at 70 eV, in brackets identification and relative abundance. GC-MS: Hewlett-Packard GC-5890 Series II, with a Mass detector and a Wiley library of spectra, DB-5, 30 m column and He as carrier gas.

Plant Material

In April of 2008, samples from the trunk and inner bark of *T. billbergii* were collected in the vicinity of Arjona, located in the Department of Bolívar, Colombia. The species was taxonomically identified by Hermes Cuadros (Universidad del Atlántico, Barranquilla-Colombia) and a voucher specimen Nº 258, deposited in the Botanical Garden "Guillermo Piñeres", Cartagena, Colombia. The name was cross-referenced with the International Plant Name Index (IPNI) and W3Tropicos.

Extraction

Powdered dry wood (5 kg) and inner bark (4 kg) were successively introduced into a Soxhlet apparatus with hexane, dichloromethane and ethanol. Evaporation of the solvents under reduced pressure produced the following residues: 2.5, 6.1, and 15.8 g from the wood and 2.8, 8.5, and 12.8 g from the bark. These extracts were evaluated through lethality tests against *Artemia salina*,[19] in order to select the more active ones for subsequent fractionation.

Fractionation of the dichloromethane trunk wood extract

Column chromatography (CC) of the CH_2Cl_2 extract of the trunk wood using eluents of increasing polarity from hexane to AcOEt-MeOH (1:1), produced 150 fractions (20 mL ea.) which were monitored by TLC and combined according to their TLC profiles to obtain 9 fractions. At this stage, all the fractions were assayed against *A. salina*. Fractions 2 and 4 showed the best LC_{50}'s and were further fractionated. Fraction 2 (280 mg) was chromatographed by column using hexane with increasing

amounts of AcOEt as eluent. The fraction obtained from hexane-AcOEt (1:1), was further purified by PTLC (CH_2Cl_2) and yielded compound I (Fig.). Fraction 4 (80 mg) was subjected to PTLC (Hexane-AcOEt, 1:1) and yielded compound II (Fig.).

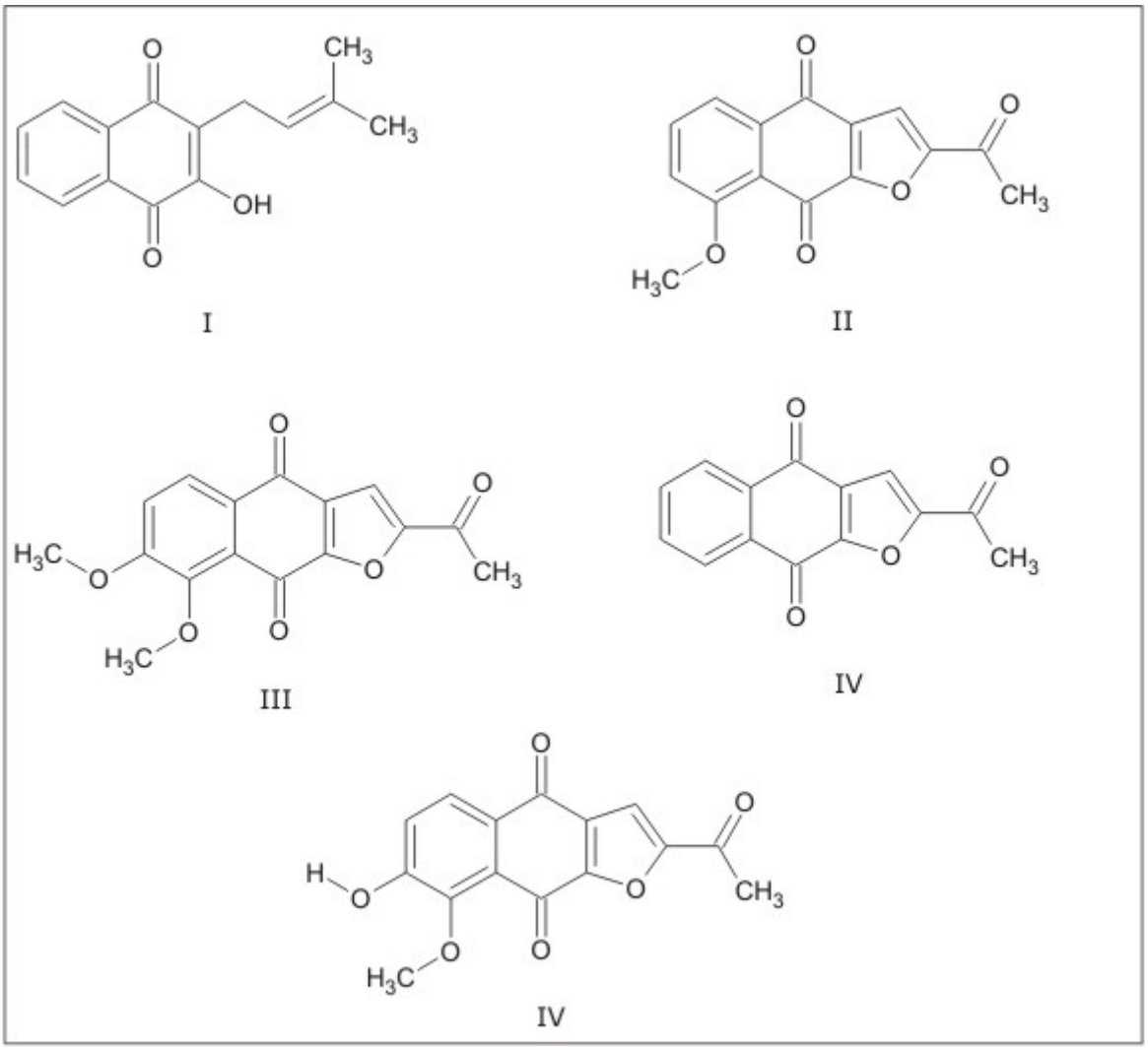

Fig. Fractionation of the dichloromethane.

Fractionation of the dichloromethane inner bark extract

Column chromatography of the CH_2Cl_2 extract of the inner bark, under the same conditions mentioned above, produced 220 fractions (20 mL ea.). They were monitored by TLC and combined to obtain 17 fractions according to their TLC profiles. Of these, fractions 3, 5, 6, 12 and 17 showed good LC_{50}'s and were assessed against *Plasmodium berghei*. These active fractions were further fractionated. Fraction 3 was purified by repeated PTLC to yield compound IV (Fig.). Fraction 5 purified by PTLC with Hexane-CH_2Cl_2 (3:7) produced compounds II and III (Fig.). Fraction 6 treated in the same manner produced compound 3, together with b-sitosterol and stigmasterol. Fraction 12 subjected to PTLC with CH_2Cl_2 produced compound V (Fig.).

Compounds 1-5 were identified using spectral data such as ^{1}H NMR, ^{13}C NMR, as well as EI-MS spectra, and by direct comparison with the data reported in the literature, as lapachol (I), 2-acetyl-8-methoxy-naphtho-[2,3*b*]-furan-4,9-dione

(II),[20] 2-acetyl-7,8-dimethoxy-naphtho-[2,3*b*]-furan-4,9 -dione (III),[21] 2-acetyl-naphtho-[2,3*b*]-furan-4,9-dione (IV),[5,9] and 2-acetyl-7-hydroxi-8-methoxy-naphtho-[2,3*b*]-furan-4,9-dione (V)[6] (Fig.). b-sitosterol and stigmasterol were separated by GC-MS and identified through comparison with their spectra in the instrument's library.

Artemia salina lethality tests

These tests were performed using the protocol reported in the literature.[19,22] Briefly, a few milligrams of brine shrimp eggs were placed for hatching in seawater. After 10-12 h, the eggs began hatching. Two days were allowed for the shrimp to mature into nauplii (shrimp can be used 48-72 h after the initiation of hatching). Stock solutions of extracts and fractions were prepared by dissolving extracts in saltwater. Working solutions of 10 to 1000 ppm were prepared by dilution and placed in culture tubes. Ten nauplii were placed into each tube. Tubes were incubated for 24 h at room temperature under illumination. Three replicates were prepared for each concentration. After 24h, the numbers of dead nauplii were counted. All results are expressed as mean LC_{50} values ± standard deviation () of at least three independent experiments.

Antimalarial activity

Estimation of antimalarial activity was performed as reported in the literature.[9] The biological activity of the fractions and isolated compounds was assessed in *Plasmodium berghei* using the inhibition of the differentiation cycle of the parasite measured through the ^{3}H-hypoxanthine incorporation. Short- term cultures of *Plasmodium berghei* followed the protocol described by Kamiyama and Matsubara (1992).[23] Chloroquine was used as control.

Statistical Analyses

The lethal concentration 50 % of *A. salina* (LC_{50}) and the inhibitory concentration 50 % (IC_{50}) of *Plasmodium berghei* were calculated on the basis of a nonlinear regression (curve fit), Statistical analyses were performed by one-way analysis of variance and Newman»Keuls multiple comparison tests. Differences were considered significant where p values were < 0.05.[24]

RESULTS

The extracts from the trunk and inner bark of *T. billbergii* were evaluated by conducting lethality tests against *Artemia salina*[19] and selecting the more active extracts for fractionation. In both cases, the CH_2Cl_2 extracts showed to be the more actives, giving lethal concentrations (LC_{50}) at 163 and 58 mg/mL, respectively (Table 1).

Fractionation of these extracts produced several active fractions (Table 2) which were assessed for antiplasmodial activity against *Plasmodium berghei* (Table 3). Posterior separation permitted the isolation and identification of the quinonic compounds 1-5, together with the triterpenoids b-sitosterol and stigmasterol.

Table 1. Toxicity of extracts and fractions of the trunk and inner bark of *Tabebuia billbergii* against *Artemia salina* larvae after 24 h of exposure

Extracts and fractions[a]	Trunk/Inner bark	LC_{50} (µg/mL)[b]	95% confidence limit (µg/mL)	
CH_2Cl_2 (TBM-10B)	Trunk	163,1	110	252
CH_2Cl_2 (TCB-1B)	Inner bark	58,0	35	79
2	Trunk	28,6	21,1	36,2
4	Trunk	51,8	39,4	64,5
3	Inner bark	55,8	37,2	72,4
4	Inner bark	28,3	15,1	48,9
5	Inner bark	76,1	45,1	82,2
9	Inner bark	123,1	97,3	176
10	Inner bark	148,5	215	98,6

[a]The extracts and fractions not listed were not toxic at the highest concentrations tested (200 µg/mL), [b]The LC_{50} values correspond to means of three independent experiments.

Table 2. *In vitro* antimalarial activity of fractions isolated of the trunk and inner bark of *Tabebuia billbergii*

Fractions	Trunk/Inner bark	IC_{50} (µg/mL)[a]	95% confidence limit (µg/mL)	
2	Trunk	>20		
4	Trunk	>20		
3	Inner bark	>20		
5	Inner bark	>20		
6	Inner bark	>20		
12	Inner bark	2,53	1,6	4,2
17	Inner bark	14,25	10,8	20,0

[a]IC_{50}= inhibitory concentration 50 %, values are means of three independent experiment.

Table 3. Anti-*Plasmodium berghei* cytotoxicity *in vitro* of naphtho-furan-diones 1-5

Compounds	IC_{50} (µM)[a]	95% confidence limit (µM)	
1	4,880	4,550	5,220
2	51,800	52,500	51,00
3	2,960	2,110	4,080
4	0,002*	0,001	0,003
5	11,800	7,340	20,600
Cloroquina	0,110	0,090	0,170

[a]IC_{50}= inhibitory concentration 50%; *p< 0,05 compared to cloroquina, a positive control. Values are means of three independent experiment.

Compounds 1-5 were identified using spectral information such as that from ^{1}H NMR, ^{13}C NMR, as well as EI-MS spectra, and by direct comparison with the data reported in the literature, as 2-hydroxy-3-(3'methyl-2'-butenyl)-naphthalen-1,4-dione (lapachol, 1), 2-acetyl-8-methoxy-naphtho-[2,3b]-furan-4,9-dione (*2*),[20] 2-acetyl-7,8- dimethoxy-naphtho-[2,3b]-furan-4,9-dione (3),[21] 2-acetyl-naphtho-[2,3b]-furan-4,9-dione (4)[5,9] and 2-acetyl-7-hydroxi-8-methoxy-naphtho-[2,3b]-furan-4,9 -dione (5).[6] b-sitosterol and stigmasterol were separated by GC-MS and identified by comparison with their spectra in the instrument's library.

The naphtho-furan-diones showed important inhibitory activity when assayed *in vitro* against *Plasmodium berghei*, especially in comparison to the activity exhibited by Chloroquine which was used as control. The IC_{50} values obtained with these pure compounds are summarized in Table 3.

DISCUSSION

The activity-guided fractionation (*A. salina*) of different extracts from the wood and inner bark of *T. billbergii* led to the isolation and identification of five quinonoid compounds. The results from the spectral data for compounds I-V isolated in this study were compared with the data reported previously in the literature for these compounds. The antimalarial activity of the fractions and isolated compounds was assayed against *Plasmodium berghei* by using the inhibition of the differentiation cycle of the parasite measured through the ^{3}H-hypoxanthine incorporation. Short-term cultures of *Plasmodium berghei* followed the protocol described by Kamiyama and Matsubara (1992).[23] Chloroquine was used as control for the experiments. The IC_{50} values obtained with various fractions and isolates from the plant are summarized in Tables 1 and 2.

Preliminary evaluation of the antiparasitic activity of compounds 1, 2, 3 and 5, suggests there is much ground for further investigation, since the values of IC_{50}'s obtained are rather encouraging. The activity observed for compound 4 deserves special attention because of its pharmaceutical potential. Of course, this requires further deep research, but we are already involved in the synthesis of some of these quinones, in order to be able to proceed with the *in vivo* tests.

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REFERENCES

1. Bernal HY, Correa JE. Especies vegetales promisorias de los países del Convenio Andrés Bello. vol. 2. Secretaría Ejecutiva del Convenio Andrés Bello (SECAB). Bogotá, Colombia: Editora Guadalupe Ltda.;1986. p. 241-4.

2. Gentry AH. Bignoniaceae. Flora de Venezuela. Vol 8. Parte 4. Instituto Nacional de Parques, Venezuela: Ediciones Fundación Educación Ambiental;1982. p. 389-91.

3. Gómez JR, Prieto JM, Heinrich M. Red Lapacho (*Tabebuia impetiginosa*) -A global ethnopharmacological commodity?. J Ethnopharmacol. 2009;121(1):1-13.

4. Weiss CR, Moideen SV, Croft SL, Houghton Peter J. Activity of extracts and isolated naphthoquinones from *Kigelia pinnata* against *Plasmodium falciparum*. J Nat Prod. 2000;63:1306-9.

5. Girard M, Kindack D, Dawson B, Ethier J-C, Awang DVC. Naphthoquinone constituents of *Tabebuia* spp. J Nat Prod. 1988;51(5):1023-4.

6. Díaz F, Medina JD. Furanonaphthoquinones from *Tabebuia ochracea* ssp. *neochrysantha*. J Nat Prod. 1996;59(4):423-4.

7. Hudson AT, Randall AW, Fry M, Ginger CD, Hill B, Latter VS, et al. Novel anti-malarial hydroxynaphthoquinones with potent broad spectrum anti-protozoal activity. Parasitology. 1985;90:45-55.

8. Fry M, Pudney M. Site of action of the antimalarial hydroxynaphthoquinone, 2-[*trans*-4-(4'-chlorophenyl)-cyclohexyl]-3-hydroxy-1,4-naphthoquinone. Biochemical Pharmacol. 1992;43(7):1545-53.

9. Pérez E, Díaz R, Estévez A, Ravelo A, García J, Pardo L, et al. Synthesis and pharmacophore modeling of naphthoquinone derivatives with cytotoxic activity in human promyelocytic leukemia HL-60 cell line. J Med Chem. 2007;50:696-706.

10. Park BS, Kim JR, Lee SE, Kim K, Takeoka GR, Ahn YJ, et al. Selective growth-inhibiting effects of compounds identified in *Tabebuia impetiginosa* inner bark on human intestinal bacteria. J Agric Food Chem. 2005;53:1152-7.

11. Boothman DA, Trask DK, Pardee AB. Inhibition of potentially DNA damage repair in human cells by b-Lapachone, an activator of topoisomerase I. Cancer Research. 1989;49:605-12.

12. Kapadia GJ, Balasubramanian V, Tokuda H, Konoshima T, Takasaki M, Koyama J, et al. Anti-tumour promoting effects of naphthoquinone derivatives on short term Epstein-Barr early antigen activation assay and in mouse skin carcinogenesis. Cancer Letters. 1997;113:47-53.

13. Bonifazi EL, Ríos-Luci C, León LG, Burton G, Padrón JM, Misico RI. Antiproliferative activity of synthetic naphthoquinones related to lapachol. First synthesis of 5-hydroxylapachol. Bioorganic Medicinal Chemistry. 2010;18(7):2621-30.

14. Frydman B, Marton LJ, Sun JS, Neder K, Witiak DT, Liu AA, et al. Induction of DNA topoisomerase II-mediated DNA cleavage by b-lapachone and related naphthoquinones. Cancer Research. 1997;57:620-7.

15. Pérez H, Díaz F, Medina JD. Chemical investigation and *in vitro* antimalarial activity of *Tabebuia ochracea* ssp. *neochrysantha*. International J Pharmacognosy. 1997;35:227-31.

16. Madhusudana R, Kingston D. Plant anticancer agents. XII. Isolation and structure elucidation of new cytotoxic quinones from *Tabebuia cassinoides*. J Nat Prod. 1982;45(5):600-4.

17. Friebolin W, Jannack B, Wenzel N, Furrer J, Oeser T, Sánchez CP, et al. Antimalarial dual drugs based on potent inhibitors of glutathione reductase from *Plasmodium falciparum*. J Med Chem. 2008;(51):1260-77.

18. Kongkathip N, Pradidphol N, Hasitapan K, Grigg R, Kao WC, Hunte C, et al. Transforming rhinacanthin analogues from potent anticancer agents into potent antimalarial agents. J Med Chem. 2010;53:1211-21.

19. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. Brine shrimp: A convenient general bioassay for active plant constituents. Planta Medica. 1982;45:31-4.

20. Wagner H, Kreher B, Lotter H, Hamburger MO, Cordell GA. Structure elucidation of new isomeric naphtho-[2,3*b*]furan-4,9-diones from *Tabebuia avellanedae* by the selective-INEPT technique. Helvetica Chimica Acta. 1989;72:659-67.

21. Zani CL, Chiari E, Krettli AU, Murta SM, Cunningham ML, Fairlamb AH, et al. Anti-plasmodial and anti-trypanosomal activity of synthetic naphtho [2,3*b*] thiophen-4,9-quinones. Bioorganic Medicinal Chemistry. 1997;5(12):2185-92.

22. McLaughlin JL, Chang CJ, Smith DL. "Bench-top" bioassays for the discovery of bioactive natural products: an update. In: Rahman A, editor, Studies in Natural Products Chemistry 9. New York: Elsevier Science Publishing Company Inc.;1991. p. 383-409.

23. Kamiyama T, Matsubara J. Application of a simple culture of *Plasmodium berghei* for assessment of antiparasitic activity. International J Parasitol. 1992;22:1137-42.

24. Finney DK. Probit analysis. 3rd ed. Cambridge, U.K.: Cambridge University Press;1971.

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