

# PHCOG MAG.: Research Article

## Inhibition of nitric oxide and interferon- $\gamma$ production by iridoids and triterpenes from the roots of *Himatanthus sucuuba*

Marinete S. Souza<sup>a</sup>, Milade dos S. Cordeiro<sup>a</sup>, Elaine C. Rosas<sup>b</sup>,  
Maria das Graças O. M. Henriques<sup>b</sup>, Antonio C. Siani<sup>b\*</sup>

<sup>1</sup>Departamento de Química Orgânica, Instituto de Química, Fundação Universidade do Amazonas, Manaus, AM, Brazil; <sup>2</sup>Laboratório de Química de Produtos Naturais e Farmacologia Aplicada, Far-Manguinhos, Fundação Oswaldo Cruz, Rua Sizenando Nabuco 100, 21041-250, Rio de Janeiro, RJ, Brazil

Corresponding Author : siani@far.fiocruz.br

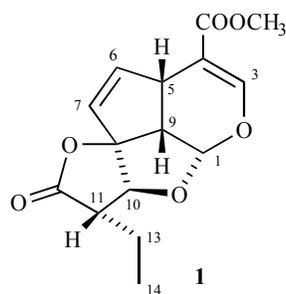
**ABSTRACT** - The iridoids  $\beta$ -dihydroplumericin (1), plumericin and isoplumericin were isolated from the roots of *Himatanthus sucuuba*, along with lupeol acetate and cinnamate. The isolated compounds and three different crude extracts from the roots exhibited *in vitro* immunoregulatory activity in the nitric oxide and interferon- $\gamma$  inhibition assays, when tested below the cytotoxic concentrations.

**KEYWORDS** - *Himatanthus sucuuba*, iridoid, triterpene,  $\beta$ -dihydroplumericin, immunoregulatory activity.

### INTRODUCTION

The latex, the powdered dried bark, or the aqueous extracts of *Himatanthus sucuuba* Spruce (M. Arg.) Woodson. (Apocynaceae) ("sucuúba", "belasco-capi") are popularly used in the Amazonian countries (Brazil, Peru and Colombia) as agents for many purposes, such as vermifuge (anthelmintic), laxative, anti-tumoral, anti-fungal, anti-inflammatory, anti-anemic, anti-ulcer, analgesic, antitussive, aphrodisiac; and to treat arthritis, boils, hernias, swellings, skin tumors, gastritis and hemorrhoids (1,2). Such a plethora of somewhat conflicting ethnobotanical information (3) may lead to the supposition of this species as possessing general immunoregulatory ability.

The iridoids fulvoplumerin, plumericin, isoplumericin and  $\beta$ -dihydroplumericin as well as the triterpenes lupeol acetate, lupeol cinnamate and  $\alpha$ -amyrin cinnamate have been isolated from latex and bark of *Himatanthus sucuuba* (3-5). Other compounds, as vanillic acid, p-coumaric acid, p-hydroxybenzoic acid, confluent acid and 2'-O-methylpentaloic acid also have been isolated from the methanolic extract of the bark; the two latter compounds from an associated lichen (6). The present study reports the isolation of  $\beta$ -dihydroplumericin (11S-13-dihydroplumericin) (1), plumericin, isoplumericin and triterpenes from the roots of *H. sucuuba*. The immunoregulatory activity of these compounds as well as crude extracts from the bark was inferred by the ability to inhibit the *in vitro* production of nitric oxide and interferon- $\gamma$ .



Roots of *Himatanthus sucuuba* were collected in the campus of the University of Amazonas, Manaus, in January 1995 and identified by the botanist A. F. Barbosa. A voucher specimen is deposited in the Herbarium of the Instituto de Ciências Biológicas da Universidade do Amazonas, Manaus, under No. 5436. All extracts were prepared by maceration at room temperature: n-hexane extract of the inner part of the root (extract A, 0.76%), acetone fraction (0.68%) from methanolic extract of the same (extract B, 3.2%), and methanol extract of the root bark (extract C, 8.6%). Extract yields were taken on dried plant basis. The triterpenes lupeol (0.52%), lupeol acetate (0.47%), and lupeol cinnamate (0.42%) were isolated from the hexane fraction of extract C, and  $\beta$ -dihydroplumericin was isolated from extract B (1.26%). All the compounds were purified by silica column chromatography. The mixture of plumericin and isoplumericin (0.39% total yield from extracts A and C) was separated by

preparative TLC (silica), eluted twice with n-hexane:ethyl acetate 8:2. A large amount of a mixture of  $\alpha$ - and  $\beta$ -amyrin cinnamates (35%) was also isolated from the chloroform extract of the hexane-defatted root bark, but it was not pharmacologically assayed. The isolated compounds were identified by spectroscopic methods and by comparing with data from the literature (3,7,8).  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  (including 2D) data for  $\beta$ -dihydroplumericin were obtained.

As the source of *ex vivo* material, male Balb/c mice (20-30 g), from the CECAL-FIOCRUZ colony, were lodged in a room with controlled temperature and lighting, with free access to lab chow and tap water. All experiments were conducted in accordance with the ethical guidelines of the International Association for the Study of Pain (9) and the institutional guidelines for animal use. Cytotoxicity was measured by the MTT method (10), and NO and IFN- $\gamma$  productions were evaluated from murine macrophages and splenocytes respectively (11). All the values were obtained as a result of triplicate experiments. Peritoneal macrophages were harvested from mice injected 3 days before with 1 ml of 3% thioglycolate. The cell viability was determined by trypan blue and the viable cells ( $2.5 \times 10^5$  cells/well) were incubated in 96-wells

plates ( $2.5 \times 10^6$  cells/mL) with the samples in different concentrations during 20h. Stock MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; 5 mg/mL; 22.5  $\mu\text{L}$ /well] was then added, and the plates incubated at 37 °C during 4 h. DMSO (150  $\mu\text{L}$ /well) was added and mixed thoroughly to dissolve the dark blue crystals and the absorbance was read at 540 nm in a plate reader (Biorad - 450). For evaluating the nitric oxide production, the macrophages obtained as described above, were incubated in a 96-wells flat bottom plate ( $2.5 \times 10^6$  cells/mL) and stimulated with or without IFN- $\gamma$  enriched media plus lipopolysaccharide (30 ng/mL), in the presence of the samples at the non-cytotoxic concentrations. After 24 h, the supernatant was recovered and the nitrite production was determined by the Greiss method (12). For evaluating the interferon- $\gamma$  production, the spleens cells were recovered from Balb/c male mice (13). The spleen mononuclear cells were isolated through Ficoll-Hypaque gradient and incubated ( $3 \times 10^5$  cells/well) with or without Concanavalin-A (5  $\mu\text{g}$ /ml) plus the extracts or the pure compounds in different concentrations. After 72 h, the supernatant was recovered and used to analyze the INF- $\gamma$  production by sandwich enzyme-linked immunosorbent assay (ELISA).

**Table 1: Inhibition of nitric oxide and interferon- $\gamma$  production by extracts and isolated compounds from *H. sucuuba* roots**

Sample*	Cytotoxicity (%)	NO inhibition (%)	IFN- $\gamma$ inhibition (%)
Extract A	0	83	0
Extract B	0	83	100
Extract C	0	84	4.5
Lupeol	0	14	62
Lupeol acetate	0	23	100
Lupeol cinnamate	0	7.0	100
Plumericin	0	80	100
$\beta$ -Dihydroplumericin 100 $\mu\text{g}$	74.6	<i>cytotoxic</i>	<i>cytotoxic</i>
$\beta$ -Dihydroplumericin 10 $\mu\text{g}$	27.9	96	98
$\beta$ -Dihydroplumericin 1 $\mu\text{g}$	22.5	2.5	92

\*Macrophages were incubated with  $\beta$ -dihydroplumericin as liposomes in phosphatidylcholine (1:1 weight) in phosphate buffered saline (sonication at 50 Hz for 5 min), at the concentration of 50 mg/mL. The other samples were administered (100  $\mu\text{g}$ /well) in Tween 20 (10 mg : 1  $\mu\text{L}$ ) suspended in PBS at a concentration of 50 mg/mL.

This represents the first chemical study of *H. sucuuba* roots. Lupeol and its esters as well as plumericin and

isoplumericin, which were also reported in the bark and latex of *H. sucuuba* (5,8), were identified by

comparison of data with those reported in literature (3).  $\beta$ -dihydroplumericin is reported for the first time in this species. All three of the crude extracts A, B and C as well as plumericin inhibited NO production by more than 80%. Extracts A and C alone were not able to inhibit IFN- $\gamma$  production. Samples that showed more than 30% of cytotoxicity were not further assayed. The activity shown by extracts A and B is probably related to the presence of plumericin and its geometric isomer. Extract A did not affect IFN- $\gamma$  at the tested dose, probably due to an insufficient concentration of those compounds. Higher degrees of IFN- $\gamma$  inhibition were observed with extracts rich in triterpene esters and iridoids.  $\beta$ -dihydroplumericin showed an evident dose-dependent immunoregulatory effect, when tested at doses below those toxic to the macrophages.  $\beta$ -dihydroplumericin was cytotoxic at 100  $\mu\text{g}/\text{mL}$  and inhibited NO and IFN- $\gamma$  at 10  $\mu\text{g}/\text{mL}$  or higher (Table 1). Its occurrence may be associated with the high toxicity reported for the roots of *H. sucuuba* (14); the hydrogenation of the exocyclic C<sub>11</sub>-C<sub>13</sub> double bond possible being responsible for enhancement in the cytotoxicity when compared to plumericin. In spite of the well-known chemical liability of the iridoids, the last five years have watched an increasing research involving the pharmacological activities of such compounds, either as glycosides or aglycone. Nevertheless, not many examples of immunoassays using these compounds are available in literature (15-17).

**$\beta$ -Dihydroplumericin (1).** White powder from CHCl<sub>3</sub>-MeOH; m.p. 150-152 °C (lit. 150-1 °C) [18]; IR bands (KBr): 1772, 1696, 1645, 1612 cm<sup>-1</sup>; <sup>1</sup>H-NMR (200 MHz, CDCl<sub>3</sub>):  $\delta$  1.10 (t, J 7.3 Hz, H-14, 3H : methyl), 1.76 (m, H-13), 2.74 (dd, J 6.7, 1.8 Hz, H-11), 3.43 (dd, J 9.5, 5.8 Hz, H-9), 3.76 (s, 3H, COOCH<sub>3</sub>), 3.97 (td, J 9.5, 2.1, H-5), 4.40 (s, H-10), 5.58 (d, J 5.8 Hz, H-1), 5.66 (dd, J 5.5, 2.1 Hz, H-7), 6.08 (dd, J 5.5, 2.1 Hz, H-6), 7.43 (s, H-3); <sup>13</sup>C-NMR (75 MHz, CDCl<sub>3</sub>):  $\delta$  176.5 (C-12), 166.6 (C-15), 152.7 (C-3), 141.4 (C-6), 126.4 (C-5), 108.6 (C-4), 106.0 (C-8), 101.4 (C-1), 86.7 (C-10), 53.8 (C-9), 51.6 (OMe), 48.8 (C-11), 37.9 (C-5), 22.7 (C-13), 11.9 (C-14). EI-GCMS m/z: 292 (M<sup>+</sup>, 30%), 231 (84), 203 (35), 193 (74), 175 (32), 139 (100). <sup>13</sup>C and <sup>1</sup>H-NMR data: corresponding to those reported for 1 isolated from *Plumeria rubra* var. *alba* (18) and *Plumeria acutifolia* (19); the  $\beta$ -orientation of the ethyl group being indicated by the H-10 singlet signal in <sup>1</sup>H-NMR.

## REFERENCES

1. P. Corrêa, *Dicionário das Plantas Úteis do Brasil e das Exóticas Cultivadas*, (Imprensa Nacional, Ministério da Agricultura, Rio de Janeiro, 1975), vol. VI, pp.154-155.
2. L.F. Villegas, I.D. Fernández, H. Maldonado, R. Torres, A. Zavaleta, A.J., Vaisberg and G.B. Hammond. Evaluation of the wound-healing activity of selected traditional medicinal plants from Perú, *J. Ethnopharmacol.* **55(3)**: 193-200 (1997) and references therein.
3. Wood C.A., Lee K., Vaisberg A.J., Kingston D.G.I., Neto C.C., Hammond G.B. A Bioactive Spirolactone Iridoid and Triterpene from *Himatanthus sucuuba*. *Chem. Pharm. Bull.*, **49(11)**: 1477-1478 (2001).
4. G.P.Perdue and R.N. Blomster. South American Plants III: Isolation of Fulvoplumierin from *Himatanthus sucuuba* (M. Arg.) Woodson (Apocynaceae). *J. Pharm. Sci.* **67(9)**: 1322-1323 (1978).
5. J.R.A. Silva, C.M. Rezende, A.C. Pinto, M.L.B. Pinheiro, M.C. Cordeiro, E. Tamborini, C.M. Young and V.S.Bolzani. Triterpenic esters from *Himatanthus sucuuba* (Spruce) Woodson, *Quim. Nova* **21(6)**: 702-704 (1998).
6. Y. Endo, H. Hayashi, T. Sato, M. Maruno, T. Ohta and S. Nozoe. Confluent Acid and 2'-O-Methylperlatoic Acid, Monoamine Oxidase B Inhibitors in a Brazilian Plant, *Himatanthus sucuuba*. *Chem. Pharm. Bull.* **42(6)**: 1198-1201 (1994).
7. M.S. Siqueira. *Estudo fitoquímico e farmacológico das raízes de Himatanthus sucuuba (Apocynaceae)*. MSc Thesis, (Universidade Federal do Amazonas, Manaus, Brazil, 1999), pp. 1-120.
8. J.R.A. Silva. *Contribuição ao estudo do látex de Himatanthus sucuuba: aspectos químicos e farmacológicos*. PhD Thesis, (Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, 2000), pp. 1-261.
9. M. Zimmerman. Ethical Guidelines for investigation of experimental pain in conscious animals. *Pain* **16(2)**:109-110 (1983).
10. T. Mosmann. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Meth.* **65(1-2)**: 55-63 (1983).
11. J.E. Lima, A.L.F. Sampaio, M.G.M.O. Henriques, T.C.B. Fidalgo. Lymphocyte activation and cytokine production by *Pisum sativum* agglutinin

- (PSA) *in vivo* and *in vitro*. *Immunopharmacol.* **41**: 147-155 (1999).
12. L.C. Green, D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok and S.R. Tannenbaum. Analysis of nitrate, nitrite, and [<sup>15</sup>N]Nitrogen in biological fluids. *Anal. Biochem.* **126(1)**:131-8, (1982).
  13. C.D. Mills, K. Kincaid, J.M. Alt, M.J. Heilman and A.M. Hill. M-1/M-2 macrophages and the Th1/Th2 paradigm, *J. Immunol.* **164(12)**: 6166-6123 (2000).
  14. R.E. Schultes. De plantis toxicariis e mundo novo tropicale commentationes. XIX. Biodynamic apocynaceous plants of the northwest Amazon. *J. Ethnopharmacol.* **1(2)**: 165-192 (1979).
  15. V.T. Mathad, K. Raj, A.P. Bhaduri, R. Sahai, A. Puri, L.M. Tripathi and V.M.L. Srivastava. *Bioorg. Studies on the Profile of Immunostimulant Activities of Modified Iridoid Glycosides. Med. Chem.* **6(5)**: 605-611 (1998).
  16. H-J. Jeong, H-Na Koo, H-J. Na, M-S. Kim, S-H. Hong, J-W. Eom, K-S Kim, T.Y. Shin and H-M. Kim. Inhibition of the TNF- $\alpha$  and IL-6 Production by Aucubin through Blockade of NF- $\kappa$ B Activation in RBL-2H3 Mast Cells. *Cytokine* **18(5)**: 252-259 (2002).
  17. W-L. Chang, H-Y. Wang, L-S. Shi, J-H. Lai and H-C. Lin. Immunosuppressive Iridoids from the Fruits of *Gardenia jasminoides*. *J. Nat. Prod.* **68(11)**: 1683-1685 (2005).
  18. G. Albers-Schönberg, H. Schmid. Über die Struktur von Plumericin, Isoplumericin,  $\beta$ -Dihydroplumericin und der  $\beta$ -Dihydroplumericinsäure. *Helv. Chim. Acta* **44**: 1447-1473 (1961).
  19. F. Abe, R.F. Chen and T. Yamauchi. *Chem. Pharm. Bull.* **36(8)**: 2784-2789 (1988).

#### CRN SEEKS BOTANICALS EXPERT

—Leading Association to Increase Emphasis on Science Behind Supplements—

The Council for Responsible Nutrition (CRN), a Washington, D.C.-based trade association representing dietary supplement industry ingredient suppliers and manufacturers, founded in 1973, is in search of a Botanicals Expert to increase the awareness of the evolving botanical research base that supports an appropriate role for plant-based dietary supplements and to use that science as the basis for influencing and responding to regulatory and legislative initiatives, industry activities and media coverage of botanical and herbal dietary supplements.

The ideal candidate will: provide scientific expertise in botany, pharmacognosy and toxicology for evaluating issues related to current practices and viewpoints of opinion leaders; evaluate proposed government or industry actions and recommend appropriate CRN positions and actions; build rapport with the botanical and natural products chemists' communities on issues and identify experts who can provide assistance to CRN on matters of concern. The ideal candidate will exercise ingenuity and initiative in developing CRN position statements on botanical and herbal product issues and submitting comments to government agencies on proposed regulatory actions that impact plant-based dietary supplements. The new scientist will also ensure that CRN's legislative or other policy positions are based on a sound scientific rationale along with analyzing emerging research results and attempts to put new data into perspective for CRN members, for regulators and legislators, for the media, and for the public.

The new scientist will support an appropriate role for plant-based dietary supplements with a variety of audiences including regulators, legislators and the media. The new scientist will be expected to: help increase the awareness of the evolving research base that supports botanical supplements; represent CRN before regulatory agencies and government institutions such as the Food and Drug Administration (FDA), the Office of Dietary Supplements, the National Center for Complementary & Alternative Medicine, and the Federal Trade Commission; interact with members of Congress in hearings and briefings; be a resource for media inquiries regarding botanical research or other issues; organize and provide staff support for CRN member working groups on botanical or regulatory issues; and serve on industry-related committees (e.g. AOAC, etc.). The new scientist will also organize botanical/regulatory components of *The Conference: CRN's Annual Symposium on Dietary Supplements*, *The Workshop: CRN's Day of Science* and other CRN-sponsored events.

The ideal candidate will possess a Doctor of Philosophy in Plant Biology, Organic Chemistry, Botany, Pharmacognosy, Toxicology or Natural Medicine or related discipline and minimum of five years relevant experience in the field of Botanical Science or Natural Products Chemistry. He or she must be familiar with current federal regulation of dietary supplements and previous dietary supplement industry or FDA experience is preferred. In addition, excellent written and oral communication skills are required with some history of publication in peer-reviewed journals. Must have the ability to multi-task and work in collegial, fast-paced, fluid environment as a team player with other staff and with member company representatives.

Interested candidates should visit <http://www.crnusa.org> for a full job description or contact Marie Hooper, CRN's Director of Administration and Human Resources, at [mhooper@crnusa.org](mailto:mhooper@crnusa.org) for more information.