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Full Length Research Paper

Cytotoxicity and antibacterial studies of iridoids and phenolic compounds isolated from the latex of *Himatanthus sucuuba*

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The latex of *Himatanthus sucuuba* (Spruce) Woodson, used popularly in the Amazon for the treatment of tumors, gastritis, inflammations and infections, was evaluated for cytotoxicity and antibacterial activities. The iridoid lactones, plumericin and isoplumericin were isolated from latex by bioassay fractionation and were found to be associated with DNA damage. Gallic acid exhibited the highest antimicrobial activity among the phenolic compounds isolated from the aqueous fraction. The compounds associated to cytotoxicity and antimicrobial activities could be responsible to the effects of this species used in traditional medicine.

Key words: *Himatanthus sucuuba*, iridoids, phenolics, cytotoxicity, antibacterial.

INTRODUCTION

In Brazil, there are about 90 genera and 850 species of the *Apocynaceae* family, divided into various formations (Souza and Lorenzi, 2005). Some species of this family such as *Himatanthus sucuuba* (Spruce) Woodson, are of therapeutic value and have a long history of use in folk medicine of Brazil. A recent review described the morphology, chemistry, pharmacology and ethnopharmacology of this species which is popularly known as *sucuuba, janaguba* and *janauba,* amongst other common names (Amaral et al., 2007).

The latex and bark of this plant are mainly used in folk medicine for the treatment of ulcers, infections, inflamematory processes and tumors (Van Den Berg, 1982; Perdue and Blomster, 1978; Schultes, 1979; Bourdy et al., 2000; Villegas et al., 1997). Previous studies of *H. sucuuba* bark and latex have shown the antifungal and antiprotozoal activity of iridoid lactones (Silva et al., 1998; Castillo et al., 2007) and the anti-inflammatory activity of triterpenoids (Miranda et al., 2000). In this study, iridoids and phenolic compounds were isolated for the first time from the latex of this species. Bioassays-guided fractionations were used to identify the compounds responsible for the antitumor and antibacterial activities of the latex from this plant.

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Abbreviations: TLC, Thin layer chromatography; **MIC,** minimum inhibitory concentration; **IC_{12},** concentration (µg/ml) is required to produce a zone of inhibition of 12 mm; **MeOH,** methanol; **EtOAc,** ethyl ethanoate; ***n*-BuOH,** n-butanol; **GC/MS,** gas chromatography coupled with mass spectrophometer; **NMR,** nuclear magnetic resonance; **MPLC,** medium pressure liquid chromatography.

MATERIALS AND METHODS

Plant material

Collection

Plant samples were collected in Santarém city, Pará State and a

voucher specimen (number 5436) was deposited at the Herbarium of the Federal University of Amazonas, Manaus, AM, Brazil.

Obtaining Latex

The latex was removed from some cuts in the bark with the help of a damp sponge. The material was collected in a bottle and then stored at 4°C.

Extraction of latex and isolation of the compounds

Liquid-liquid extraction was performed on the latex of *H. sucuuba* as described previously (Barreto et al., 2007). The hexane fraction (12.0 g) was separated on a Sephadex LH-20 column, and eluted successively with hexane, dichloromethane (CH_2Cl_2) and methanol (MeOH). The hexane fraction was also subjected to preparative thin layer chromatography (TLC) (Hexane: EtOAc, 6:4) in order to isolate a mixture of plumericin and isoplumericin (8:2, 5.0 mg, 0.04% of the extract). Plumericin and isoplumericin were identified by spectroscopic methods (NMR and GC/MS) and by comparison with literature data (Trost et al., 1986; Abdel-Kader et al., 1997). The aqueous fraction was successively partitioned with $CHCl_3$ and after lyophilized. This powdered fraction (2.4 g) was submitted to reverse phase C-18 medium pressure liquid chromatography (MPLC) with water up to water/MeOH (1:1), flow 6 ml/min. 26 fractions of 40 ml were obtained and combined in two principal fractions: A (0.63 g) and B (1.8 g). The fraction A was chromatographed on Sephadex LH-20 column with water/MeOH (7:3) as eluent, for the isolation of catechol (39.5 mg). The fraction B was subjected to Sephadex LH-20 chromatography with MeOH as eluent and also on HPLC column (twice) RP-18 (Shimadzu, 45 x 250 mm, 10 µm particle size) and water/MeOH (6:4) as eluent, for the isolation of three compounds: gallic acid (16.6 mg), myricetrin (3.9 mg) and quercitrin (4.7 mg). The compounds were identified by spectroscopic methods and by comparison with data in the literature (Chanwitheesuk et al., 2007; Mabry et al., 1970). The flavonoids were also identified by UV spectroscopy using shift reagents (Mabry et al., 1970).

Antibacterial activity

Microorganisms

The microorganisms used in this study were *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus haemolyticus* (ATCC 2737), *Proteus mirabilis* (MRSA), *Shigella sonnei* (ATCC 25931), *Salmonella typhimurium* (ATCC 13311) and *Escherichia coli* (ATCC 25922). All MRSA bacteria were identified by traditional biochemical tests and according to National Committee for Clinical Laboratory Standards (NCCLS) (Machado et al., 2003).

Disc diffusion method

The antibacterial activity of the samples was determined using the disc diffusion method (Machado et al., 2003). Varying amounts of sample (25, 50, 100, 250, 500 and 1000 µg) were applied to sterile filter paper discs (6 mm in diameter). Antibacterial activity was determined by measuring the diameter (d) of the inhibition zone formed around the disk (considered 10 - 18 mm).

Minimum inhibitory concentration (MIC)

Culture conditions, media preparation and minimum inhibitory concentration (MIC) assays were undertaken according to methodology adopted from National Council for Clinical Laboratory Standards (1993). Concentrations ranging from 15.5 to 500 µg/ml were used for each sample. The MIC is defined as the lowest concentration of the sample at which visible growth of the microorganism is completely inhibited.

Mechanism-based yeast bioassay for DNA damaging activity

The assay was evaluated using genetically engineered mutants of yeast of *Saccharomyces cerevisiae* as described previously (Gunatilaka et al., 1992). The IC_{12} is defined as the concentration in µg/ml that is required to inhibit growth over a 12 mm diameter in a 100 µl well after 48 h incubation at 37°C. Camptothecin (RS188N and rad52) and streptonigrin (rad52.top1) were used as control drugs.

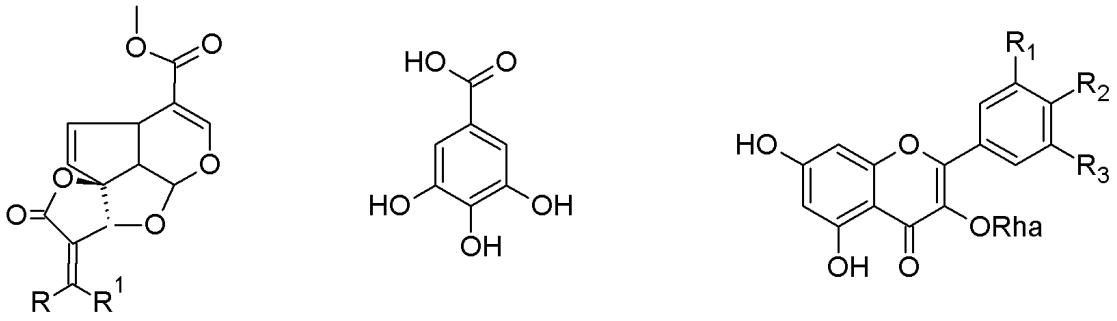
RESULTS AND DISCUSSION

Bioassay fractionation of the latex extract was used to identify substances that could be related to the ethnopharmacological use of *H. sucuuba*. The fractionation was performed as follows: *n*-BuOH was added to the crude latex of this species to promote polyisoprene precipitation. The organic (*n*-BuOH) and aqueous fractions were separated, evaporated and assayed against mutant strains of *S. cerevisae.* This assay utilizes DNA repair or recombination deficient mutants of the yeast *S. cerevisiae* for the screening of compounds, which induce DNA damage. This mechanism-based yeast assay depends on the different responses of DNA repair-deficient and repair proficient yeast (*S. cerevisiae*) strains to the sample. The major DNA repair pathway is RAD52 pathway associated with the repair of double strand break and meiotic recombination (Gunatilaka et al., 1994). A mutant rad52 repair-deficient strain, rad52.top1, with the additional deletion of the DNA topoisomerase I gene is also available and can detect agents that produce DNA damage specifically by interacting with DNA topoisomerase (Gunatilaka et al., 1994). An extract is considered active if it shows selective activity against one or more repair-deficient yeasts and has an IC_{12} less than 2000 µg/ml (Gunatilaka et al., 1994). The results are shown in Table 1. Only the *n*-BuOH fraction exhibited activity with an IC_{12} = 1641 µg/ml (rad52.top1). The *n*-BuOH fraction was then partitioned with a solution of MeOH (80%) and hexane. Enhanced activity was exhibited by the hexane fraction, with an IC_{12} = 542 µg/ml (rad52.top1). Column and preparative TLC were performed on the hexane fraction and the activity of isolated compounds was determined. The iridoid lactones (plumericin (1) and isoplumericin (2) showed relevant activity against rad52.top1 (IC_{12} = 32.8 µg/ml). The activity of the iridoids against rad52 yeast strains was approximately four times lower (IC_{12} = 112.3 µg/ml) and nine times less effective against repair-proficient strains (RS188N, IC_{12} = 289.0 µg/ml) than against rad52.top1. These results indicated, for the first time, that this mixture

Table 1. Activity of the fractions and iridoids from *H. sucuuba* against mutant strains of *S. cerevisae*.

Test samples	Zone of inhibition (mm)			IC_{12} (µg/ml)	
	RS188N	rad52	rad52.top1	rad52	rad52.top1
Aqueous	11.0	10.0	10.5	7160	4787
n-BuOH	11.0	12.5	13.0	1910	1641
MeOH (80%)	8.0	9.0	10.0	3104	2225
Hexane	10.0	12.0	15.5	1434	542
Plumericin and Isoplumericin	19.0	23.0	29.0	112.3	32.8

IC_{12}, the concentration (µg/ml) is required to produce a zone of inhibition of 12 mm.



1 Plumericin R = H; R^1 = CH_3

2 Isoplumericin R = CH_3; R^1 = H

3 Gallic acid

4 Myricetrin R_1=R_2=R_3=OH

5 Quercitrin R_1=R_2=OH, R_3=H

Figure 1. Chemical structures of phenolic and iridoids compounds isolated from *H. sucuuba*.

of iridoids could be associated with the DNA damaging activity of the latex extract and that it could be topoisomerase II inhibitor. The observed activity is important not only because it corroborates the ethnopharmacological use of the species, but also because it indicates a possible synergistic or additive action due to the two iridoids since plumericin alone results in less cytotoxicity activity (IC_{12} = 70 µg/ml, rad52.top1) (Wood et al., 2001) than that observed in mixture (IC_{12} = 32.8 µg/ml) .

The antimicrobial activities of the hexane, chloroform and aqueous fractions of *H. sucuuba* latex were tested using the disc diffusion method with seven microorganisms and only the last fraction exhibited activity (d = 17 mm). The antimicrobial activity of the aqueous fraction was observed against five bacteria at a concentration of 500 µg/ml (*Proteus mirabilis* and *Escherichia coli*) and 350 µg/ml (*S. aureus, S. epidermis* and *S. haemolyticus*). Among the phenolic compounds isolated from the aqueous fraction, gallic acid (3), a hydroxybenzoic acid, showed the most potent antimicrobial activity (MIC values: 31 µg/ml for *S. aureus* and *S. epidermidis,* 62 µg/ml for *P. mirabilis* and *S. haemolyticus* and 125 µg/ml for *E. coli*). Gallic acid has been previously reported to possess anti-inflammatory, antifungal and antibacterial activities (Chanwitheesuk et al., 2007). The flavonoids, myricetrin (4) and quercitrin (5), were also isolated from the aqueous fraction, but they did not show antibacterial activity at any of the concentrations tested. The chemical structures of iridoids and phenolic compounds 1 - 5 are illustrated in Figure 1.

Conclusion

The results of this phytochemical work on the isolation and the identification of the active compounds of the latex of *H. sucuuba* provided scientific validation for the popular use of this species, and also confirmed a better activity of iridoids mixture on the DNA in a new plant material source, which is easier to obtain and less difficult to the plant than the bark removal.

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