

Callus culture of *Aspidosperma ramiflorum* Muell. Arg.: growth and alkaloid production

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ABSTRACT. Callus culture of *Aspidosperma ramiflorum* was established in Murashige and Skoog medium. Callus were supplemented with 1 mg.L⁻¹ 2,4-dichlorophenoxyacetic acid, 1.5 mg.L⁻¹ benzylaminopurine, 30 g L⁻¹ sucrose and 10 g L⁻¹ agar. Cultures accumulated the same major alkaloids, ramiflorine A and ramiflorine B, present in the stem bark of the parent plant. The alkaloid contents of 10-methoxy-geissoschizol, ramiflorine A and ramiflorine B, of bark and callus cultures were quantitatively compared by HPLC.

Key words: Apocynaceae, *Aspidosperma ramiflorum*, monoterpenoid alkaloids, ramiflorine A, ramiflorine B, tissue culture.

RESUMO. Cultura de calos de *Aspidosperma ramiflorum* Muell. Arg.: crescimento e produção de alcalóides A cultura de calos de *Aspidosperma ramiflorum* foi estabelecida em meio MS. Os calos foram suplementados com 1mg.L⁻¹ de 2,4-dichlorophenoxyacetic ácido, 1.5 mg.L⁻¹ benzylaminopurine, 30 g.L⁻¹ de sacarose e 10 g.L⁻¹ de agar. As culturas acumularam os mesmos alcalóides principais, como ramiflorina A e ramiflorina B, presentes nos galhos da planta adulta. Os conteúdo alcaloídico de 10-metoxi-geissoschizol, ramiflorina A e ramiflorina B, das cascas do tronco e das culturas de calos foram comparados quantitativamente por HPLC.

Palavras-chave: Apocynaceae, *Aspidosperma ramiflorum*, alcalóides monoterpenóides, ramiflorina A, ramiflorina B, cultura de tecidos.

Species of *Aspidosperma* are a source of bioactive indole alkaloids. However, according to one study, *in vitro* cultures have not produced alkaloids of pharmaceutical interest (Stöckigt *et al.*, 1994). *Aspidosperma ramiflorum* Muell. Arg., commonly known as 'guatambu', is a tree which reaches up to 12-30m in height, native to forests in south-eastern Brazil (Lorenzi, 1992). Some indole alkaloids have been previously isolated from stem bark (Reis *et al.*, 1996), and the alkaloid extract of bark showed antimicrobial activity against gram-negative bacteria (Oliveira, 1999). *A. ramiflorum*, a heavy tree and a long life cycle, is seriously threatened with extinction. It is collected from wild forests rather than grown in plantations, making the drug supply rather uncertain. Therefore many attempts have been made to establish cell culture systems of these rare plants, which would allow a continuous supply of plant material (Verpoorte *et al.*, 1993). The aim of this study has been to investigate the potential of

obtaining pharmaceutically important metabolites from *in vitro* cultures of *Aspidosperma ramiflorum*.

Material and methods

The procedure to obtain *A. ramiflorum* Muell. Arg. callus culture started with twenty-day-old seedlings obtained from certified seeds acquired at IPEF-ESALQ-USP (Piracicaba, Brazil). Leaves and hypocotyls of the seedlings were cut into small pieces (5-8 mm), surfaces sterilised with 1% NaOCl for 10 min and then washed three times with sterile water. Callus tissue growth was induced on Murashige and Skoog medium-MS (Murashige and Skoog, 1962), supplemented with agar (10 g.L⁻¹), sucrose (30 g.L⁻¹), 2,4 dichlorophenoxyacetic acid (2,4D - 1 mg.L⁻¹) and benzylaminopurine (BA - 1,5 mg.L⁻¹), and agar as solidifier (1 g.L⁻¹). After 4 weeks, callus material that had developed at the edge of the cutting was excised and transferred to fresh medium. Callus was incubated for 16 hours daily

photoperiods (2000 lux from a cool white fluorescent tubes) at 25°C and subcultured every four weeks. After a period of five subcultures, a brown friable callus (C1) and light green to yellowish coloured friable callus (C2) were obtained.

The extraction and purification of alkaloids from callus culture were carried out according Verpoorte *et al.* (1998), with some modifications. Freeze-dried callus material was blended in an Ultra-Turax with 5% acetic acid, and cells were removed by filtration. The acid solution was adjusted to pH 9.0 with NH₄OH and extracted with CH₂Cl₂. The organic layers were washed with water and concentrated under vacuum to yield the crude alkaloid fraction that was then weighed to determine total alkaloid production. Fraction was further analysed and compared with the alkaloids isolated from the stem bark of the plant using TLC run on silica gel GF₂₅₄ developed with CHCl₃:AcOEt: triethylamine (49,5:49,5:1,0) in an NH₃ atmosphere. For HPLC analysis, the crude extract was dissolved in CH₂Cl₂:MeOH (80:20) and 10 µL were injected onto a Waters µ-Bondapak RP-18 (reverse phase, 4.6 mm x 250 mm) column at 40°C. Solvent A was 100 mmol.L⁻¹ ammonium formate in 0.12% octanesulfonic acid (v/v), formic acid and acetonitrile (88:4:8, v/v), while solvent B of 100 mmol L⁻¹ aqueous ammonium formate containing 0.12 % octanesulfonic acid (v/v)/ formic acid / acetonitrile (64: 4: 32, v/v). The separation was carried out by mixture of solvent A and a progressively increasing amount of B (0; 10; 40; 90 and 100 %) for 60 minutes. The flow rate was 1.3 mL.min⁻¹. The effluent was monitored with photodiode-array detector with windows at 222 nm and 254 nm and mass spectra analyses.

Results and discussion

The callus cultures accumulated two of the major alkaloids reported for the intact plant (Reis *et al.*, 1996), namely ramiflorine A and ramiflorine B (Figure 1). Quantification of the different alkaloids in intact plant and callus culture are shown in Table 1. The HPLC chromatograms of standard alkaloids isolated from *A. ramiflorum* intact plant extract and from callus cultures extracts are shown in Figure 2.

The callus culture C1 accumulates less alkaloids than the parent plant (15%) and C2 showed only traces of alkaloids. In callus tissue cultures of several species the development of certain levels of

differentiation has been reported to be important for the successful production of phytochemicals by cell cultures (Verpoorte *et al.*, 1993).

There are many examples in the literature demonstrating a relation between differentiation and secondary metabolites accumulation. Hiraoka and Tabata (1974) showed that alkaloid concentration in mature plant and callus tissue of *Datura innoxia* was 0.10% and 0.01% of dry weight, respectively (10:1). The present study showed that in *A. ramiflorum* the ratio of alkaloid concentration between mature plants and morphologically undifferentiated cells of callus was 4:1 (C1) and 6:1 (C2) (Table 1). The mass obtained in total alkaloid extract (Table 1) was different from the total mass sum of the measured alkaloids for HPLC. This fact might have occurred due to a retention of other alkaloids, other than the 10-methoxy-geissoschizol, (10 MG) ramiflorine A (RA) and ramiflorine B (RB) types, in the HPLC column; they may be different compounds, non-alkaloids types, carried jointly in the extraction process.

Table 1. Alkaloid yields (% W/DW) in stem bark and in *in vitro* cultures of *Aspidosperma ramiflorum*

Sample*	Total alkaloid yield**	10MG	RA	RB
Stem	1.160	0.07	0.150	0.270
Callus C1	0.294	absent	0.008	0.006
Callus C2	0.190	absent	traces	traces

* Alkaloid determinations were made from callus after 30 days of growth. ** Percentage values, related to the dried material

We may conclude that in spite of the lowest concentration of alkaloids obtained from callus, the culture medium and the cultivation conditions established in this work assure the obtaining of alkaloids from *A. ramiflorum* with antimicrobial activity. The optimisation of these conditions, through further investigations regarding changes in the culture medium composition, the addition of precursors to the culture medium, stress factors, treatment of elicitors and immobilisation of cells, will be accomplished and will increase production of alkaloids.

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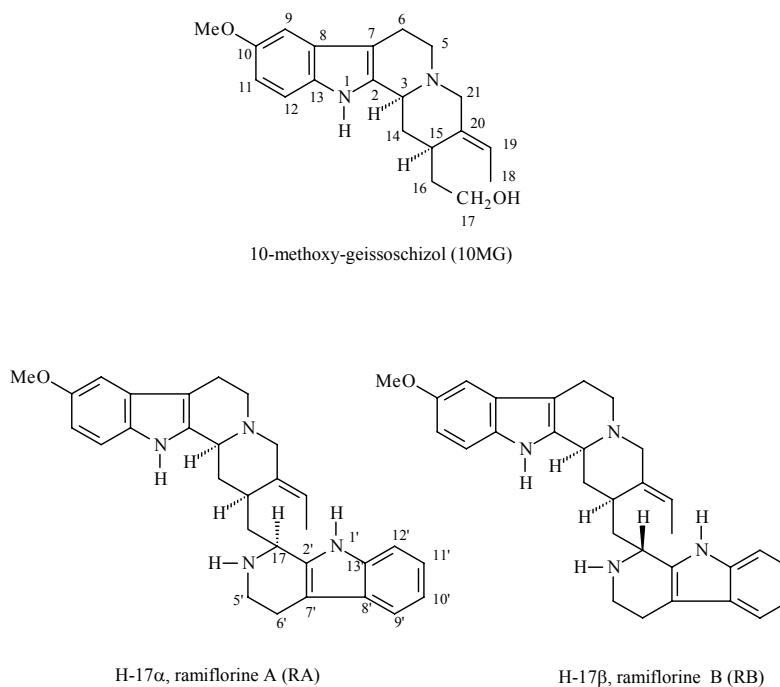


Figure 1. The structures of major alkaloids isolated from stem bark of *Aspidosperma ramiflorum* Mull. Arg.

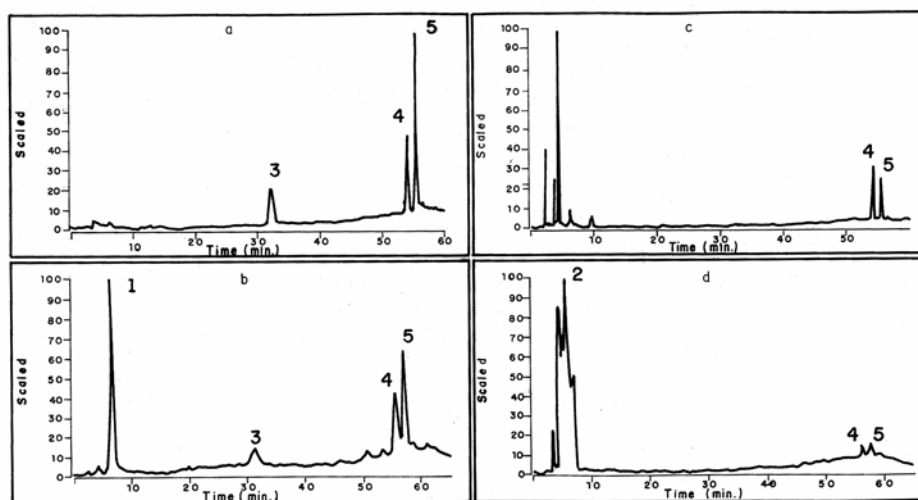


Figure 2. HPLC chromatograms of (a)- standard alkaloids isolated from *A. ramiflorum*; (b)- intact plant extract; (c)- callus extract C1; (d)- callus extract C2. 1- tryptamine; 2- tryptophan; 3 - 10-methoxy-geissoschizol, 4- ramiflorine A and 5- ramiflorine B

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