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Original article

Seasonal influence and cytotoxicity of extracts, fractions and major compounds from *Allamanda schottii*

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A B S T R A C T

The aim of this research was to evaluate the fractions obtained from the leaf, stem and roots of *Allamanda schottii*, Pohl, Apocynaceae, responsible for the cytotoxicity, using several cell lines. Cytotoxicity was correlated with the season the part of the plant, and the major compounds were assessed. The ethanol extracts of leaves, stems and roots obtained at different seasons were evaluated in the human erythromyeloblastoid leukemia cell line (K562). Subsequently the ethanol extracts and dichloromethane fractions collected in winter were evaluated in mouse fibroblast cell line (*Mus musculus*) (L929), cervix adenocarcinoma (HeLa), human pre-B leukemia (Nalm6), as well as K562 cell line. The compounds plumericin, plumieride and ursolic acid isolated from ethanol extracts of the stems were evaluated in the same cell lines, as well as on breast adenocarcinoma cell line (MCF-7), and *Mus musculus* skin melanoma cell line (B16F10). The chromatographic profiles of the dichloromethane fractions were obtained by high performance liquid chromatography. The results revealed that the season during which *A. schottii* was collected, and the part of the plant analyzed, influence the cytotoxicity on the K562 cells tested. On the other hand the dichloromethane fractions, mainly from the stems and roots, are responsible for the cytoxicity on the cells tested. These results may be associated with the seasonal variation of plumericin in these parts of the plant. This information is in accordance with the HPLC analysis. The results clearly show the potential for the phytotherapeutic use of this species, and suggest that the cytotoxic activity observed may be due to the presence of plumericin, or to minor compounds not yet identified. The seasonal influence on the production of secondary metabolites was verified.

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Introduction

The search for substances with antitumor potential has significantly expanded the study of natural products. Plants have provided an extraordinary source of new compounds with therapeutic activity and the secondary metabolites represents an efficient alternative for obtaining new drugs. Plants have an important role as a source of antitumoral agents, and over 67% of anti-cancer drugs currently in use are derived from natural sources (Cragg and Newman, 2012). It is important to note that the production of these metabolites in plants is related to adaptive processes in response to factors such as climatic differences, stress levels, water availability, light, and natural predators (Gobbo-Neto and Lopes, 2007). The amount and nature of these compounds is not constant throughout the year, and they are not produced by all parts of the plant. These aspects are important considerations to guide studies that aim to the isolation of natural products. In addition, bio-directed studies could potentially reduce the number of stages required to identify active compounds in plants.

Allamanda, Apocynaceae, is a genus of climbing shrubs, comprised by ten species that are distributed throughout Brazil. These species have been used mainly as ornamental plants, for instance, *A. cathartica* L., *A. schottii* Pohl and *A. blanchetii* A.DC. (Endress and Bruyns, 2000; Gonçalves and Lorenzi, 2007).

The ethnopharmacological use of *Allamanda* genus is related to its latex as a scabicide and in lice control. The infusion of the flowers is purgative and is also used as an anti-helminthic, and as a topical agent it is used to reduce hyperthermia. The stems are used against hepatic tumors (Correa Jr et al., 1984; Moreira, 1985; Rahayu, 2001; Lorenzi and Matos, 2002). *Allamanda cathartica* L. has long been used for different purposes, including the treatment of hepatic tumors, as it has low acute toxicity in rats > 30g/kg (Lopes et al., 2009).

Plants belonging to the *Allamanda* genus are known to produce iridoids, mainly plumericin, which has been documented to have antineoplastic, antileukemic, anti-inflammatory, antimicrobial, and antileishmanial activities (Anderson et al., 1988; Hamburger et al., 1991; Castillo et al., 2007; Kuete and Efferth, 2011).

The iridoid glycoside, plumieride has gained interest because of its antidermatophytic and antitumor activities (Tiwari et al., 2002; Dobhal et al., 2004). In addition to iridoids, other classes of secondary metabolites produced by this genus are terpenes, flavonoids and coumarins (Anderson et al., 1988; Ganapaty et al., 1988; Kardono et al. 1990; Schmidt et al., 2006).

The ethanol extract of the roots of *A. cathartica* has been shown to be active in vivo against leukemic cells P-388 and *in vitro* against cells derived from human nasopharyngeal carcinoma (KB) (Kupchan et al., 1974). Our group has previously observed an anti-proliferative effect of the ethanol extract from different parts of *A. schottii* and *A. blanchettii* (leaf, stem and root) on human erythromyeloblastoid leukemic cells (K562), as well as on bone marrow endothelial cells (BMEC) and human umbilical cord endothelial cells (HUVEC). The results demonstrated the cytostatic and cytotoxic effect of the ethanol extract of *A. schottii* roots, at concentrations of < 80 and < 8 µg/ml, respectively. The cytotoxic effects of the leaf and stem extracts were at least ten times lower than these values (Schmidt et al., 2006). Therefore it is of great importance to investigate the antitumoral effects of this genus.

In this context the aim of this research was to evaluate the extracts and fractions obtained from the leaf, stem and root of *A. schottii* responsible for cytotoxic activities, using several cell lines. The cytotoxicity to K652 cells was analyzed in relation to the season of the year and the part of the plant used, and the major constituents were elucidated.

Materials and methods

Plant material

Different parts (leaves, stems, and roots) of *Allamanda schottii* Pohl, Apocynaceae, were collected in Blumenau, a city in the state of Santa Catarina, Brazil, on March 23, June 19, September 17 and December 22, 2006 corresponding to the end of summer, autumn, winter and spring, respectively. The plant material was classified by Prof. Oscar Benigno Iza (Universidade do Vale do Itajaí), and voucher specimens were deposited at the Barbosa Rodrigues Herbarium (HBR, Itajaí), under identifier HBR 52525. All plants analyzed were planted on the same day under the same environment, and collected after four years. After plant collection, each part was separately dried at room temperature.

Preparation of extracts and fractions

The dried vegetal material (100 g) of each part (leaves, stems and roots) of *A. schottii* collected at the end of each season was macerated with 95% ethanol at room temperature, for seven days. Solvent removal was carried out under reduced pressure at temperatures below 45°C, until the desired concentrations were achieved, in order to obtain the ethanol extracts. A fraction of each extract was dissolved separately in methanol and water (90:10) and was successively partitioned using *n*-hexane, dichloromethane and ethyl acetate to obtain the respective fractions, after the removal of solvents. The samples were stored under refrigeration and protected from light until analysis.

Isolation and identification of components

The ethanol extract of the stems (15.1 g) collected at the end of winter (September) of 2006 was chromatographed in a silica-gel column with *n*-hexane, ethyl acetate, and ethanol as solvents.

The fraction eluted with *n*-hexane and ethyl acetate (8:2) yielded, after crystallization, a mixture of β-sitosterol and stigmasterol (36.5 mg). The fraction eluted with *n*-hexane and ethyl acetate (7:3) yielded plumericin (15.2 mg). Elution with *n*-hexane and ethyl acetate at a ratio of 6.5:3.5 yielded ursolic acid (62.4 mg), and at a ratio of 6:4 the sterol 3-*O*-β-glucopyranosyl-sitosterol (9.2 mg) was obtained. The fraction eluted with ethyl acetate and ethanol (8:2) yielded plumieride (39.4 mg). In the subsequent fractions mixtures of sugars were isolated.

All the compounds were identified based on their spectral data, by comparison with the literature (Malheiros et al. 1997, Schmidt et al., 2006) and direct comparison with original samples.

LC Analysis

The analysis was conducted using a high performance liquid chromatography (HPLC) system (Waters) equipped with a 600-F pump and 717 plus autosampler with an in-line degasser (AF) and coupled to a UV-Vis detector (PDA 2996). A C18 column (25 cm, 4.6 mm i.d.; 0.5 µm film thickness and 100 A) was used (Luna, Phenomenex) at 25°C. The gradient system used consisted of a mixture of solvents A (acetonitrile 10%), B (methanol) and C (water acidified with phosphoric acid at pH 3.54), starting with 10% B and 80% C at a flow rate of 0.8 ml/min. The linear solvent gradient increased to 20% B and 70% C in 10 min; 35% B and 55% C in 10 min; 50% B and 40% C in 15 min and 70% B and 20% C in 20 min maintained until the end of the run. After each analysis, 45% of mobile phase B was pumped and maintained for 10 min to re-equilibrate the system for baseline stability. UV-Vis spectra were recorded at a wavelength range of 200-400 nm (detection wavelength was 230 nm). The solvents used were HPLC grade (high purity), filtered (0.2 µm, Schleicher & Schuell, Maidstone, Kent, UK) and degassed by sonication before use. The samples of the dichloromethane and ethyl acetate fraction (1.0 mg/ml), and standard plumieride and plumericin (100 µg/ml) were dissolved using a mixture of the solvents A:B:C at proportion of 10:10:80. Each solution was then filtered through a 0.45 µm membrane filter and 20 µl aliquots were analyzed in triplicate.

Cytotoxicity assay

Cell viability was assessed by performing 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Mosmann, 1983) assays to determine the IC_{50} concentrations of the studied agents on the probit model (Finney, 1971). In these assays, non-tumoral L929 fibroblast cells (*Mus musculus*), B16F10 melanoma (*Mus musculus*), HeLa from cervix adenocarcinoma and mammary carcinoma cells MCF-7 (Homo sapiens), all from the Cell Bank of Rio de Janeiro-BCRJ, were plated in 96-well plates. Human erythromyeloblastoid leukemia (K562) and human pre-B leukemia (Nalm6) cell lines were plated at a density of 10,000 cells per well. The cells were treated with the ethanol extracts of the roots, stems and leaves, with the *n*-hexane, dichloromethane and ethyl acetate fractions, and with the isolated compounds plumericin, ursolic acid and plumeiride in MEM culture medium (Invitrogen/GIBCO) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% antibiotic PSN (penicillin, streptomycin and neomycin) solution (Invitrogen/GIBCO). Cells were cultured in 96-well plates, and grown for 48 h at 37°C and 5% CO_2. For negative controls (100% viability) the cells were incubated with the maximum amount of the diluent and MEM used. Paclitaxel (antitumor, melanoma and adenocarcinomas), Gleevec (K562) and vincristine (Nalm-6) were used as positive controls. The concentration leading to 50% inhibition of viability (IC_{50}) after 48 h was determined by measuring the absorbance at wavelength of 570 nm as an indicator of MTT reductase activity (Mosmann, 1983), using an Asys Hitech microplate reader. The viability of the treated cells was expressed as a percentage relative to the viability of control vehicle-treated cells. Each experiment was performed in triplicate and independently repeated at least three times.

Statistical analysis

The results are expressed as mean values ± SD from separate experiments. Group differences were determined by analysis of variance (ANOVA). Statistically significant differences were indicated by ANOVA, and the values were compared using the Tukey's test. The differences were considered statistically significant at $p < 0.05$.

Results and discussion

Selection of the cytotoxic extract and fractions

To evaluate the cytotoxic activity of *Allamanda schottii* Pohl, Apocynaceae, human erythromyeloblastoid leukemia (K562), human pre-B leukemia (Nalm6), cervix adenocarcinoma (HeLa) cell lines and non-tumoral murine fibroblast (L929), were incubated with the ethanol extracts of the roots, stems and leaves collected in September, which is the end of winter in Brazil. After 48 h, cell viability was determined by the MTT assay. The IC_{50} values (µg/ml) are listed in Table 1. The values of total degree of inhibition to extracts established by Fouche et al. (2008) were used as a reference for data analysis of IC_{50}.

The ethanol extract of the roots had an IC_{50} value of 11.20 ± 3.50 µg/ml for the HeLa cells. The stem and leaf extracts were most active against leukemic cell Nalm6, with IC_{50} values of 30.00 ± 4.00 and 30.80 ± 1.10 µg/ml, respectively. The stem and leaves extracts were not active against K562 cells (IC_{50} > 100 µg/ml) nor the non-tumoral cells (L929).

These results indicate that cell lines differ in their sensitivity toward the same plant extract. Studies carried out by Schmidt et al. (2006) observed a more pronounced cytostatic and cytotoxic activity of the ethanol extract of roots from *A. schottii* in comparison with extracts of the stems, and a negligible effect of leaf extracts, against K562 leukemic cells, bone marrow endothelial cells (BMEC) and human umbilical cord endothelial cells (HUVEC). These differences in activity could be related to seasonal influence. The results found by Schmidt et al. (2006), when compared with our study, confirms that seasonal effects influence cytotoxicity.

The extracts that gave IC_{50} values below 100 µg/ml, in at least one tumor cell line, were partitioned with *n*-hexane, dichloromethane (DCM) and ethyl acetate (EtOAc) and the fractions were then evaluated in the same cells. As observed in Table 1 the main bioactive compounds were found in the DCM fractions in all parts of the plant studied, there being a significant reduction in the IC_{50} values for cells in which the extracts were active. Surprisingly, the DCM fractions were also active against the K562 cells, to which the extracts were not active.

In the case of the DCM fraction of the roots there was no significant difference in the IC_{50} value when compared to the control drugs paclitaxel, gleevec and vincristine in relation to

Table 1
Cytotoxic effect (IC_{50}) of extracts and fractions of different parts of the plant *Allamanda schottii* collected in winter against L929, HeLa, K562 and Nalm6 cell lines.

Compounds	IC_{50} (µg/ml)			
	L929	HeLa	K562	Nalm6
Ethanolic extract of roots	55.80 ± 8.10	11.20 ± 3.50	> 100	109.00 ± 0.40
n-Hexane fraction	-	-	> 100	38.40 ± 4.00
Dichloromethane fraction	30.50 ± 3.50	6.30 ± 3.00	1.00 ± 0.80	1.20 ± 0.80
Ethyl acetate fraction	-	-	> 100	35.00 ± 7.10
Ethanolic extract of stems	> 100	> 100	> 100	30.00 ± 4.00
n-Hexane fraction	-	-	45.00 ± 3.50	46.00 ± 8.20
Dichloromethane fraction	65.60 ± 9.50	14.50 ± 3.00	4.90 ± 1.20	0.20 ± 0.01
Ethyl acetate fraction	-	-	41.00 ± 3.30	7.40 ± 1.30
Ethanolic extract of leaves	> 100	> 100	> 100	30.80 ± 1.10
n-Hexane fraction	-	-	55.30 ± 6.10	74.40 ± 1.30
Dichloromethane fraction	> 100	53.70 ± 0.10	46.80 ± 1.20	6.40 ± 2.30
Ethyl acetate fraction	-	-	> 100	28.50 ± 1.30
Paclitaxel	3.84 ± 0.01	3.06 ± 0.54	-	-
Gleevec	-	-	0.10 ± 0.04	-
Vincristine	-	-	-	0.06 ± 0.04

Not available (-). Data are expressed as mean ± standard deviation of three determinations.

HeLa, K562 and Nalm6 cells, respectively ($p > 0.05$). Similar IC_{50} values were observed for the DCM fraction of the stems compared to gleevec and vincrisctine in relation to K562 and Nalm6 cells, respectively ($p > 0.05$), or in the DCM fraction of the leaves and vincristine in relation to Nalm-6 cells ($p > 0.05$). These outstanding results indicate that the DCM fraction elicits a similar activity to commercial drugs, strongly suggesting the presence of bioactive compounds in this fraction, which may be acting in isolation or in synergy to produce the considerable effect observed.

Identification of active compounds in DCM fraction

Due to the observed cytotoxic activity described above, the ethanol extract of the stems, collected at the end of winter, was fractioned using open column chromatography. From this procedure a mixture of β-sitosterol and stigmasterol, plumericin, plumieride, ursolic acid, 3-*O*-β-glucopyranosyl-sitosterol and a mixture of sugars were obtained. The compounds were analyzed by nuclear magnetic resonance (NMR) and their chemical shifts were compared with those found in the literature or with data of the standards previously isolated (Ganapaty et al., 1988, Abdel-Kader et al., 1997, Malheiros et al., 1997, Schmidt et al., 2006).

To determine the relationship between the cytotoxicity of the *A. schottii* extracts, fractions and their composition, the principal compounds isolated, plumericin, plumieride, and ursolic acid, were evaluated using different cell lines. The results are presented in Table 2.

The cytotoxic effects of plumericin and ursolic acid suggest that these compounds are cytotoxic to HeLa and Nalm6 cells, respectively observed in the DCM fractions from different parts of the plant. In the K562 cells, the isolated compounds did not contribute to the effects observed. Other minor compounds still not identified may be co-responsible for the activity shown. This appears to be a cytotoxic synergy operating in the DCM fraction.

The activity of the isolated compounds toward B16F10 melanoma and breast adenocarcinoma cells MCF-7 was also evaluated. The best results were found for plumericin and ursolic acid in the case of B16F10 cells with IC_{50} values of 3.80 ± 1.70 and 13.60 ± 1.30 µM, respectively. However, comparatively, paclitaxel was more cytotoxic to this cell line ($p < 0.01$). Although the activity against B16F10 cells was lower than that of the reference drug, the results observed against melanoma cells are of considerable interest, since plumericin could be used as a model for new derivatives to improve the effectiveness of cytotoxicity studies in relation to this highly invasive and metastatic type of cancer.

Abdel-Kader et al. (1997) documented low cytotoxicity of plumericin and isoplumericin against a lung carcinoma (M109) cell line (IC_{50} 100 µg/ml). Anderson et al. (1988) isolated the iridoids plumericin, isoplumericin, allamandin and allancin from *A. schottii* and these compounds showed antitumoral activity against KB cells, confirming the importance of the use of different cell models in cytotoxic studies. Additionally, Gomes (2008) observed no evidence of mutagenicity in *Salmonella typhimurim* tests for plumericin, with or without metabolic activation with S9 and using the strains TA98, TA97a, TA100 and TA102. This result could be considered a determining factor for future in vivo studies with this iridoid. Another determining factor is the bioavailability of the compounds. According to Liao et al. (2005), the ursolic acid concentration in rat plasma, after oral administration, is extremely low; this implies that ursolic acid has high binding activity in organs and low blood distributions, and/or it is metabolized by the gut wall and liver, and/or it is poorly absorbed by the intestine. No information about the plumericin bioavailability was found in current literature.

Table 2
Cytotoxic effect (IC_{50}) of compounds plumericin, plumieride and ursolic acid isolated from *Allamanda schottii* against, MCF-7, B16F10, L929, HeLa ,K562, Nalm6 cell lines.

Compounds	IC_{50} µM					
	MCF-7	B16F10	L929	HeLa	K562	Nalm6
Plumericin (**2**)	121.7 ± 60.0	3.8 ± 1.7	161.9 ± 25.5	24.5 ± 13.4	111.1 ± 27.6	92.4 ± 10.7
Plumieride (**1**)	> 212	> 212	> 212	> 212	> 212	> 212
Ursolic acid	-	13.6 ± 1.3	-	-	> 219	66.8 ± 24.8
Paclitaxel	1.5 ± 0.6	0.4 ± 0.4	4.5 ± 1.6	3.6 ± 0.5	-	-
Gleevec	-	-	-	-	0.5 ± 0.1	-
Vincristine	-	-	-	-	-	0.15 ± 0.06

Not available (-). Data are expressed as mean ± standard deviation of three determinations.

Although plumericin plays an important role in the activity of the DCM fraction, other active compounds are also significant contributors, indicating synergy, which supports the phytotherapeutic use of the fraction rather than the isolated compounds. As observed in our results, the toxicity of species of the *Allamanda* genus is also related to the ursolic acid content. This compound was evaluated in relation to melanoma B16 by Saady et al. (1996) and showed significant cytotoxicity. It has also been documented to have activity as a suppressor of tumorigenesis, inhibitor of tumor promotion and suppressor of angiogenesis. The cytotoxicity may be related to the inhibition of DNA replication, activation of caspases, and induction of Ca^{2+} release (Hsu et al., 2004; Yim et al., 2006). Additionally, ursolic acid reportedly caused a 76% reduction in the number of micronuclei induced by mitomycin D (Guevara et al., 1996).

Plumieride was analyzed in all cell lines and did not present any sign of cell cytotoxicity by MTT. Dobhal et al. (2004) modified the plumieride structure, to enhance its activity. The results showed that, in the case of the fibrosarcoma (RIF) tumor cell line, the native molecule had an IC_{50} value of 49.5 µg/ml, but the pentacetate analogue showed improved activity (IC_{50} 19.5 µg/ml). The hydrolysis of the methylester did not make any significant difference to the efficacy (IC_{50} 22 µg/ml). When the log *p* values were increased (from propyl to dodecylamide analogues), a more effective activity (IC_{50}11.8 µg/ml) was observed.

Seasonal influence on cytotoxicity

Considering that seasonal changes can affect the production of secondary metabolites, *A. schottii* was harvested at the end of each season throughout 2006, and each of the extracts obtained from the roots, stems and leaves were fractioned with *n*-hexane, DCM and AcOEt. The DCM fractions relating to the four seasons were evaluated using leukemia cell lines K562. This cell line was selected due to its previously described resistance to apoptosis, and because it is a good indicator of the cytotoxic compounds of interest. The results are presented in Fig. 1.

For the root and stem DCM fractions, IC_{50} values of below to 10 µg/ml were observed, independently of the season, except for the stem of DCM fraction obtained from plants collected in spring, with IC_{50} values of up to 30 µg/ml. The IC_{50} values for the DCM fractions of the leaves were between 20 and 40 µg/ml. These results confirm the relation between the chemical composition or concentration of active principles at different seasons and their cytotoxic effect. The variability observed in the concentration of compounds could be related to environmental conditions such as climatic differences, stress levels, water availability, light, and natural predators (Gobbo-Neto and Lopes, 2007).

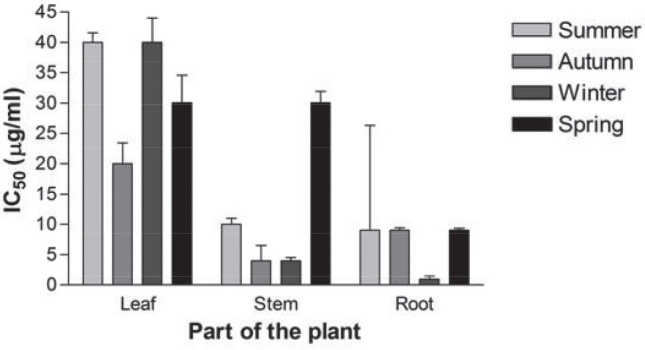


Figure 1 – Season and toxicity of dichloromethane fractions of *Allamanda schottii* on K562 cells (IC_{50} in µg/ml).

To define the chemical composition of the DCM fractions obtained from the different parts of the plants collected at different seasons of the year, the HPLC analysis was conducted as an alternative means to analyze the quality the plant extracts. The chromatograms obtained are presented in Fig. 2

The chromatographic method developed in this study was used to evaluate the production of secondary metabolites present in *Allamanda schottii*, iridoids, which are the target of this study. The dichloromethane fraction of stems obtained in winter was analyzed in a series of mobile phases with different solvent systems to obtain a chromatogram with good peak separation. The phase with the best chromatogram consisted of a mixture of methanol, acetonitrile and ultrapure water acidified with phosphoric acid at pH 3.54. The absorbance at wavelength 230 nm was measured, since this is the best wavelength to obtain iridoids.

The chromatographic profiles of *A. schottii* were very similar for each part examined at different seasons, differing in most cases only in terms of some peaks' intensities. Since there is a direct relationship between chromatographic peak area

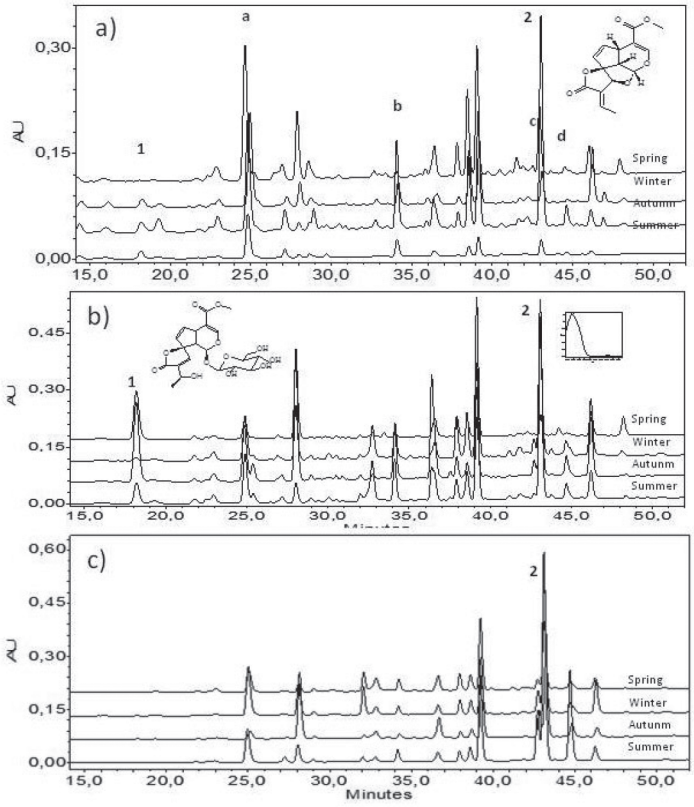
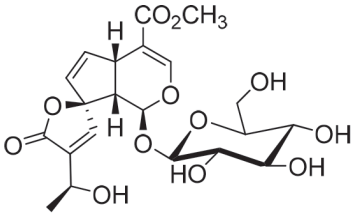


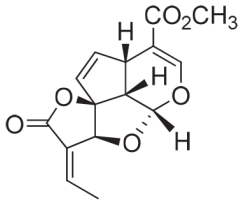
Figure 2 – Chromatographic profiles of dichloromethane (DCM) fractions of *Allamanda schottii* collected at different seasons, acquired by HPLC λ= 230 nm. a) DCM fractions of leaves, b) DCM fractions of stem, c) DCM fractions of root (plumieride (**1**) and plumericin (**2**), a, b, c and d iridoids not identified). For HPLC conditions see experimental procedures.

and analyte concentration, a difference in the phytochemical concentrations could be observed.

A total of 24 peaks at between 15 and 52 min were detected in the chromatograms of the DCM fractions (Fig. 2) at a wavelength of 230 nm. The identification of the iridoids was carried out by comparison of the UV spectra and retention time of previously isolated iridoids plumieride (**1**) and plumericin (**2**), injected under the same analysis conditions. The plumieride presented RT 18.3 ± 1.0 min and plumericin RT 43.0 ± 1.0 min (peak max. 215 nm). The peaks related to the two substances were identified by numbers **1** and **2**, respectively, on the chromatograms of the dichloromethane fractions evaluated. The UV spectra for peaks a, b, c and d in the dichloromethane fraction were similar to plumericin and plumieride (data not shown). Therefore, it can be concluded that these metabolites are iridoids. The iridoids detected mainly in the dichloromethane fraction, especially in the case of this species, were aglycones. These iridoids, in general, were found mainly in the roots and stems, and in lower concentrations in the leaves. Plumericin (**2**) was found in all parts of the plant collected at all seasons, and the highest concentration was found in the roots. The influence of seasonality on this iridoid can be observed in the leaf extracts since during the summer, this iridoid was obtained in lower proportions.



1



2

Several iridoid aglycones have been isolated from plants of the genus *Allamanda*, including allamandin, allancin, fulvoplumierin and protoplumierin (Kupchan et al., 1974; Anderson et al., 1988; Tan et al., 1991). The iridoids exhibit several types of pharmacological activities including anti-inflammatory, analgesic, antibacterial, antifungal and antitumor action, as well as sedative, tranquilizing and sleep-inducing properties, also they are uses for the treatment of hepatic dysfunctions (Ghisalberti, 1988; Dewick, 2002; Dinda et al., 2007).

The relation between the chromatographic profiles and toxicity of the DCM fractions against K562 leukemic cells may be associated with the presence of the iridoid plumericin. The qualitative analysis of plumericin by chromatography can provide information on the cytotoxic activity, since higher concentrations of this substance are related to increased

activity. The roots collected in winter gave the best results against K562, and this fraction contained a high concentration of plumericin. The concentration in the stems is similar to that of the roots, except for the stems collected in spring. The leaves were found to contain lower concentrations of this metabolite, mainly in plants collected during summer.

As shown in Table 2, the iridoid glycoside plumieride showed cytotoxic effect at concentrations greater than 212 µM (> 100 µg/ml). These values are quite high when compared to the activity observed for plumericin against, for example, to B16F10 cells (3.8 µM). Plumieride was detected in dichloromethane spring, autumn and summer stems fractions and in the ethyl acetate fraction from the stems collected in autumn, winter and spring (chromatograms of the ethyl acetate not shown). The dichloromethane and ethyl acetate fractions analyzed presented IC_{50} values inferior to 50 µg/ml, especially to leukemic cells K562 and Nalm-6 (except the ethyl acetate fractions of roots and leaves against K562 cells). These results indicate that plumieride does not contribute to the cytotoxic activity observed in these fractions. Plumericin can contribute, in part, to this activity. This compound exhibited a cytotoxic effect at concentrations 3.8 ± 1.7 µM to B16F10 cells

The results for the cytotoxicity of *A. schotti* extracts need to be correlated with the season of the year, the fraction and the part of the plant evaluated, as these three factors are determinant in terms of cytotoxicity. The presence of plumericin and ursolic acid in the roots, as well as in other parts of the plant, particularly in the DCM fraction, appears to be a major factor responsible for the cytotoxicity observed. The results clearly show the phytotherapeutic potential use of the plant, due to synergy or the presence of minority compounds not yet identified, as well as the seasonal influence on the production of secondary metabolites.

Authors' contributions

AM, FGN, RAY, VCF contributed in plant collection and identification, confection of herbarium, running the laboratory work, analysis of the data and drafted the paper. AF, JFW, ran the laboratory work, analysis extractions of active principles and biological studies. CW, FDT, MSM, AM contributed to chromatographic analysis and SLB, GCF, AEN, RAF contributed in biological studies analysis of the data and drafted the paper. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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