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# *In vitro* cultivation of *Vismia japurensis*: Isolation of the new anthrone 1,8,10-trihydroxy-3,10-dimethyl-9(10H)-anthracenone

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**Abstract:** *Vismia japurensis* Reichardt is a plant of ecological and chemical importance from which a variety of bioactive substances have been isolated. The current study aimed to establish *in vitro* cultures of this species as a source of secondary metabolites. Appropriate decontamination treatments and germination tests were performed and, after *in vitro* culture establishment, the propagated plants were multiplied in a sterile environment to increase the biomass of available experimental material. Seeds showed low contamination and a high germination percentage on Woody Plant Medium (WPM) supplemented with gibberellic acid (both at concentrations of 5 and 10 mg/L). *V. japurensis* nodal segments rapidly regenerated when first grown in WPM and then transplanted to Murashige and Skoog medium (MS). After 60 days in MS medium, the propagated plants were removed, lyophilized, and extracted with hexane and methanol. The hexane extract was fractionated via open column chromatography, and the substance isolated was purified by high performance liquid chromatography. Structural determination of the isolated substance was carried out using one and two-dimensional nuclear magnetic resonance and mass spectrometry. The isolated substance was identified as 1,8,10-trihydroxy-3,10-dimethyl-9(10*H*)-anthracenone, which, based on the conducted literature search, is reported for the first time.

**Key words:** Bioprospection, chromatography, phytochemistry, tissue culture.

## INTRODUCTION

*Vismia japurensis* (Hypericaceae) is a shrubby plant species, popularly known in Amazonian Brazil as *lacre, purga de vento* or *picharrinha*. These names are also attributed to other species of this genus (Di Stasi & Hiruma-Lima 2002). It is considered a pioneer species, commonly encountered in early successional phases of Amazonian secondary forests. Its ecological importance lies in regeneration of degraded areas, including bioremediation (Monaco et al. 2003, Silva et al. 2008, Mota & Santana 2016).

Literature surveys reveal that a wide variety of secondary metabolites, including bioactive compounds with potential therapeutic use, has been isolated from wild *V. japurensis*. Bioactive isolates include, for example, the triterpenes friedelin and friedelanol, that exhibit antimicrobial, antidiabetic, antiangiogenic, anti-Newcastle disease virus effects and potent anti-inflammatory, analgesic and antipyretic activities. Other bioactive isolates are vismiaquinone A, which shows antiplasmodic and anti-inflammatory activity, and acetylvismone B, which is reported to have antiproliferative, anti-inflammatory, antifungal and antitumoral properties (Miraglia et al. 1981, Pinheiro et al. 1984, Cassinelli et al. 1986, Kuete et al. 2007, Noungoue et al. 2009, Tamokou et al. 2009, Antonisamy et al. 2011, Kuete & Efferth

2011, Rodanant et al. 2017, Pereira et al. 2018, Sunil et al. 2021, Credo et al. 2022).

The phytotoxic action of hexane and methanol extracts from *V. japurensis* plants cultivated *in natura* and *in vitro* was evaluated by Lima et al. (2022), and showed that the hexane extract was largely responsible for reduction in root growth of *in vitro* seedlings of *Lactucca sativa*. So, *V. japurensis* is a promising species to be included in bioprospecting studies due to its important biological activities and agronomic potential.

To reduce the indiscriminate and predatory collection of wild plants of medicinal importance, biotechnological approaches can be applied to plant tissue culture to enable the *in vitro* establishment of species for later phytochemical exploration (Fumagali et al. 2008).

It is possible to obtain plant improvement, propagate species of interest and produce secondary metabolites of pharmaceutical relevance from cells, tissues and/or organs established *in vitro* (Dias et al. 2016, Chandran et al. 2020). Different types of plant explants, such as leaves, petioles, roots and seeds can be used for *in vitro* establishment (Grattapaglia & Machado 1999). Accordingly, once the desired development stage of a species has been determined, it should be possible to increase production of the substance of interest. There are several examples of the successful application of this method, such as: the *in vitro* production of vinblastine and vincristine by callus of *Catharanthus roseus* (L.) G. Don., artemisinin production from callus and root cultures of *Artemisia annua* L. and vismiones from seedlings of *Vismia guianensis* (Aubl.) Pers. (Pasqua et al. 1995, Liu et al. 2002, Fatima et al. 2015).

The limitation in establishing woody species *in vitro* is caused by such factors as culture oxidation, caused by the phenolic substances produced and released in response to tissue injuries, as well as by contamination from both epiphytic and endophytic microbiota (Sato et al. 2001, Cordeiro et al. 2004, Couto et al. 2004). As an attempt to resolve these problems, substances with antibiotic and antioxidant activities have been tested in decontamination processes and seeds have been used as contamination-free plant material to facilitate *in vitro* establishment (Souza et al. 2011).

Due to the phytochemical and pharmacological potential of *V. japurensis*, the present study aimed to establish *in vitro* cultures of tissues derived from this species, using different sources of explants and evaluating different decontamination treatments. The study also aimed to fractionate extracts of *in vitro* cultured plant tissues and to isolate secondary metabolites.

## MATERIALS AND METHODS

### Chemicals and culture media conditions

Chemicals and deuterated solvents were purchased from Sigma-Aldrich/Merck (São Paulo, Brazil), HPLC grade solvents were purchased from Tedia (Brazil), and other solvents were distilled in the laboratory from commercial grade materials (Araguaia, Brazil). Culture media were purchased from Kasvi® (Campinas, Brazil). The culture media used in the experiments were WPM (wood plant medium) and MS (Murashige and Skoog) with varied macronutrient compositions (Murashige & Skoog 1962, Lloyd & McCown 1981). Both had 3% sucrose as the carbon source, and 0.8% agar as the gelling agent. The pH was adjusted to 5.7 ± 0.1 and cultures were kept in a growth room at a temperature of 26 ± 2 °C under a 16:8 hour (white light/dark) photoperiod, with an light intensity of 40 to 50 µmol/$m^2$.s.

## Plant material

Leaves and fruits of *V. japurensis* were collected within the urban area of Manaus (3°02'59.9"S; 60°01'50.4"W) in December, 2018. Collections were made under IBAMA permit number 16970-1 and SISGEN registry number: AF64920.

## Surface sterilization of the explants

Twelve surface sterilization treatments on leaves and three on seeds of *V. japurensis* were tested. In each treatment, the composition, concentration and combinations of decontaminating agents, as well as exposure time were varied (Table I). Experimental design was completely randomized, consisting of 20 replications (for leaves) and 165 (for seeds). Each repetition consisted of a test tube with a leaf or seed. Evaluations of the percentage of tubes contaminated with fungi and bacteria were carried out daily for leaves and every thirty days for seeds (four evaluations in total) for a period of ninety days.

## Seed germination

Decontaminated seeds were placed in WPM and MS media with 5 and 10 mg/L of gibberellic acid ($GA_3$) and kept in a growth room. The number of germinated seeds was evaluated every 30 days over a period of 90 days. Each treatment consisted of 55 repetitions.

## Treatments for seedling multiplication

*In vitro* germinated seedlings were used for sprout induction. Each seedling was transferred to 16 × 160 mm test tubes, each containing 5 mL of culture medium. Tests were performed in three media: 1) WPM, 2) MS and 3) MS medium with 50% reduction of nitrate, using separately the cytokinins 6-benzylaminopurine (BAP) and kinetin (KIN). Concentrations of 1 and 5 mg/L

**Table I. Chemical agents used in the decontamination of *Vismia japurensis* leaves and seeds.**

| Pre- decontamination | | | Decontamination | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Decontaminating solution | | 70% ethanol | ethanol (70%) | CaClO (0.5%) | CaClO (1%) | CaClO (2%) | CaClO (4%) | NaClO (0.5%) | NaClO (1%) | NaClO (2%) | NaClO (2.5%) |
| **LT1** | 24 h | - | 3 min | - | - | - | 10 min | - | 10 min | - | - |
| **LT2** | 24 h | - | 5 min | - | - | - | 5 min | - | - | - | 10 min |
| **LT3** | 2 h | - | 3 min | - | - | - | - | - | - | 15 min | - |
| **LT4** | 2 h | - | 4 min | - | - | - | - | - | 10 min | - | - |
| **LT5** | 4 h | - | 3 min | - | - | - | - | - | 10 min | - | - |
| **LT6** | 4 h | - | 3 min | - | - | - | - | - | - | 15 min | - |
| **LT7** | 4 h | 5 min | 3 min | 40 min | - | - | - | 30 min | - | - | - |
| **LT8** | 4 h | 5 min | 3 min | - | 15 min | - | - | - | 15 min | - | - |
| **LT9** | 4 h | 5 min | 3 min | - | - | 20 min | - | - | - | 15 min | - |
| **LT10** | 5 h | 5 min | 3 min | 40 min | | - | - | 30 min | | - | - |
| **LT11** | 5 h | 5 min | 3 min | - | 15 min | - | - | - | 15 min | - | - |
| **LT12** | 5 h | 5 min | 3 min | - | - | 20 min | - | - | - | 15 min | - |
| **ST1** | 1 h | 5 min | 1 min | - | - | - | - | - | - | 5 min | - |
| **ST2** | 2 h | 5 min | 1 min | - | - | - | - | - | - | 5 min | - |
| **ST3** | 1 h | - | 3 min | - | - | - | - | - | - | - | - |

**Legend: LT: Leaf decontamination treatment, ST: Seeds decontamination treatment. Decontamination solution = Mancozeb® 2 g/L + Streptomycin 100 mg/L.**

of both cytokinin BAP and cytokinin KIN were evaluated, constituting different treatments.

The experimental design was completely randomized, with 20 replications per treatment. Cultures were evaluated for 30 days, with mean growth and shoot number ± standard deviation (SD) being calculated every 7 days. To assay the most efficient treatment in multiplication, data from the final evaluation were subtracted from those of the initial evaluation (values obtained directly after inoculation). The data were subjected to analysis of variance (ANOVA), followed by a Tukey test for separation of means, using a 95% confidence interval = 0.05 alpha value, with calculations made using GraphPad Prism software (version 7.00, March 31, 2016).

### Increased multiplication of *in vitro* propagated plants

To increase biomass, established seedlings were multiplied from nodal segments in WPM medium without addition of plant hormones. Cultures were kept in a growth room at a temperature of 26 ± 2 °C, under a 16:8 hour (white light/dark) photoperiod. Following shoot initiation, each shoot was individually transplanted into test tubes and bottles with MS medium. After 60 days the propagated plants were collected, washed to remove any adhering culture medium and lyophilized.

### Plant extraction and chemical fractionation

The plant material was lyophilized, macerated, and extracted with hexane for 20 minutes in an ultrasonic bath (Unique®, model USC-2800: US Frequency, 40 kHz, São Paulo), and then filtered. This procedure was repeated for a total of 8 sequential extractions using hexane. Subsequently, the plant material was dried and subjected to extraction with methanol, resulting in a total of 6 methanol extractions, carried out sequentially. All extractions had the ratio of 1 gram of plant material to 30 mL of solvent. The obtained extracts were concentrated using a rotary evaporator (Fisatom, model 550, São Paulo) and dried in a ventilated fume hood. Methanol extract was stored in freezer for future work.

Hexane extract was analyzed by comparative thin layer chromatography (TLC - Macherey-Nagel, Model Aluminum chromatosheet for TLC Alugram), using the solvents hexane, DCM and acetone in different combinations for elution, with the plates subsequently being developed using physical and chemical developers; the extract was also analyzed by hydrogen nuclear magnetic resonance spectroscopy ($^1$H-NMR).

The hexane extract (230 mg) was fractionated by open column chromatography (OPC) (h x Ø: 26 x 2 cm) of silica (proportion of 1 g of extract/100 g of silica), using the eluents in a combination of increasing order of polarity: hexane/DCM 7:3, 6:4, 5:5, 4:6, 3:7, 2:8; DCM 100%; DCM/acetone 95:5, 9:1, 8:2, 1:1; 100% acetone; acetone/MeOH 9:1, 1:1 and MeOH 100%. Fractions 27 (2 mg) and 28 (3 mg) were pooled, totaling 5 mg; fractions 29-36 were also pooled (8 mg) (Figure 1).

For HPLC analysis, the fractions were dissolved in MeOH and fractionated using a $C_{18}$ luna analytical column (Phenomenex®; 250 × 4.6 mm) for the stationary phase, with an injection volume of 15 µL and flow rate of 1 mL/min, and elution with 100% isocratic MeOH, with monitoring conducted at 254 and 280 nm. For purification, samples were dissolved in 150 µL of MeOH, using a semi-preparative column $C_{18}$ (Phenomenex®; 250 × 10 mm) with an injection volume of 50 µL and a flow of 4.7 mL/min, 100% isocratic MeOH, with 254 and 280 nm as the monitoring wavelengths.

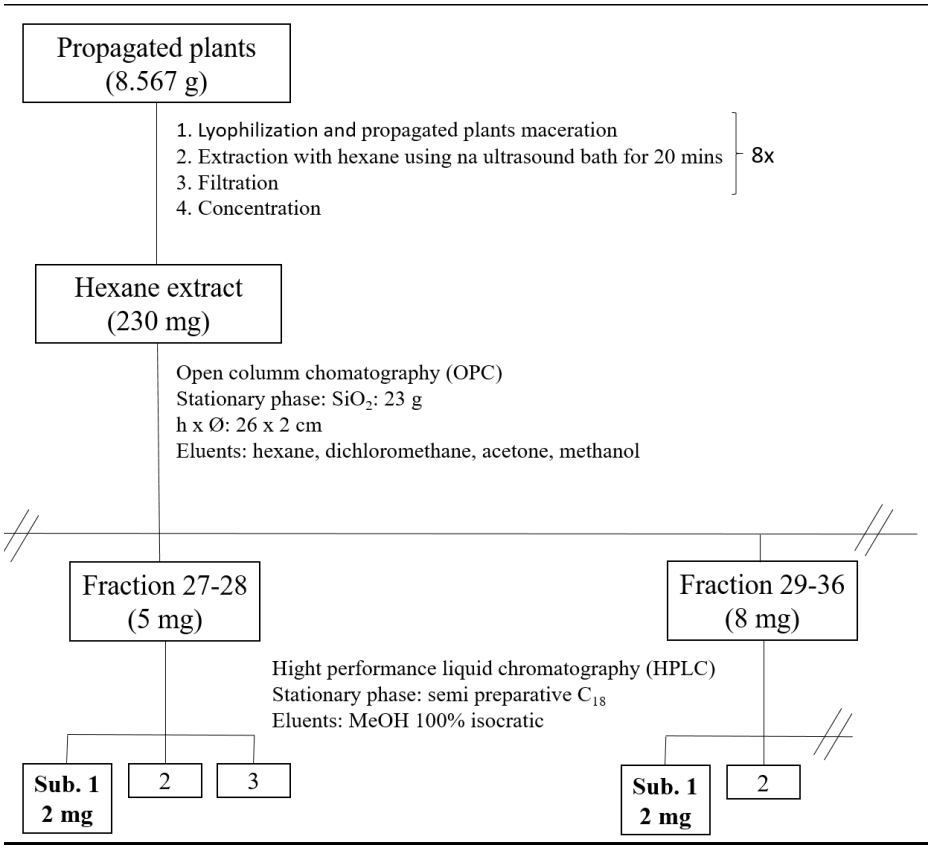


**Figure 1. Flowchart of *in vitro Vismia japurensis* extraction and chemical fractionation.**

### Nuclear magnetic resonance and mass spectrometry analysis

The isolated substance was dissolved in deuterated chloroform ($CDCl_3$), and subjected to $^{1}H$ and $^{13}C$ nuclear magnetic resonance analysis. $^{1}H$, $^{13}C$ one and two-dimensional NMR spectra were obtained with a Bruker (BioSpin AG spectrometer, model Fourier 300 Ultrashield) operating at 300 MHz for the $^{1}H$ core and at 75 MHz for the $^{13}C$ core. Tetramethylsilane (TMS) was used as internal standard. High resolution mass spectra were obtained with the micrOTOF-Q mass spectrometer model (ESI-TOF Mass Spectrometer, Bruker Daltonics), operating in positive and negative modes.

## RESULTS AND DISCUSSION

### Surface sterilization of the explants

Leaf explants were all contaminated by fungi three days after attempted disinfestation treatments. However, seeds showed lower amount of fungal contamination (> 93% of aseptic seeds). Treatments 1 and 2 presented the highest percentages of seeds free from bacterial contamination (98.8% and 97.6%, respectively) (Table II). Since the first two treatments were similar in contamination incidence, treatment 1 was established as the most suitable due to its immersion time in the fungicide solution being reduced by one hour.

The lower infestation levels shown by seeds compared to leaves can be explained by the physical barrier that the fruit itself presents. This barrier reduces direct contact of the seeds with the general microbial diversity present in the environment; consequently, the outermost layers of the fruit have greater amounts of fungi (Kobayasti et al. 2011).

Low bacterial contamination is possibly related to the presence of sodium hypochlorite

**Table II. Percentage of contamination free seed.**

| | Fungus free | | | Bacteria free | | |
|---|---|---|---|---|---|---|
| | ST1 | ST2 | ST3 | ST1 | ST2 | ST3 |
| $EV_1$ | 93.9% | 97.57% | 94.54% | 98.8% | 97.6% | 69.7% |
| $EV_2$ | 93.9% | 94.54% | 93.93% | 98.8% | 97.6% | 67.3% |
| $EV_3$ | 93.9% | 94.54% | 93.93% | 98.8% | 97.6% | 67.3% |

**Legend: ST1, ST2 and ST3: different disinfestation treatments evaluated; $EV_1$: 30 days after disinfestation, $EV_2$: 60 days after disinfestation, $EV_3$: 90 days after disinfestation.**

in the laminar flow decontamination step. Chlorine-based decontaminating agents, as well ethanol, are the most commonly used in this process, in addition to fungicides and bactericides, where concentration, combination and time of exposure to chemical agents varies depending on explant resistance (Pereira et al. 2015, Huang et al. 2021).

The abundance of fungi in Amazonian species means it is difficult to establish species *in vitro* using leaf explants. In the specific case of *V. japurensis*, since the leaves are hairy, effective decontamination without the tissue being compromised for regeneration becomes infeasible. The use of seeds, however, does allow plant material to be obtained without contamination (Souza et al. 2011).

## Seed germination

Seeds inoculated on WPM medium showed higher germination rates across the evaluation period, increasing the percentage of germinations by adding $GA_3$, reaching 50% of germination after 90 days of cultivation in WPM medium with 10 mg/L of $GA_3$. In MS medium, even with the addition of $GA_3$, only about 6% of the seeds germinated at the end of 90 days (Table III).

*In vitro* seed germination has become an excellent option for obtaining aseptic seedlings (Resende et al. 2021). The germination process is usually triggered by the absorption of water, occurring at the same time as the liberation of gibberellins by the embryo, thus stimulating the synthesis of hydrolytic enzymes involved in the breakdown of starch, so causing the nutritional reserves to be absorbed and transported to regions of embryo development and growth (Lavagnini et al. 2014).

The addition of gibberellin to culture media can contribute to breaking dormancy in some species, thus speeding up the germination process. As for the reduction in germination capacity in MS medium, there are several similar examples in the literature, including from studies with *Lychnophora pinaster* (Asteraceae) and *Melissa officinalis* (Lamiaceae), in which these species also presented delay and reduction in germination rate when inoculated in MS medium (Souza et al. 2003, Reis et al. 2008).

Possibly, in response to the greater amount of nutrients and vitamins in the MS medium, the water potential was altered, reducing the availability of water for the imbibition process of *V. japurensis* seeds and hindering germination. Among the evaluated treatments, the WPM medium with 10 mg/L of $GA_3$ was the most adequate for the germination of *V. japurensis* seeds.

## *In vitro* propagated plant multiplication

Propagated plants grew continuously during the four weeks of evaluation; however, the average growth values (height) and the number of released shoots were not significantly different among themselves within the same treatment. Thus, the data from the final evaluation was

**Table III. Germination percentage of seeds *Vismia japurensis* in different culture media and $GA_3$ concentrations.**

| | WPM media | | | MS media | | |
|---|---|---|---|---|---|---|
| | 0 mg/L of $GA_3$ | 5 mg/L of $GA_3$ | 10 mg/L of $GA_3$ | 0 mg/L of $GA_3$ | 5 mg/L of $GA_3$ | 10 mg/L of $GA_3$ |
| $EV_1$ | 6.6% | 17.5% | 24.2% | 0% | 0% | 0% |
| $EV_2$ | 11.5% | 25.4% | 36.9% | 1.8% | 1.8% | 2.4% |
| $EV_3$ | 16.3% | 33.9% | 50.3% | 2.4% | 6% | 3.6% |

**Legend: $EV_1$: 30 days after inoculation, $EV_2$: 60 days after inoculation, $EV_3$: 90 days after inoculation.**

subtracted from the initial value to compare growth and multiplication rates between the different evaluated treatments.

Of the three culture media evaluated, WPM and MS with nitrate reduction were more suitable for the *in vitro* multiplication of *V. japurensis* from seedlings. In the WPM medium without the addition of cytokinins (control), the plants showed higher growth values (average height 0.83 ± 0.29 cm), but no shoot was observed. More shoots were released when cytokinins BAP and KIN were added to WPM and MS media with nitrate reduction (Table IV).

Several species require the addition of growth regulators inorder for shoots to grow. Among the most used cytokinins are BAP and KIN. Their efficiency is affected by the stage of plant development and the applied dose. Since rooting was not the objective of *V. japurensis* culture, and since the joint addition of auxins and cytokinins to the culture medium could lead to callus formation, it was decided to evaluate only cytokinins, because these are directly related to aerial part formation.

Of the evaluated cytokinins for propagated plant multiplication of *V. japurensis*, BAP caused the highest number of shoots, but these shoots showed little growth and aerial development (Figure 2). The combination of WPM medium with KIN (1 and 5 mg/L) led to excellent shoot formation. Thus, KIN was qualitatively established as the best hormone to induce shoots in *V. japurensis* providing shoots with good aerial part development (Figure 2).

BAP and KIN have been previously in the culture of plant tissues with mixed results. The literature presents several plant species for which the addition of BAP to the culture medium for multiplication is not beneficial. In these plants, BAP causes a reduction in the rate of formation of explants, a reduction in the number of shoots per explant and a decrease in the elongation of the shoots (Garlet et al. 2011, Morais et al. 2014). In contrast, other researchers found that the addition of cytokinin KIN provided sprouts having greater shoot elongation/length (Bekircan et al. 2018, Oliveira et al. 2019).

Although cytokinins are essential in shoot multiplication of several species, excessive concentrations can generate toxicity and reduce organogenesis. In the present work, two concentrations of cytokinins (1 and 5 mg/L) were evaluated to obtain an initial response from the plants and to define whether intermediate values needed to be evaluated. No significant difference in results was observed for the evaluated concentrations, except for the use of KIN in MS medium, in which only the concentration of 5 mg/L promoted the formation of shoots (Table IV).

Although the main objective of the multiplication phase is to produce a large amount of *in vitro* propagated plants, the qualitative aspects also need to be evaluated when considering the success of tissue culture

**Table IV.** Mean growth and shoot number ± standard deviation of mean (SD).

| Cytokinins mg/L | MS 100 % | | MS 50 % | | WPM | |
|---|---|---|---|---|---|---|
| | Growth (cm) | nº of shoots | Growth (cm) | nº of shoots | Growth (cm) | nº of shoots |
| 1 BAP | 0.57 ± 0.2 a | 2.4 ± 0.5 a | 0.27 ± 0.9 a | 3.6 ± 1.3 a | 0.16 ± 0.2 a | 4.6 ± 1.2 a |
| 5 BAP | 0.44 ± 0.13 ab | 2.6 ± 0.9 a | 0.20 ± 0.1 a | 3 ± 1.5 ac | 0.29 ± 0.2 ab | 5.4 ± 1.7 a |
| 1 KIN | 0.62 ± 0.15 a | 0 b | 0.27 ± 0.09 a | 0.4 ± 0.8 b | 0.47 ± 0.3 b | 3.9 ± 1.7 ab |
| 5 KIN | 0.76 ± 0.33 ac | 2 ± 0.5 ac | 0.26 ± 0.1 a | 2.4 ± 1.5 c | 0.31 ± 0.19 ab | 3.7 ± 1.4 ab |
| Control | 0.57 ± 0.2 a | 0 b | 0.20 ± 0.1 a | 0 b | 0.83 ± 0.29 c | 0.1 ± 0.3 c |

**Legend: Control: culture medium without addition of cytokinins. Means followed by the same letter in the columns do not statistically differ from each other by the Tukey test (0.05 alpha value).**

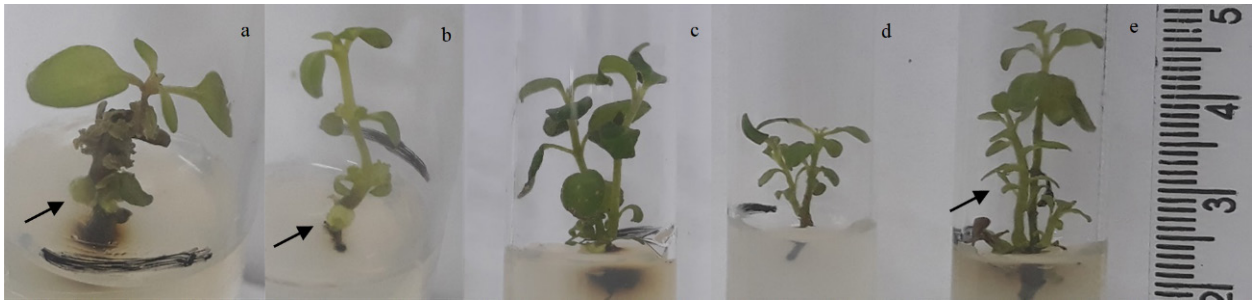


**Figure 2. Comparison of shoot development of seedlings cultivated in WPM medium with BAP and KIN cytokinins after 21 days of cultivation. a) and b) with 5 mg/L of BAP; c), d) and e) with 5 mg/L of KIN.**

(Grattapaglia & Machado 1999). Thus, even though MS medium is used for tissue culture of miscellaneous plant species, it was possible to establish WPM culture medium (which has a lower nitrogen content and a higher sulphur concentration) as the most suitable for the multiplication of *V. japurensis*, especially in association with cytokinin KIN. This medium is also suitable for a variety of other woody species (Kielse et al. 2009, Oliveira et al. 2013, Bezerra et al. 2014, Nowakowska et al. 2019, Sreelekshmi & Suma 2019).

It was possible to confirm that *V. japurensis* nodal segments in WPM medium show rapid growth, even without the need for plant hormones. Consequently, it was decided to increase the number of propagated plants *in vitro* for extraction using nodal segments inoculated in WPM medium, being later transferred to MS medium.

The production of secondary metabolites can be altered depending on the culture medium and growth regulators used; and cytokinins added for physiological purposes can act as culture elicitors (Govindaraju & Arulselvi 2018). A study carried out with *Aloe arborescens* Mill showed that the choice of the type of cytokinin and the exogenous concentration provided in the tissue culture influenced the proliferation of buds and also the *in vitro* production of secondary metabolites. Buds regenerated with cytokinins presented increased amounts of iridoids, phenolics, flavonoids and tannins (Amoo et al. 2012).

Such characteristics and elicitors, which are often not considered, can alter the chemical production of *V. japurensis*, which justifies the chemical difference presented between *in vitro* and *in situ* plant extracts presented in the work by Lima et al. (2022).

## Substance isolation and identification

Fractions 27-28 and 29-36 obtained from open column fractionation of the hexane extract were fractionated separately by HPLC. The fraction corresponding to the first eluted peak from each sample were collected separately. Peak 1 from the 27-28 fraction had a retention time of 4.35 minutes, while that for the 29-36 fraction was 3.99 minutes. The UV spectra of both were similar, showing absorbance between 250 and 400 nm, which suggested they were the same substance. An $^{1}$H-NMR analysis was performed separately and also indicated that they were the same substance.

The substance isolated appeared as a white crystal, with a total mass of 4 mg. The $^{1}$H-NMR spectrum showed two signals at δ 12.19 (*s*, 1H) and 12.29 (*s*, 1H), indicating the presence of two chelated hydroxyls, and at δ 2.42 (*s*, 3H), and δ 1.64 (*s*, 3H) indicative of two methyls (Figure S1). The δ 2.42 signal suggests a methyl group linked to the aromatic ring. In the region of aromatic ring hydrogens a triplet at δ 7.57 (*J* = 8 Hz; 1H), a doublet of doublets at δ 7.41 (*J* = 8, 1.1 Hz; 1H), two broad singlets at δ 7.24 (1H) and 6.76 (1H) and a doublet of doublets at δ 6.94 (*J* = 8, 1.1 Hz, 1H) were observed (Table V).

From the NMR signals and the correlations with COSY (correlation spectroscopy), HMBC (heteronuclear multiple bond correlation) and HSQC (heteronuclear single quantum coherence) it was inferred that the substance is an anthrone. $^{13}$C-NMR data were compared to the anthrone chrysophanol isolated by Tamano & Koketsu

**Table V. $^{1}$H and $^{13}$C NMR data of 1,8,10-trihydroxy-3,10-dimethyl-9 (10*H*)-anthracenone.**

| Position | Observed $^{13}$C | $^{1}$H (ppm) | HMBC δ | COSY |
|---|---|---|---|---|
| 1 | 162.82 | - | H-12.19 | |
| 2 | 117.32 | 6.76 (*bs*, 1H) | H-12.19, H-7.24, H-2.42 | H-2.42, H-7.24 |
| 3 | 149.17 | - | H-2.42 | - |
| 4 | 117.84 | 7.24 (*bs*, 1H) | H-6.76, H-2.42 | H-2.42, H-6.76 |
| 5 | 116.47 | 7.41 (*dd*, 1H, *J* 8; 1.1 Hz) | H-6.94 | H-7.57 |
| 6 | 137.09 | 7.57 (*t*, 1H, *J* 8 Hz) | | H-6.94, H-7.41 |
| 7 | 117.12 | 6.94 (*dd*, 1H, *J* 8; 1.1 Hz) | H-12.28, H-7.41 | H-7.57 |
| 8 | 162.61 | - | H-12.28, H-7.57 | |
| 9 | NO | | | |
| 8a | 113.31 | - | H-12.28, H-7.41, H-6.94 | |
| 9a | 111.21 | - | H-12.19, H-7.24, H-6.76 | |
| 4a, 10a | 150.36 | - | H- 7.57, H-1.64 | |
| 10 | 71.21 | | H-7.41, H-7.24, H-1.64 | |
| Me/C10 | 22.57 | 2.42 (*s*, 3H) | H-7.24 | H-6.76, H-7.24 |
| Me/C3 | 38.48 | 1.64 (*s*, 3H) | | |
| OH/C8 | | 12.28 | | |
| OH/C1 | | 12.19 | | |

**Legend: NO: not observed; *s*: singlet, *bs*: broad singlet, *dd*: double doublet, *t*: triplet.**

(1982), and the $^{1}$H-NMR data were compared to those of Lo et al. (2012). The signals obtained are in accordance with the cited references, differing from an anthrone isolated from *V. japurensis* propagated plants *in vitro* only by the presence of a methyl and a hydroxyl at carbon number 10. It was not possible to visualize the chemical shift of carbon 9 in the obtained spectra. According to a bibliographic survey carried out using Scifinder this is the first report of this substance, which should be called 1,8,10-trihydroxy-3,10-dimethyl-9(10*H*)-anthracenone. Comparison of the NMR data for this molecule with chrysophanol, the closest structure found in literature is given as supplemental material (Figure S4, Table SI).

Mass spectrometric analysis showed a value of *m/z* of 271.0956 in positive mode and *m/z* 269.0821 in negative mode, confirming the molecular formula $C_{16}H_{14}O_4$ (270 u) (Figures S2-S3). Figure 3 shows the molecular structure of 1,8,10-trihydroxy-3,10-dimethyl-9(10*H*)-anthracenone, and the correlations of their hydrogens and carbons. The $^{1}$H-NMR and mass spectra are available as supplementary material, as well as the NMR data comparison with the closest substance found in literature: chrysophanol.

## CONCLUSIONS

The aseptic establishment of *V. japurensis* was possible from seeds through the use of sodium hypochlorite in the decontamination stage. Of the three culture media evaluated, WPM medium stood out as the most suitable in both

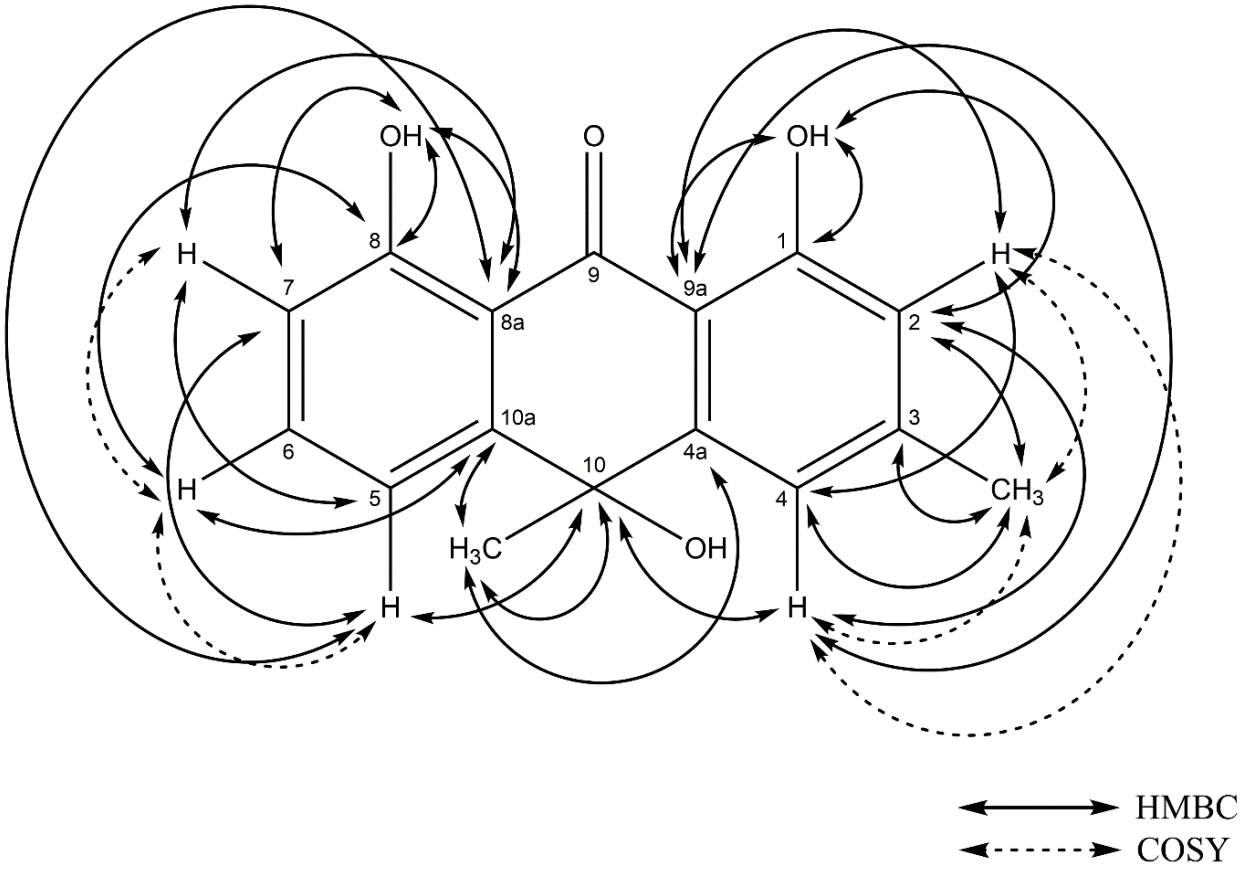


**Figure 3. HMBC and COSY correlations observed for 1,8,10-trihydroxy-3,10-dimethyl-9(10*H*)-anthracenone.**

the germination and multiplication stages for the studied species.

Chemical fractionation allowed the isolation of a new anthrone: 1,8,10-trihydroxy-3,10-dimethyl-9(10*H*)-anthracenone. Based on a literature survey, this is the first report of this substance. This work shows the great potential of *in vitro* tissue culture to yield new substances.

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## SUPPLEMENTARY MATERIAL

**Table SI.**
**Figures S1-S4.**

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## Author contributions

The authors of this publication contributed to it in the following ways: 1 – Laísley Martins Lima: performed all experimental work (dissertation thesis), which formed the basis for the article manuscript, and also , wrote the article; 2 – Weison Lima da Silva: assisted in chemical analysis and fractionation techniques; 3 – Julio Cezar de Souza: helped in the planning of the research and supervised plant cell cultures; 4 – Cecilia Veronica Nunez: work advisor, supported in planning and conducting the study, found financial support and helped writing the article. The authors declare that they have no conflict of interest.

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