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Sâmia Torres Silva

Federal University of Lavras: Universidade Federal de Lavras

Rafael Marlon Alves de Assis

Federal University of Lavras: Universidade Federal de Lavras

Adriane Duarte Coelho

Federal University of Lavras: Universidade Federal de Lavras

Simony Carvalho Mendonça

Federal University of Lavras: Universidade Federal de Lavras

Alexandre Alves de Carvalho

Federal University of Lavras: Universidade Federal de Lavras

José Eduardo Brasil Pereira Pinto (✉ jeduardo@ufla.br)

Federal University of Lavras: Universidade Federal de Lavras <https://orcid.org/0000-0002-1141-7907>

Suzan Kelly Vilela Bertolucci

Federal University of Lavras: Universidade Federal de Lavras

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Accumulation of podophyllotoxin in root culture of *Hyptis suaveolens* (L.) Poit: a potential natural lignan for clinically useful anticancer drugs

Sâmia Torres Silva¹, Rafael Marlon Alves de Assis², Adriane Duarte Coelho², Simony Carvalho Mendonça¹, Alexandre Alves de Carvalho², José Eduardo Brasil Pereira Pinto^{2*}, Suzan Kelly Vilela Bertolucci¹.

✉ José Eduardo Brasil Pereira Pinto
jeduardo@ufla.br

¹Laboratory of Phytochemistry and Medicinal Plants, Department of Agriculture, Federal University of Lavras, Zip Code 37200-900, Lavras, Brazil.

²Laboratory of Plant Tissue Culture and Medicinal Plants, Department of Agriculture, Federal University of Lavras, Zip Code 37200-900, Lavras, Brazil.¹

Abstract

Podophyllotoxin (PTOX) is a natural antiviral, antirheumatic and anticancer molecule but its chemical synthesis is expensive. The present study aimed to develop and validate an analytical method by HPLC for the quantification of PTOX in roots of *Hyptis suaveolens*, as well as to evaluate the culture of its roots *in vitro* in liquid medium supplement with different concentrations of Indole-3-butyric acid (IBA), 1-Naphthaleneacetic acid (NAA), vitamins and myo-inositol. The analytical method was developed and validated. Root culture was used for biomass production and PTOX content was quantified using the developed analytical method after successful validation. The parameters that confirmed the analytical method were selectivity (peak purity: > 99%), system suitability ($R_s = 2.92$; $N = 7064$; $k = 1.23$; $A_s = 1.31$; $DPR = 0.61\%$), intra-day and inter-day precision ($DPR = 2.43\%$ and 2.96% , respectively), linearity ($R^2 = 0.997$), recovery percentage (90.47% to 101.85%), limit of quantification (5.25 ng) and limit of detection (0.5 ng). Root culture in MS medium containing 1 mg L^{-1} IBA + 0.5 mg L^{-1} NAA showed the highest root dry weight (248.76 mg) and the highest PTOX concentration in the root ($179,97 \mu\text{g g}^{-1}$). The vitamins and myo-inositol in the medium produced 198.88 mg of root dry weight and $6.01 \mu\text{g g}^{-1}$ of PTOX. The roots cultured in liquid MS medium supplemented with 1 mg L^{-1} of IBA + 0.5 mg L^{-1} of NAA maximized the root biomass and PTOX content. The adequate balance of vitamin and myo-inositol supplementation in liquid MS culture medium increased the production of root dry weight and PTOX accumulation.

Keywords: *In vitro*, growth regulator, vitamins, HPLC-DAD analytical validation, aryltetralin lignan

Introduction

Podophyllotoxin (PTOX) is a highly valued aryltetralin lignan that is used worldwide for its antimitotic properties and as a prototype of molecules used in the treatment of cancer and genital warts caused by human papillomavirus (HPV) (Castro *et al.* 2015; Kumari *et al.* 2017). PTOX is a natural precursor for the production of chemotherapeutic agents, including the semisynthetic derivatives etoposide, etoposide phosphate and teniposide, among others. These compounds provide treatment for lung, cervical and testicular cancer, as well as neuroblastoma, hepatoma and leukemias (Li *et al.* 2018; Shah *et al.* 2021). The chemical synthesis of PTOX is difficult due to the presence of four contiguous chiral centers and a *trans*-lactone portion sensitive to the bases (Shah *et al.* 2021). Because its chemical synthesis is a complex process, the price of PTOX obtained by this form is high. Thus, obtaining it through natural sources is more economically viable (Kumari *et al.* 2017).

PTOX occurs in species of the genera *Podophyllum*, *Sinopodophyllum*, *Nepeta*, *Salvia*, *Thymus*, *Linum*, among others. In the literature, plants that are PTOX sources are overexploited and may be at risk of extinction, as is the case with the species *Podophyllum peltatum* and *Podophyllum hexandrum* (Ardalani *et al.* 2017; Kumar *et al.* 2015; Kumari *et al.* 2017; Shah *et al.* 2021). To meet growing pharmaceutical needs, studies to identify new forms of production, such as the tissue culture using organs are needed to circumvent this problem (Ardalani *et al.* 2017; Kumari *et al.* 2017). Another alternative explored have been cell suspension cultures but produce only small amounts of PTOX (max. 0.65% dry weight) (Seegers *et al.* 2017).

Many medicines are derived from plants, and the health of the world's population is dependent on medicinal species. Biotechnology with tissue culture, has been gaining prominence in the production of pharmaceutical compounds of interest to the drug industry (Hussain *et al.* 2022). *Hyptis suaveolens* (L.) Poit (Lamiaceae), known as Bamburral or weed-

straw, is a species native to tropical America but has a wide distribution and is considered an invasive and medicinal plant (Padalia *et al.* 2014). *H. suaveolens* has podophyllotoxin (PTOX) in its roots (Velóz *et al.* 2013). However, with the species *Hyptis suaveolens* it has few works. Therefore, this species can be an alternative for the production of the active compound. It is a species found a lot in Brazil.

Recent plant research advancements depict that to produce biologically active compounds, the cultivation of root cultures is a great alternative to other methods. According to Isah *et al.* (2018) in many plants grown *in vitro*, the yield of secondary metabolites may be higher in the roots than in other plant parts. They may be transformed or unrooted root crops, with the possibility of commercial application (Gonçalves and Romano 2018; Hussain *et al.* 2022). The advantages of using *in vitro* root culture for biomass production and PTOX production over the traditional use of plant raw materials, allow a product to be obtained all year round regardless of external climatic and soil conditions while preserving the natural habitats of valuable medicinal plants. Besides that, shorter time to cultivate the roots and the ease of extraction and purification of preparations, the enhancement of the biosynthesis of the necessary substances with the help of elicitors, process automation.

However, the composition of plant culture media is an important parameter for both growth and specialized metabolite production. The influence of culture medium and various other culture parameters (hormone, carbon source and concentration, elicitation and precursor feeding) on aryltetralin lignan production was evaluated by Renouard *et al.* (2018) in hairy root cultures of *Linum flavum* (L.). The highest PTOX accumulation and exudation yields were measured using Gamborg B5 medium supplemented with sucrose at a 6% (w/v) concentration. However, supplementation with 1 mg/mL naphthaleneacetic acid (NAA) negatively influenced PTOX production. Silva *et al.* (2022) reported that adventitious root formation and PTOX

induction in *H. suaveolens* leaves grown in semi-solid MS medium only happen in plants grown in the dark and under monochromatic red LED light.

Myo-inositol is one of the most used vitamins in the culture medium and belongs to the vitamin B complex. Plant, yeast and fungal growth, and in humans' inositol is essential (Farlex 2010). Myo-inositol play important role in plants, because participates in the transport of indole-3-acetic acid (IAA) and controls growth and cellular expansion IAA-induced (Loewus and Murthy 2000). In addition, play others roles such as growth regulation, membrane biogenesis, signal transduction, ion channel physiology and membrane dynamics (Loewus and Murthy 2000). Myo-inositol in combination with IBA significantly increased root fresh and dry matter of cherry rootstock and medicinal plant *Hyoscyamus niger* (Hong *et al.* 2010; Sarropoulou *et al.* 2013; Sarropoulou *et al.* 2015). The increase in the root fresh matter can be attributed to an increase in cell volume and/or expansion of new cells. Also, the elevated levels of myo-inositol were very effective in stimulating root formation in both apple and pear shoots (Toma 2012). The most widely used vitamins are those of B group, thiamine (vitamin B1), nicotinic acid (niacin or vitamin B3), pyridoxine (vitamin B6) are considered essential ingredients of plant tissue culture media. Vitamins are considered important components which induce plant cell growth also their role in stimulated the bioactive metabolites (George *et al.* 2008; Pérez *et al.* 2004).

Another very important point to consider in the development of studies involving the measurement of secondary metabolites is the use of a rigorous analytical method. In order for PTOX obtained from plant tissue culture to be used by the pharmaceutical industry, it is necessary to validate the analytical method used. The validation of an analytical method is the process of demonstrating that the procedure used for the quantitative analysis of a substance of interest presents precision, accuracy and reliability of the results (Patil *et al.* 2017). Although there are analytical methods using liquid chromatography validated for the measurement of

PTOX in the literature (Brandão *et al.* 2017; Sharma *et al.* 2018), the application of any analytical procedure has intrinsic specificity within a particular matrix. Methods need to be validated or revalidated (Patil *et al.* 2017). Revalidation is required when the scope of the validated method has changed, for example, if operating conditions have changed (Patel *et al.* 2011). Approval of products by regulatory authorities requires the applicant to prove control of the entire development process. Method validation results in official testing methods and quality control (Ravisankar *et al.* 2015).

Thus, the objective of this study was to develop and validate an analytical method by HPLC for the quantification of PTOX in roots of *Hyptis suaveolens*, as well as to evaluate the *in vitro* culture of roots of the species in liquid medium with different concentrations of IBA (Indole-3-butyric acid) and NAA and with different concentrations of vitamins and myo-inositol.

Materials and methods

Plant material

To validate the analytical method by HPLC for PTOX quantification, roots of *Hyptis suaveolens* mother plants grown in a greenhouse or adventitious roots from *in vitro* cultivation were used.

The root donor *Hyptis suaveolens* seedlings for the analytical validation and *in vitro* cultivation experiments were established from seed inoculation of the mother plants. Before asepsis, the mucilage of the seeds was removed using lime and water. Then, they were immersed in 50% aqueous sodium hypochlorite solution and kept under constant stirring for 20 min. In a laminar flow chamber, they were washed five times with autoclaved distilled water. The culture medium for seed inoculation was MS (Murashige and Skoog, 1962) with half the

original concentration of salts, 30 g L⁻¹ of sucrose, and 5.5 g L⁻¹ of agar, with the pH adjusted to 5.7 ± 0.1.

Vouchers of the mother plants were deposited in the ESAL Herbarium (Herbarium of the Department of Biology of the Federal University of Lavras, Lavras-MG, Brazil) under registration 20475 and in the SPF Herbarium (Herbarium of the University of São Paulo, São Paulo-SP). Brazil) under the collection name HB Andrade 1 (SPF).

Development of the analytical method

Chemicals

Chloroform stabilized with amylene (Synth[®] and Fmaia[®]), ethyl acetate (Vetec[®]), absolute ethyl alcohol (99.8%; Alphatec[®]), phosphoric acid (Vetec[®]) and sodium nitrite (97%) were all grade for analysis. For the preparation of phosphate buffer, anhydrous monobasic potassium phosphate (Sigma–Aldrich[®]), anhydrous dibasic potassium phosphate (Qhemis) and potassium hydroxide (Alphatec[®]) were used. Acetonitrile (JT Baker[®]) and methanol (AppliChem Panreac[®]) HPLC grade and podophyllotoxin (PTOX) (Sigma-Aldrich[®], HPLC-declared purity ≥ 98%) were used for liquid chromatography.

Extractive optimization and sample preparation

Several PTOX extraction methods have been described in the literature (Brandão *et al.* 2017; Velóz *et al.* 2013; Zheljazkov *et al.* 2013). Based on these studies, an effective condition was first determined to extract the maximum amount of PTOX from the dehydrated roots of *H. suaveolens*. Extracts of a single batch of *H. suaveolens* roots were prepared by ultrasound-assisted extraction without or with pretreatment with 25 mM potassium phosphate buffer pH 7, followed by extraction with distinct solvents (ethyl acetate/chloroform/absolute ethanol; duty cycle (1 and 2); time sonication (15 and 30 min) as described in Table S1. The extracts were

analyzed by HPLC-PDA, and the peak areas of PTOX obtained in the exploratory run condition were submitted to ANOVA, and the means were compared by the Tukey test ($p < 0.05$).

The established extraction method comprised the extraction of 25 mg of roots in two 30-min sonication cycles. In the first cycle, the roots were sonicated with 5 mL of 25 mM phosphate buffer, pH 7, followed by another 15 min of sonication with 5 mL of chloroform. The solution was vacuum filtered in a Büchner funnel and transferred to a 15 mL test tube. For the second sonication cycle, 3 mL of the aqueous fraction of the first cycle was collected, repeating the same operation as the first. From the organic phase of each cycle, 1.25 mL of extract was taken, combined and concentrated in a rotary evaporator at 40 °C.

The residue was dissolved in 1 mL MeOH (HPLC-grade). Then, the sample was transferred to a microtube and subjected to sonication for 2 min, followed by centrifugation at 10,000 rpm for 10 min. The supernatant was automatically injected into the chromatograph.

Chemical stability of PTOX in samples

The chemical stability of PTOX in the samples ($n = 6$) was evaluated by maintaining the solutions in the chromatograph carousel at room temperature (23 ± 2 °C), which were analyzed sequentially at intervals of 0, 6, 12, 18, 24, 30, 36 and 42 h using exploratory running conditions. The PTOX concentrations were calculated at each time interval and subjected to analysis of variance by the F test ($p < 0.05$); the means of the data were compared by Tukey's test ($p < 0.05$).

Development and establishment of the chromatographic condition

The chromatographic analyses were carried out using an Agilent 1200 liquid chromatography system (Agilent Technologies®, Waldbronn, Germany) equipped with a quaternary pump (G1311A) with a degasser (G13222A) and ALS autosampler (G1322A) and

a thermostat-controlled column compartment TCC (G1316A). A variable-wavelength ultraviolet detector (G1315D) was used to obtain the chromatogram at 210 nm. The equipment was controlled by OpenLAB software version A07.04, build 04.07.28.

The development of the chromatographic conditions for the quantification of PTOX started from the analysis of an exploratory profile comprised of a linear gradient obtained from samples of *H. suaveolens* roots. The elution conditions were developed and optimized by adjusting the chromatographic parameters regarding the proportions of the organic modifier, temperature, flow, elution mode and gradient slope. The established chromatographic conditions were performed in a C18 reversed-phase analytical column (Eclipse XDB-C18, Agilent Technologies®, USA) with silica-based packing (150 mm × 4.6 mm ID, 5 µm) and a precolumn (12, 5 mm × 4.6 mm ID, 5 µm). Ten microliters of sample were injected into the column at a temperature of 25 °C. Water (A) and acetonitrile (B) were used as eluents, both containing 0.1% (v/v) phosphoric acid, at a constant flow rate of 1.0 mL/min as follows: linear gradient elution from 50% B to 70% B in 2.5 min, followed by 70% B to 95% B up to 5 min; isocratic elution of 95% B for 1 min and then returned to initial conditions in 2 min. A 2-min equilibrium time was allowed between injections, showing a short overall run time (10 min). PTOX peak identification was based on UV data recorded online by DAD at 210 nm, as well as on the co-injection of standard.

System suitability

The parameters of system suitability (System Suitability Test) were calculated (resolution (*R* s), capacity coefficient (*k*), asymmetry factor (*A* s), number of theoretical plates (*N*) and relative standard deviation (RSD) of retention time (RT) of the peak corresponding to PTOX). The data were obtained using the *System Suitability* of the OpenLab EZChrom®

software, according to *The United States Pharmacopoeia* (2006) (USP) and the results were expressed as the mean value of six determinations.

Method validation

The parameters followed were according to international conference on harmonization (ICH 1996) and Brazil (2017) guidelines. OpenLab EZChrom[®] software version A.04.07 (Agilent) was used for data acquisition and processing. Statistical analyses were performed using Microsoft Excel[®] (Microsoft Corporation, 2003), GraphPad Prism[®] version 6.0 (Mac, GraphPad Software, La Jolla, CA) and R.

Selectivity

The analytes were analyzed by spectral homogeneity of the peak corresponding to PTOX at 210 nm to determine the purity of the peaks. This analysis was performed by overlapping spectra recorded in the ascending, apical and descending regions of the peaks simultaneously. The PTOX peak was considered pure when the spectral similarity indices were $\geq 95.0\%$. System suitability was assessed by the outcomes for resolution (R_s), retention factor (k), asymmetry factor (A_s), number of theoretical plates (N), and RSD of the retention time of peaks. The results were expressed as the mean value of six determinations.

Linear regression, precision, limit of quantification (LOQ) and detection (LOD), and recovery

Standard PTOX solutions ($25 \mu\text{g mL}^{-1}$) were prepared in methanol. Three calibration curves were obtained by plotting the injected mass of PTOX (6.25–200.00 ng) against the peak area on three separate days using linear regression. The curves obtained were statistically compared by covariance analysis ($p < 0.05$), and the mean equation of the three curves was used for quantitative PTOX calculations. The intra-day precision was evaluated at 100% of the

working concentration by reference to the RSD values of sample solutions analyzed on the same day (n = 6). The inter-day precision (n = 12) was carried out with 2 analysts on 2 different days to analyzing sample solutions. To determine the LOQ and LOD values standard solutions were injected at decreasing concentrations into the HPLC equipment in quintuplicate. Peak area with an RSD below 5% as the concentration of the LOQ, whereas a sign-to-noise ratio (S/N) of 2 was defined as LOD (AOAC 2000; Brazil 2017). Known standard solutions were added to *H. suaveolens* root powder to determine recovery, this was done before extraction at three different concentration levels within the analytical concentration range of the curve.

Robustness

Six samples were prepared to analyze the established conditions and the column temperature variation (24 and 26°C), flow rate (0.9 and 1.1 mL/min) and phosphoric acid concentration in the mobile phase (0.09 and 0.11%). Following the determinations of RDC ANVISA 166, the concentrations of PTOX were compared by ANOVA, and the means were compared by Tukey's test ($p < 0.05$) (Brazil 2017). The spectral purity of the peak corresponding to PTOX was also evaluated as described for selectivity evaluation.

In vitro culture of *H. suaveolens* roots in liquid medium with IBA and NAA

Fresh roots (0.4 g) from 45-day-old seedlings were inoculated in 40 mL of liquid MS culture medium containing 30 g L⁻¹ sucrose, at pH 5.7 (Murashige and Skoog 1962). The treatments were as follows: 1) control: liquid MS medium without regulator, 2) 0.5 mg L⁻¹ IBA + 0.5 mg L⁻¹ NAA; 3) 0.5 mg L⁻¹ IBA + 1 mg L⁻¹ NAA; 4) 1 mg L⁻¹ IBA + 0.5 mg L⁻¹ NAA; 5) 0.5 mg L⁻¹ IBA; 6) 1 mg L⁻¹ IBA; 7) 0.5 mg L⁻¹ NAA; and 8) 1 mg L⁻¹ NAA. The experimental design was completely randomized (CRD) with 10 replicates per treatment.

The roots were kept in a shaker at 80 rpm in the absence of light for 42 days. The variables analyzed were the fresh and dry weight of the initial and final roots (time zero and at 42 days, respectively), fresh weight growth index (FWGI = root fresh weight final (FRWF) - root fresh weight initial (RFWI)/RFWI, dry weight growth index (DWGI = root dry weight final (RDWF) - root dry weight initial (RDWI)/RDWI, PTOX content in the initial and final dry roots, and PTOX content present in the culture medium.

The initial dry weight (11.69 mg) was obtained from the dehydration of 30 samples of 0.4 g of roots. The amount of PTOX in the initial dry roots ($159.50 \mu\text{g g}^{-1}$) was obtained from the triplicate analysis from a sample composed of 30 root samples.

For the analysis of PTOX in the culture medium, after vacuum filtration, it was stored in a freezer until analysis. To prepare these samples, after thawing, a 1 mL microtube of liquid culture medium + 500 μL of chloroform was collected. The samples were vortexed for 1 min (vortex) and centrifuged at 10,000 rpm for 5 min. The organic phase was separated and evaporated to dryness under a gas exhaust hood. The residue was resuspended in 250 μL of HPLC grade methanol, vortexed for 30 seconds and automatically injected into the HPLC system.

In vitro culture of *H. suaveolens* roots in liquid medium with different concentrations of vitamins and myo-inositol

Roots of 46-day-old seedlings were used as explants. The liquid MS culture medium (40 mL) was supplemented with 1 mg L^{-1} IBA, 0.5 mg L^{-1} NAA and 30 g L^{-1} sucrose, with the pH adjusted to 5.7 ± 0.1 . Four treatments were performed: T1) MS without vitamins and without myo-inositol; T2) MS with $\frac{1}{2}$ of the vitamins (thiamine, nicotinic acid and pyridoxine 0.25 mg L^{-1} of each) and $\frac{1}{2}$ of the myo-inositol (50 mg L^{-1}); T3) MS with the original concentration of vitamins (thiamine, nicotinic acid and pyridoxine 0.5 mg L^{-1} of each) and myo-inositol (100 mg

L⁻¹); and T4) MS with twice as many vitamins (thiamine, nicotinic acid and pyridoxine 1.0 mg L⁻¹ of each) and twice as many myo-inositol (200 mg L⁻¹), and each treatment consisted of 10 replicates. The experiment was evaluated at 44 days regarding the fresh weight (RFW) and dry weight (RDW) of roots and the PTOX content in the dry roots.

Statistical analyses

The extractive optimization and tissue culture data were analyzed by simple analysis of variance (ANOVA) and Tukey and Scott Knott tests, respectively. Differences between the evaluated groups were considered statistically significant when $p < 0.05$. The statistical analyses of the validation of the method were performed in the programs Microsoft Excel[®] (Microsoft Corporation, 2003), GraphPad Prism[®] version 6.0 (Mac, GraphPad Software, La Jolla, CA) and R software.

Results and discussion

Development of the analytical method

To achieve the best extraction efficiency for PTOX in *H. suaveolens*, several extraction conditions, based on sample extracts reported in the literature, were optimized by analyzing different extracted samples. The experimental parameters included nonbuffered and buffered pretreatment samples; extraction with different solvents (ethyl acetate/chloroform/absolute ethanol); duty cycle (1 and 2); and sonication time (15 and 30 min).

The PTOX extraction method described by Brandão *et al.* (2017) consumes 2 h of extraction with ethyl acetate and requires a C18 Sep-Pak cartridge for preconcentration of the sample, which increases the analysis costs. The method of Velóz *et al.* (2013), although simple, consumes 6 days of extractive procedure. The methodology of Zheljazkov *et al.* (2013) requires equipment and techniques for extract drying that are unusual and economically unfeasible for

most analytical laboratories. Therefore, compared to the methodologies described in the literature, the condition of sample preparation for *H. suaveolens* roots has the advantages of being an easily executable procedure in routine analyses in analytical laboratories due to the availability of reagents and equipment, with non-excessive time consumption, low cost and effectiveness for the extraction of the constituent of interest. In addition to the data being statistically evaluated by ANOVA and Tukey's test ($p < 0.05$) (for detail see Figure S1 in supplementary material), the efficacy of the optimized extraction conditions was further evaluated by recovery studies, as shown in the next section.

The chemical stability of the samples was assessed by the sequential analysis of sample solutions ($n = 6$) stored at room temperature for up to 42 h. There was no significant difference ($p > 0.05$) in the assayed concentration within the evaluated period, as shown in Table S2.

The development of the chromatographic conditions for the quantification of PTOX in *H. suaveolens* started from an exploratory analysis employing a linear gradient of 5 to 95% acetonitrile for 70 min. To ensure the shortest possible analysis time, selectivity and adequate resolution of PTOX with neighboring peaks ($R_s > 1.5$), changes were made in the proportions of the organic modifier, temperature, flow, elution mode (isocratic and linear gradient) and gradient slope. The established chromatographic conditions resulted in a shorter analysis time (10 min) and lower baseline fluctuation, selectivity and resolution greater than 1.5 for the quantification of the PTOX peak. The overlap of the UV spectra of the sample peak at 2.73 min and the peak of the PTOX standard attested to the identity and purity of the analytical peak. Figure 1 shows the typical chromatographic profile of the *H. suaveolens* root sample and the spectral homogeneity analysis.

The reliability of the chromatographic conditions was ensured by system suitability tests, whose outcomes were in agreement with values specified by Epshtein (2020), except for retention factor ($k = 1.23$, Table 1). However, this was not a problem because the peak had good

resolution ($R_s = 2.92$) at a low retention time ($RT = 2.73$). According to Epshtein (2020), the capacity coefficient can be arbitrarily considered an additional characteristic of the chromatographic system resolution.

Method validation

Selectivity

The spectral purity of the PTOX peak in the sample solution was assessed by UV/PDA. The spectra were evaluated in the ascending, apex, and descending regions of the target peaks. The similarity value calculated by the EZChrom[®] software was 99%. The results showed that they were identical to their respective standards, indicating that other compounds did not coelute.

Linear regression, precision, limit of quantification (LOQ), limit of detection (LOD), and recovery

The calibration curve of the mass versus peak area plot for PTOX was linear over the established range (6.0–200.0 ng) with an r^2 of 0.997 for the regression equation ($y = 14.258x + 3.020$). The calibration curves obtained on three consecutive days were statistically similar ($p > 0.05$). The method showed repeatability ($n = 6$) and intermediate precision ($n = 12$) results for precision, as seen in Table 2. The validation manual of the Association of Official Analytical Chemists (AOAC 2013) recommends that repeatability values be estimated as a function of the analyte concentration in the study sample because analyses using diluted analyte concentrations may lead to greater variation in the responses, and in these cases, coefficients of variation higher than those conventionally used can be accepted. The concentration of PTOX in the sample was approximately 0.03%. According to the AOAC (2013) guide for analytes with concentrations of 0.01%, the maximum RSD value of 4% is accepted. For more accurate calculations, the guide also provides the equation $RSD (\%) = 2C^{-0.15}$, where C is the analyte concentration in mass

fraction. Applying this formula, there was a DPR of 3.38% for the validating method, and thus, it was concluded that the DPR value found (2.43%) was in accordance with the recommendation of the AOAC. Very low values for the limit of quantification (LOQ) and limit of detection (LOD) show that the high method sensitivity (Table 2). The recovery range of 90.47% to 101.85% for samples spiked with standard PTOX, show that the method validation is accurate (Table 3). Three concentration levels of standards solution were added before the extraction procedure (high, medium, and low) according to ANVISA (Brazil 2017). For quantitative analysis there is no fixed value to consider recovery acceptable, since it depends on the analyte concentration in the sample, according to the AOAC (2013) guidelines. Podophyllotoxin concentrations ranging from 0.01% to 0.1% w/w was used to carried out the recovery test. For analytes with this concentration range, recovery rates of 85-110% and 90-108%, respectively, are recommended. Therefore, the recovery values were acceptable for the method.

Robustness

Among the experiments where variations were introduced in the analytical conditions established for the method, the PTOX levels showed no significant difference ($p > 0.05$). Small variations in the analysis conditions do not compromise the reliability of the results. Therefore, the validation method proved to be robust under column temperature, flow rate and phosphoric acid concentration in the mobile phase.

In vitro culture of *H. suaveolens* roots in liquid medium with IBA and NAA

Supplementation with different concentrations of IBA and NAA significantly affected the production of roots and PTOX in *H. suaveolens*. The highest accumulation of fresh and dry root material was observed in treatments 2 (0.5 mg L⁻¹ IBA + 0.5 mg L⁻¹ NAA), 4 (1 mg L⁻¹

IBA + 0.5 mg L⁻¹ of NAA) and 6 (1 mg L⁻¹ of IBA) (Table 4, Figure 2). Among these, treatment 4 accumulated the highest final fresh (6.49 g) and dry (248.76 mg) weight, with a fresh growth index of approximately 12.2 times and a dry growth rate 14.1 times higher than the control (Table 4). Considering the initial root dry weight (11.69 mg) and the final root dry weight of treatment 4 (248.76 mg), proportional to the final root dry weight obtained in the study by Velóz *et al.* (2013), treatment 4 of the present study had a production of root dry weight 21.3 times higher than that of the previous study. In the study by Velóz *et al.* (2013) 10 g of fresh roots of *H. suaveolens* were grown in liquid MS medium without supplementation with growth regulators for 35 days, resulting in 15.8 g of fresh roots and 2.10 g of dry roots.

The type, concentration and combination of auxin greatly influences the processes of induction and proliferation of adventitious root culture. IBA and NAA are the most suitable auxins for the induction and development of adventitious root cultures of medicinal plants (Rahmat and Kang 2019). IBA, compared to other types of auxin, is a hormone that is more suitable for the adventitious root culture of *Centella asiatica* (L.) Urb. (Ling *et al.* 2009) and *Podophyllum hexandrum* Royle (Rajesh *et al.* 2012). NAA also has a significant influence on the induction and proliferation of adventitious root cultures of *Boesenbergia rotunda* (L.) Mansf. (Azhar *et al.* 2018), *Fagonia indica* Burm.fil. (Khan *et al.* 2017) and *Mondia whitei* (Hook.fil.) Skeels (Baskaran *et al.* 2016).

The combination of IBA and NAA is also very successful for the *in vitro* growth of adventitious roots of medicinal plants. For example, a combination of 1.0 mg/L NAA and 2.0 mg/L IBA was selected as the optimal media for root induction in *Zingiber zerumbet* Smith adventitious root cultures (Alwakil *et al.* 2022) and an IBA of 1.0 mg/L and an NAA of 1.0 mg/L were suitable for *Luffa acutangula* (L.) Roxb. (Umamaheswari *et al.* 2014).

Regarding PTOX accumulation, the control treatment (T1, without growth regulators) had the highest concentration of PTOX in the roots (211.66 µg g⁻¹). However, this treatment

was the one that exuded the lowest PTOX content in the culture medium ($0.06 \mu\text{g mL}^{-1}$) (Table 5). However, due to the direct relationship between biomass accumulation and secondary metabolite content, treatment 4, which accumulated more fresh and dry weight, had the highest PTOX content. Treatment 4 accumulated $44.99 \mu\text{g}$ of PTOX in the roots and $29.15 \mu\text{g}$ in the medium, and consequently, had the highest sum of these amounts ($74.14 \mu\text{g}$). Compared to the PTOX content observed by Velóz *et al.* (2013) in roots, the content of treatment 1 was 44% higher and that of treatment 4 was 33.3% higher, indicating a substantial improvement in PTOX accumulation. Velóz *et al.* (2013) did not observe PTOX excretion from the roots into the medium. In turn, in *in vitro* cultures, plantlets and calluses obtained through 30 hormonal combinations, Velóz *et al.* (2013) observed very low accumulations of PTOX only in the following combinations: NAA (1 mg/mL)+Kinetin-Kin (0.06 mg/mL); NAA (0.4 mg/mL)+Kin (0.2 mg/mL); and NAA (0.4 mg/mL)+6- Benzylaminopurine-BAP (0.06 mg/mL), respectively, with values of 0.042 , 0.018 and $0.049 \text{ mg PTOX/g}_{\text{DPM}}$. In the present study, in the absence of growth regulators or in the presence of different concentrations of IBA and NAA, contents ranging from 54.44 to $211.66 \mu\text{g g}^{-1}$ were observed (0.05 to 0.21%). Adventitious roots were induced in leaf explants and nodal segments of *H. suaveolens* and NAA was the best PGR to induce adventitious roots' emergence in leaves, whereas IBA was the best one for in nodal segment (Silva *et al.* 2022). However, PGR NAA inhibited the synthesis of PTOX, but IBA induced this secondary compound (Silva *et al.* 2022). The induction of adventitious roots in *Podophyllum peltatum* was optimal on $1/2$ MS medium lacking NH_4NO_3 and supplemented with $14.78 \mu\text{M}$ IBA, also was observed PTOX production (Anbazhagan *et al.* 2008). The accumulation of PTOX in the hairy roots of *H. suaveolens* was greatest when using MS at three-quarters strength without hormones and supplemented with 2 L^{-1} thiamine (Bazaldúa *et al.* 2019).

In the study by Nikule *et al.* (2020) with calli of the species *Phyllanthus tenellus*, it was shown that the accumulation of polyphenolic lignans phyllanthin, hypophyllanthin and niranthin was variable according to auxin and cytokinins incorporated into the medium. The lignan content was higher in calli grown in MS medium supplemented with 2.0 mg L⁻¹ NAA. According to Anjum *et al.* (2017), information on the biosynthesis, accumulation and regulation of PTOX in *in vitro* plant cultures is limited. In their study, the authors found that NAA increased the production of lignans in callus cultures of *Linum usitatissimum* L.

Figure 3 shows that in the treatment that was not supplemented with growth regulators (treatment 1), there was no darkening of the medium. In the presence of growth regulators, the media was a light yellow to brownish red color, indicating differences in their chemical profiles by HPLC-HPLC, without affecting the purity of the peak of interest. In a culture of lateral shoots of *H. suaveolens* grown in liquid MS medium enriched with 100 mg L⁻¹ myo-inositol and different concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (Kin) or BAP maintained at 25 °C in a culture room with a 16-h photoperiod, Velóz *et al.* (2013) observed that the tissue was phenolized and died during the first week of culture. Additionally, in the study by Velóz *et al.* (2013) the treatments with a low NAA (0.4 and 1 mg/mL) but a high 6-BAP (0.5 mg/mL) were phenolized.

The chemical synthesis of podophyllotoxin is very complicated and depend upon the supply of raw materials. In addition to, plants grown from *P. hexandrum* rhizome take at least 4 to 5 years to produce raw material for the production of podophyllotoxin and plants from seeds would take even longer (Moraes *et al.* 2002). Market demand for podophyllotoxin is increasing and human overexploitation results in endangered natural resources. Since the natural supply is limited, there is a possibility of employing plant cell or organ cultures for the biotechnological production of podophyllotoxin. Wink *et al.* (2005) reported a production of 130 mg L⁻¹ of podophyllotoxin from cell suspension and root culture in several species of

Linum. Traditionally, PTOX has been extracted from rhizomes of two major sources, *Podophyllum hexandrum* and *P. peltatum*. The first species is preferred because its resin is richer in PTOX, with a content of about 4.3% dry weight against 0.25% in *P. peltatum* (Giri and Lakshmi Narasu 2000; Gordaliza *et al.* 2004; Wink *et al.* 2005; Yousefzadi *et al.* 2010). Wild roots of *H. suaveolens* showed a concentration of 0.05 mg PTOX per g of dry plant material and in root culture extracts was 0.12 mg PTOX per g of dry plant material (Velóz *et al.* 2013). In the present study PTOX content for in vitro root culture extracts was 0.18%. Therefore, *Hyptis suaveolens* species can be an alternative for the production of the active compound.

In vitro culture of *H. suaveolens* roots in liquid medium with different concentrations of vitamins and myo-inositol

The highest accumulation of root fresh and dry weight was observed in cultures with liquid MS medium at half the indicated concentration of vitamins and myo-inositol and in standard liquid MS medium. However, in twice the amount indicated in the formulation of the MS medium, they were detrimental to growth. However, the liquid MS medium without vitamins and without myo-inositol had the highest accumulation of PTOX ($7.50 \mu\text{g g}^{-1}$), followed by the MS treatment with half of vitamins and half of myo-inositol ($6.01 \mu\text{g g}^{-1}$) (Table 6). There was no detectable amount of PTOX in the culture medium. In the *in vitro* cultivation of *H. suaveolens* roots, Velóz *et al.* (2013) supplemented liquid MS medium with 100 mg L^{-1} myo-inositol in the absence of regulators and obtained 36.7% growth at the end culture period (35 days) in comparison with the inoculum.

The addition of vitamins and myo-inositol to the culture medium was essential for root growth due to the functions these constituents have. According to George *et al.* (2008), vitamins are necessary for plant cells as essential intermediates or metabolic catalysts. Myo-inositol is

related to cell wall biogenesis, auxin storage, phytic acid synthesis, oligosaccharide synthesis and stress tolerance (Sharma *et al.* 2020).

The production of lignans by plant tissues is related to the defense and regulation of growth. Its biosynthesis is adjusted so that plants can adapt to their development conditions (water conditions and reactions to stresses) (Behr *et al.* 2018). The changes in the concentration of vitamins and myo-inositol indicated that it did not cause a sufficient stress condition for the exudation of PTOX by the root in the culture medium.

Conclusion

In conclusion, a simple and rapid HPLC method was developed and validated for PTOX quantification in adventitious root cultures of *H. suaveolens*. The developed analytical method is specific, sensitive, linear, precise, accurate, and robust. The present investigation shows better results than predictions report on the growth and accumulation of PTOX of adventitious root culture in *H. suaveolens*. The herb may emerge as one of the alternative sources of this natural lignan for clinically useful anticancer drugs. The synergistic applications of plant growth regulators (IBA and NAA) enhanced the fresh and dry root weight and PTOX content. The maximum production of this ariltetralin lignan was obtained in *in vitro* root culture of *H. suaveolens* in liquid MS medium supplemented with 1 mg L⁻¹ IBA + 0.5 mg L⁻¹ NAA for 42 days (root culture was 0.18%). The addition of vitamins and myo-inositol to the culture medium was necessary for root growth but not for PTOX accumulation. The cultivation of roots in liquid medium with 50% of the original concentration of vitamins and myo-inositol of the liquid MS culture medium was more suitable for the species, simultaneously presenting a greater accumulation of root dry weight and PTOX. The adventitious root culture protocol should be useful for the application of biotechnological approaches for improving the *in vitro* production of this bioactive lignan.

517

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524

525 **Author contribution**

526 All authors contributed substantially to the work reported. STS, SKVB, and JEBPP
527 conceived the study, designed the experiments; RMAA, ADC and AAC, performed
528 experiments, analyzed data; STS, SCM and SKVB did the chemical analyses. STS, SKVB, and
529 JEBPP wrote the manuscript. All authors read and approved the manuscript.

530

531 **Data availability**

532 The authors confirm that the data supporting the findings of this study are available
533 within the article.

534

535 **Declarations**

536 **Ethics approval and consent to participate** Not applicable.

537 **Consent for publishing** Not applicable.

538 **Code availability** Not applicable.

539 **Conflict of interest** The authors declare no competing interests.

540

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Table 1. System suitability parameters assayed for the developed HPLC-PDA method.

Peak	Parameters ^a						
	<i>Rs</i>	<i>As</i>	<i>N</i>	<i>k</i>	RT	RSD	3-Point purity
PTOX	2.92	1.31	7064	1.23	2.73	0.61%	0.99
Recommended values*	$R_s \geq 1.5$	$0.8 \leq 1.5$	≥ 2000	$K > 2$	-	$\leq 2\%$ for $n \geq 5$	0.95

^aEvaluated parameters: resolution (*Rs*), tailing (*T*), number of theoretical plates (*N*), capacity coefficient (*k*) and relative standard deviation (RSD) of the retention time (RT, *n* = 6) recorded for PTOX peak. * Values recommended by the Epshtein (2020), except for 3-point purity.

Table 2. Validation parameter determination coefficient (n = 3), LOQ (ng), LOD (ng), repeatability (n = 6) and intermediate precision (n = 12) for PTOX in *Hyptis suaveolens* in vitro root powder (for chromatographic conditions, see Fig. 1).

Validation parameters	Response values
Ranges of linearity (ng)	6.25-200.00
Regression equation ¹	y=14,258x+3,020
Determination coefficient (r ²) ¹	0.997
LOQ ²	≥ 5,25 ng
LOD ³	≥ 0,5 ng
Repeatability - Av. content (RSD)	76.58 (2.43%)
Intermediate precision - Av. content (RSD)	75.93 (2.96%)

Legend: Av. = average. RSD = relative standard deviation. 1Av. of 3 analytical curves. 2LOQ follows the criteria of the Association of Official Analytical Chemists (AOAC, 2000), DPR ≤ 5%. 3LOD follows RDC 166/2017 criteria (BRAZIL, 2017), S/N ratio ≥ 2:1.

Table 3. Percentage recoveries of PTOX spiked at three concentrations levels (high, medium, and low) in dry root powder of *Hyptis suaveolens* (n = 3 for each level).

Compound	Level	Amount added (ng)	Recovery (%)	RSD (%)
PTOX	Low	15.00	90.47	4.46
	Medium	60.00	98.03	4.46
	High	93.40	101.85	4.78

Table 4. Growth of *Hyptis suaveolens* roots grown *in vitro* in liquid MS medium supplemented with different combinations of IBA and NAA. RFWI (root fresh weight initial), RDWI (root dry weight initial), RFWF (root fresh weight final), RDWF (root dry weight final), FWGI (fresh weight growth index); DWGI (dry weight growth index).

Trat.	IBA mg L ⁻¹	NAA mg L ⁻¹	RFWI (g)	RDWI (mg)	RFWF (g)	RDWF (mg)	FWGI	DWGI
1	0.0	0.0	0.4	11.69	0.90 d	28.55 f	1.25 d	1.44 f
2	0.5	0.5	0.4	11.69	3.92 b	168.98 b	8.79 b	13.45 b
3	0.5	1.0	0.4	11.69	3.51 b	148.88 c	7.78 b	11.74 c
4	1.0	0.5	0.4	11.69	6.49 a	248.76 a	15.21 a	20.28 a
5	0.5	0.0	0.4	11.69	2.11 c	109.28 e	4.28 c	8.35 e
6	1.0	0.0	0.4	11.69	4.46 b	161.78 b	10.16 b	12.84 b
7	0.0	0.5	0.4	11.69	3.55 b	107.80 e	7.88 b	8.22 e
8	0.0	1.0	0.4	11.69	2.30 c	131.46 d	4.75 c	10.25 d

The means followed by the same letter in the column do not differ from each other, according to the Scott Knott test, at the 5% probability level.

Table 5. PTOX accumulation in roots (PTOX in the root), in the culture medium (PTOX in the medium), root dry weight (RDW), accumulated in the total weight of the dry roots of the treatment (PTOX TRDW), accumulated in the total volume of the remaining culture medium of the treatment (PTOX TVM) and sum of PTOX TRDW + PTOX TVM.

Treat.	IBA (mg L ⁻¹)	NAA (mg L ⁻¹)	PTOX In the root (µg g ⁻¹)	PTOX In the medium ¹ (µg mL ⁻¹)	RDW (g)	PTOX TRDW (µg)	PTOX TVM (µg)	PTOX TRDW + TVM (µg)
1	0.0	0.0	211.66 a	0.06 g	0.03 e	6.35 c	2.19 g	8.54 d
2	0.5	0.5	72.20 g	0.40 d	0.17 b	12.27 b	15.89 d	28.16 c
3	0.5	1.0	86.04 f	0.33 e	0.15 c	12.90 b	13.34 e	26.24 c
4	1.0	0.5	179.97 b	0.73 a	0.25 a	44.99 a	29.15 a	74.14 a
5	0.5	0.0	108.06 d	0.50 b	0.10 d	10.80 b	20.12 b	30.92 b
6	1.0	0.0	54.44 h	0.45 c	0.16 b	8.71 c	18.13 c	26.84 c
7	0.0	0.5	144.76 c	0.41 d	0.11 d	15.92 b	16.53 d	32.45 b
8	0.0	1.0	100.15 e	0.29 f	0.14 c	14.02 b	11.79 f	25.81 c

¹ Total value corresponding to the value obtained in 1 Erlenmeyer flask.

The means followed by the same letter in the column do not differ from each other, according to the Scott Knott test, at the 5% probability level.

Table 6. Root fresh weight (RFW), dry weight (RDW), PTOX content and accumulated total weight of dry roots of the treatment (PTOX TRDW) in *H. suaveolens* roots grown in MS liquid medium with different concentrations of vitamins and myo-inositol for 44 days.

Treatments	RFW (mg)	RDW (mg)	PTOX (µg g ⁻¹)	PTOX TRDW (µg)
MS without vitamins and myo-inositol	1306.80 b	115.66 c	7.50 a	0.86 b
MS half vitamins and myo-inositol	1999.20 a	198.88 b	6.01 b	1.19 a
MS	2397.60 a	258.90 a	4.54 d	1.17 a
MS double vitamins and myo-inositol	1123.20 b	131.94 c	5.09 c	0.67 c

The means followed by the same letter in the column do not differ from each other, according to the Scott Knott test, at the 5% probability level.

Figures Legends

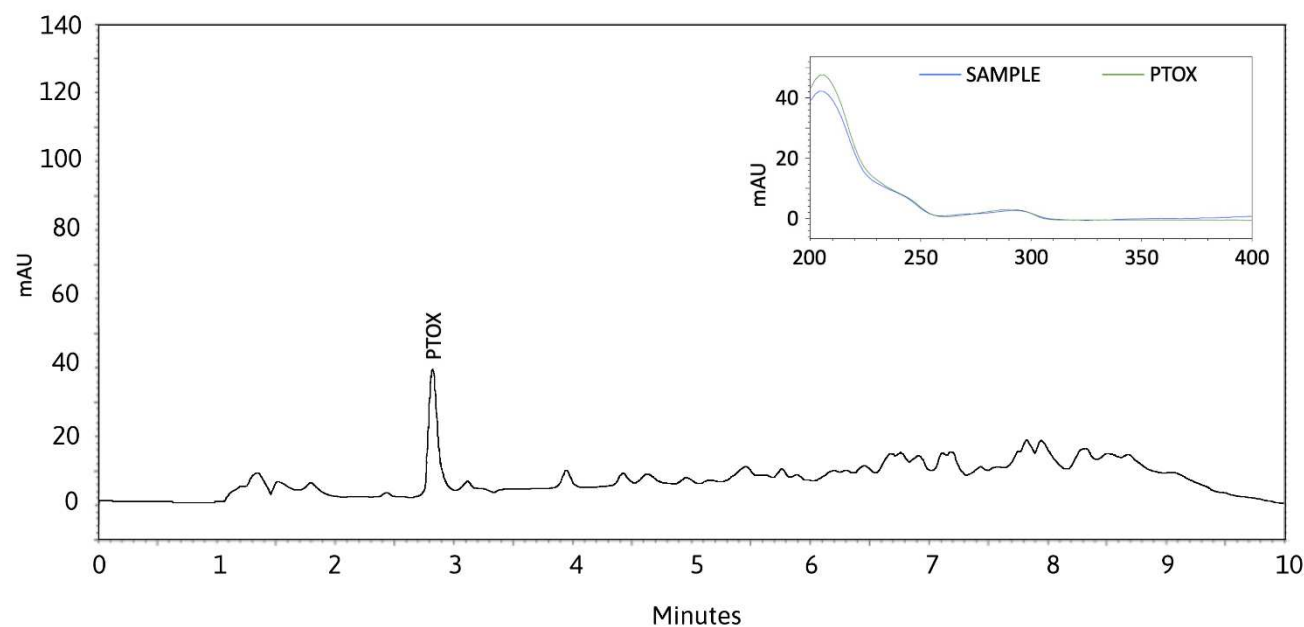
Figure 1. Typical RP-HPLC-UV/PDA chromatogram obtained for dry root powder of *Hyptis suaveolens* and UV-spectra overlap of pure PTOX standard and 2.73 min peak sample.

Figure 2. Roots of *Hyptis suaveolens* after *in vitro* culture for 42 days in liquid MS medium supplemented with different concentrations of IBA and NAA (mg L^{-1}): T1 = 0.0 (control); T2 = 0.5 + 0.5; T3 = 0.5 + 1.0; T4 = 1.0 + 0.5; T5 = 0.5 + 0.0; T6 = 1.0 + 0.0; T7 = 0.0 + 0.5; and T8 = 0.0 + 1.0.

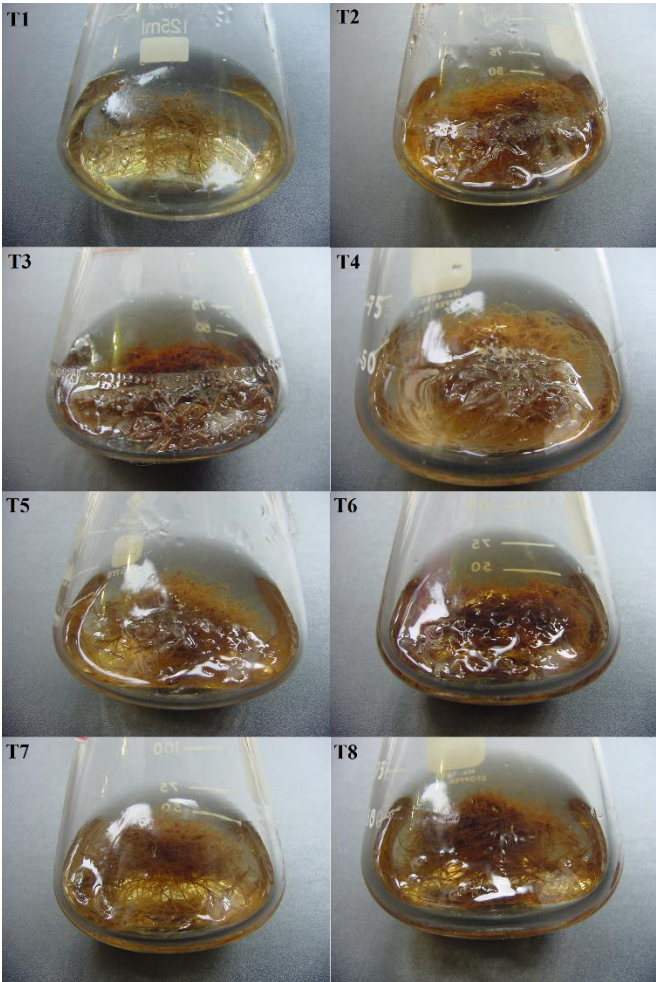
Figure 3. Chromatographic profiles of MS liquid culture medium samples after *in vitro* cultivation of *H. suaveolens* roots and their physical appearance. Culture medium supplemented with different concentrations of IBA and NAA as follows in mg L^{-1} : T1 = 0.0; 0.0 (control)/T2 = 0.5; 0.5/T3 = 0.5; 1.0/T4 = 1.0; 0.5/T5 = 0.5; 0.0/T6 = 1.0; 0.0/T7 = 0; 0, 5/T8 = 0; 1.0.

Figures

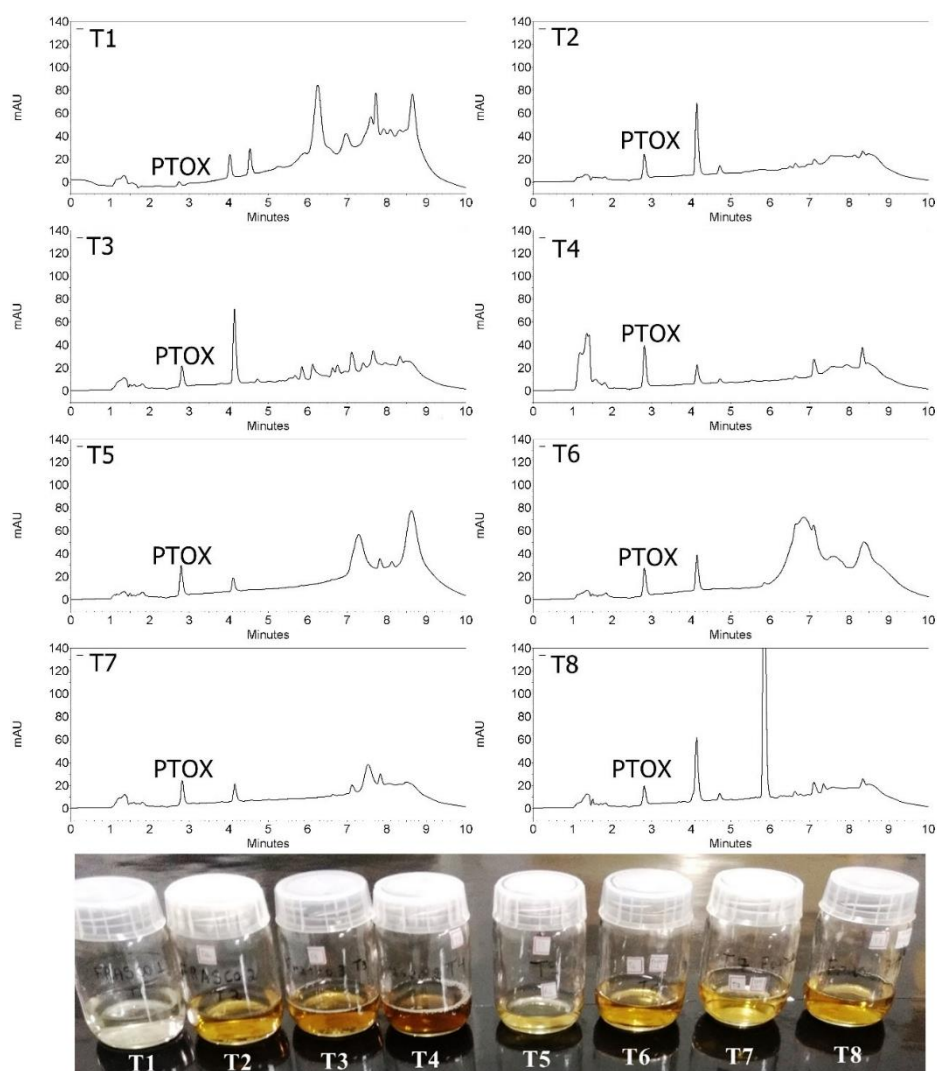
Figure 1



907 **Figure 2**



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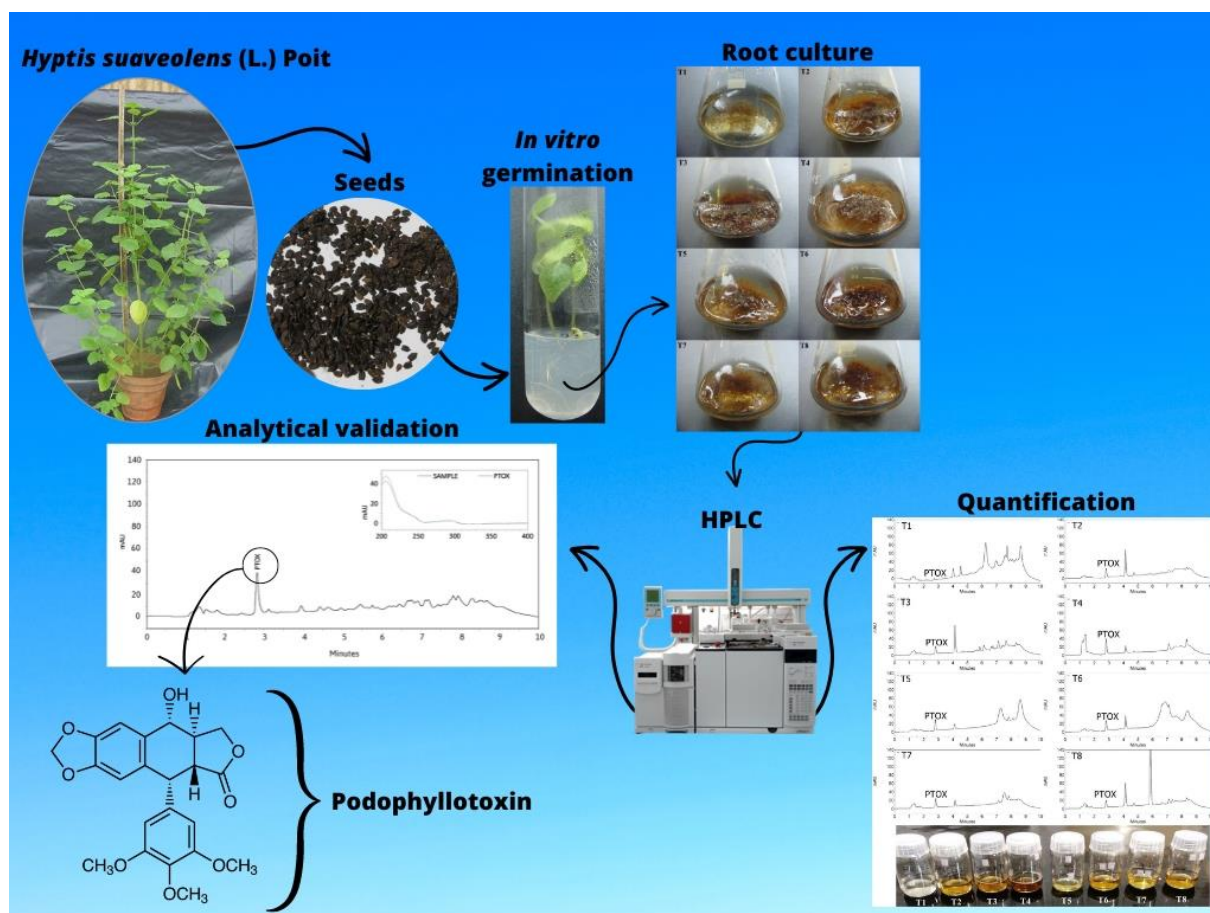
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