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Podophyllotoxin-Like Lignans Production through Hairy Roots of *Hyptis suaveolens*

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Abstract. The wild shurb *Hyptis suaveolens* also known as "chia" was used as food and medicine since prehispanic times in Mexico, and nowadays is employed to treat several ailments. The anticancer lignan podophyllotoxin (PTOX) was recently reported in this plant, and its highest accumulation occurs in roots. This work reports the establishment of several lines of hairy root cultures of *H. suaveolens* producing PTOX. Three *Agrobacterium rhizogenes* strains (ATCC 15834, K599 + pGus-GFP$^+$, and ATCC 15834 pTDT) were used to induce hairy roots. The strain 15834 of *Agrobacterium rhizogenes* carrying its wild type Ri plasmid was transformed by introducing the TDT binary vector which contains the Tomato Threonin Deaminase gene. Hairy roots induced by infection with this new strain, showed red fluorescence; while those roots induced by infecting with K599+pGus-GFP$^+$ showed green fluorescence as well as the expression of GUS gene. The transformed roots derived from explants infected with *A. rhizogenes* ATCC 15834 wild type exhibited the characteristic morphology of hairy roots. Fourteen root lines out of more than 100 lines obtained were selected. Cytotoxicity, fluorescence, GUS gene expression, growth rate and morphology, were used as selection criteria. Cytotoxic activity of different extracts obtained from these hairy roots against four cell lines of human carcinomas (KB, HF6, MC7 and PC3) was determined by the sulfurodamine B method. The cytotoxic values (IC_{50}) of some root extracts were lower than 20 µg/mL, and some others even lower than 4 µg/mL. PTOX-like lignans, as β-peltatin and 6-methoxy podophyllotoxin (6-MPTOX), were identified by LC/MS.

Keywords: Cytotoxicity, *Hyptis suaveolens*, Hairy roots, Podophyllotoxin, Fluorescent roots.

INTRODUCTION

During the last three decades the interest around secondary plant metabolites has increased. Advanced spectrometric and spectroscopic techniques, have contributed in an important way to the identification of bioactive plant metabolites. The scientific study of a putative medicinal plant starts with the ethnobotanical selection of the species, and the use of several assays to determine its pharmacological activity[1]. The scientific investigation is complemented by instrumental analysis techniques to identify, quantify, and standardize the content of the active ingredients[2]. In order to improve the production of specific plant compounds, some biotechnological procedures have been developed, such as *in vitro* cell tissue and organ cultures techniques as well as molecular biology methods[3]. Several patented drugs currently in use for the treatment of various diseases including cancer are obtained from plant species[4-6]. Cancer is the leading cause of death worldwide and the second in the American Continent[7], representing a public health problem. In Mexico, the cancer mortality rate varies between sexes and ages: in women, it is highest at age ranges of 30 to 64 years old; whereas in men, the rate is higher in the ages of 20-29, and 65 and older[8]. The highest organ-frequency affected by this disease is lung, followed by stomach, liver, and breast. Approximately 72% of cancer deaths registered in 2007 occurred in low and middle income countries. Worldwide deaths from cancer are still rising and it is estimated that will be of 12 million by 2030[9].

More than seventy percent of the anti-cancer drugs come from natural sources, as plants, microorganisms or marine organisms[10]. Global reports indicate that more than 3000 plant species are used for the treatment of different types of cancers[11]. In Mexico, depending on the source consulted, the number of plant species with medicinal use can vary between 3000 and 5000[12-15]. Nevertheless, it has been documented that around 300 species belonging to 90 families, are used against cancer-related symptoms [16].

Several compounds obtained from medicinal plants are used on cancer-chemotherapy, some of the most widely employed are three semisynthetic derivatives of podophyllotoxin (PTOX): etoposide, teniposide and etopophos[17, 18]. Podophyllotoxin is a natural aryltetralin lignan, usually obtained from *Podophyllum emodi*; however its overuse has threatened this plant to its extinction. Other genera referred as natural alternative sources of PTOX are*: Linum, Juniperus, Hyptis, Teucrium, Nepeta, Dysosma, Jeffersonia, Thymus* and *Thuja*[19]. Nowadays the amount of PTOX obtained from producer plants, are insufficient to supply its increasing demand.

Hyptis suaveolens or "chia" is a wild shrub, which was used in prehispanic Mexico as food and medicine, and nowadays used to treat other ailments. The presence of PTOX was recently reported in this plant [20]. In order to enhance the production of PTOX*, in vitro* cultures of *H. suaveolens* plantlets was established; and the effect of several plant growth regulators was determined. As the higher accumulation of PTOX in this plant occurs in its roots[21], hairy roots cultures represent an adequate biotechnological strategy that offers good genetic stability, and the possibility to increase the production of putative pharmacologically active compounds.

The objective of this work was the establishment hairy roots cultures of *Hyptis suaveolens* that accumulate podophyllotoxin or its cytotoxic analogs.

METHODS

Plant material. *H. suaveolens* was collected in Merida, Yucatan, Mexico in 2006. A plant specimen was deposited in the herbarium of the Scientific Research Center of Yucatan, with the voucher number CICY 7086. The seeds were carefully washed under running tap water for 10 min and surface-sterilized by immersing in

70% (*v/v*) ethanol for 1 min and in 1.5 % (*v/v*) solution of sodium hypochlorite (commercial bleach) for 5 min. Sterilized seeds were immediately rinsed three times with sterile distilled water for 5 min and placed in glass vessels containing Murashige and Skoog medium, amended with sucrose 30 g/L, and 2.5g/L water-phytagel for germination. The cultures were maintained in a growth chamber at 24 ± 2°C with a photoperiod of 16/8 hours light/darkness, with an energy light source of 1.3 mol/ m^2/ s. The *in vitro* multiplication of plantlets was done by placing explants, 3cm length of internodal segments in same conditions.

ATCC 15834 strain transformation. Through DNA extraction from *A. rhizogenes* K599 pTDT the TDT plasmid was obtained and then electroporated, into the strain of interest (pTDT refers to a binary vector containing the gene coding for Threonine Deaminase of Tomato that confers red fluorescence to transformed plant tissue). The ADN PUREGENE® (Gentra systems) protocol was used to extract DNA from K599 pTDT strain. The electroporation was done by mixing 50 l of competent cells of the wild type strain ATCC 15834 with 2 l of DNA extracted from K599 pTDT. The electroporation was done at 1.8 Kv with a pulse.

Hairy roots induction. To induce *H. suaveolens* hairy roots, internodal segments were infected with the three strains of *Agrobacterium rhizogenes*: ATCC 15834 wild type, K599+pGus-GFP$^+$ (with the green fluorescent protein and Gus gene), and ATCC 15834 pTDT which was generated by genetic transformation as described above. Hairy roots lines were selected according to their fluorescence, growth rate and cytotoxicity values.

Cytotoxic lignan extraction. The extraction of PTOX and other cytotoxic lignans, was done with 80:20 v/v methanol/dichloromethane[22].

Identification of podophyllotoxin and its analog lignans. The UFLC/ESI-HRMS and MS/MS system consisted of an Ultra-Fast Liquid Chromatograph Prominence system (Shimadzu, Champs-sur-Marne, France) coupled with (i) a dual-wavelength UV–vis detector (SPD-20A, Shimadzu) and (ii) a high-resolution mass spectrometer (HRMS) Ultima Global hybrid quadrupole/time-of-flight (Q-TOF) instrument (Waters-Micromass, Guyancourt, France), with a pneumatically assisted electrospray (ESI) ion source (Z-spray) and an additional sprayer (Lock Spray) for the reference compound. LC–ESI–MS were recorded in the negative ion mode. The capillary voltage was ±3.5 kV and a cone voltage range from ±20 to ±60 V was used.

One milliliter of each sample (20 L for MS/MS analyses) was loaded onto an XR-ODS (monofunctional C_{18}) 2.0 mm i.d. × 50 mm, 2.2 m column (Shimadzu, Champs-sur-Marne, France). The flow-rate was 0.3 mL/min. The gradient timetable with 0.2% aqueous acetic acid (v/v; A) and 0.2% acetic acid in acetonitrile (v/v; B) was on four steps as the following (A:B): 0-20 min 100:0; 20-55 min 82:18; 55-80 min 70:30; and 80-90 min 0:100; and staying stable for 5 min (end of mass acquisition). The gradient went back to the initial conditions (100:0) in 5 min and was maintained for 5 more min before the next injection. Samples were cooled at 20°C in the tray; the column was run at 30°C.

Data analysis. LC/MS data was reprocessed to identify alkaloid peaks using MassLynx 4.0 SP4 software (Waters-Micromass, Guyancourt, France). An automated integration method was used on molecular ions, which enabled reproducible analyses.

Cytotoxic evaluation. Sulforhodamine B (SRB) bioassay was used to determine cytotoxicity of crude extracts. Through this method the cytotoxic activity of the hydroalcoholic extract (HA) of the selected hairy root lines of *H. suaveolens* was measured against a panel of human carcinoma cells lines consisting of KB (nasopharyngeal), HF-6 (colon), MCF-7 (breast), and PC-3 (prostate). The half maximum inhibitory concentration (IC_{50}) of the HA extract was calculated for each cell line, and the values were compared with those displayed by camptothecin, and podophyllotoxin used as positive controls.

RESULTS

The germination of plantlets from sterilized seeds supplied enough plant material to induce hairy roots. Explants obtained from leaves and internodal segments were suitable for hairy root formation using the *A. rhizogenes* strains mentioned above. The strain ATCC15834 wild type was suitable to induce hairy roots from leaves explants, although addition of plant growth regulators (PGR) was necessary and was dose-dependent. The highest percentage (7.5 %) of hairy roots formation from leaves was obtained by addition of 2 mg/L α-naphthalene acetic acid (NAA), and 1 mg/L 6-benzylaminopurine (BAP). Higher doses reduced this percentage and increased the time of root sprouting, while lower doses did not gave root formation at all. Two root lines obtained from leaves were selected **(Figure 1).**

Addition of PGR was not necessary to induce hairy roots from internodal explants. The percentage of hairy roots induction was 15 % when these explants were used **(Figure 2)** and the growth rate was higher. Results obtained using the *A. rhizogenes* strain K599 pTDT were negligible (data not shown). The ATCC 15834 pTDT strain, became the "best root inducer". As expected, induced roots by this modified strain showed red fluorescence from the expression of Threonine Deaminase of Tomato **(Figure 3)**. Interestingly, the highest percentage of roots induction (21 %) was obtained by infecting internodal segments with this modified strain. **(Figure 3).**

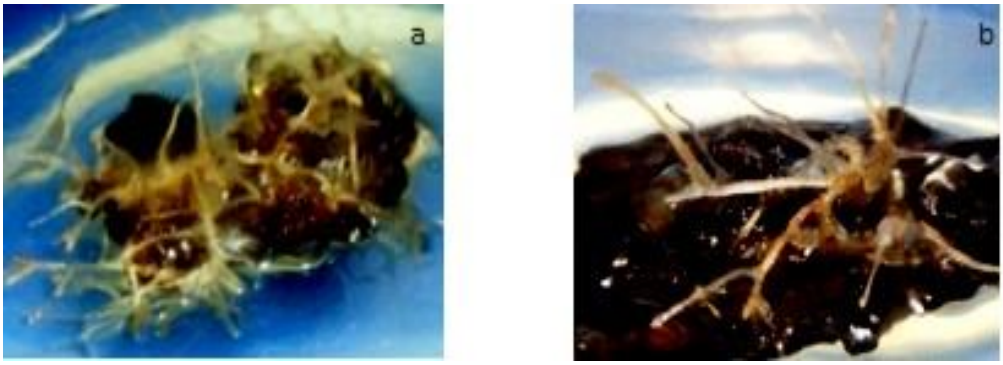

Figure 1. Typical morphology of hairy roots of *H. suaveolens* emerging from leaves infected with the ATCC15834 *A. rhizogenes* strain, the explants were placed on MS medium culture supplemented with ANA, and BAP (2:1 mg/ L, respectively).

Figure 2: Roots 30 days after of the explants infection (internodal segments of *Hyptis suaveolens*) infection with ATCC 15834.

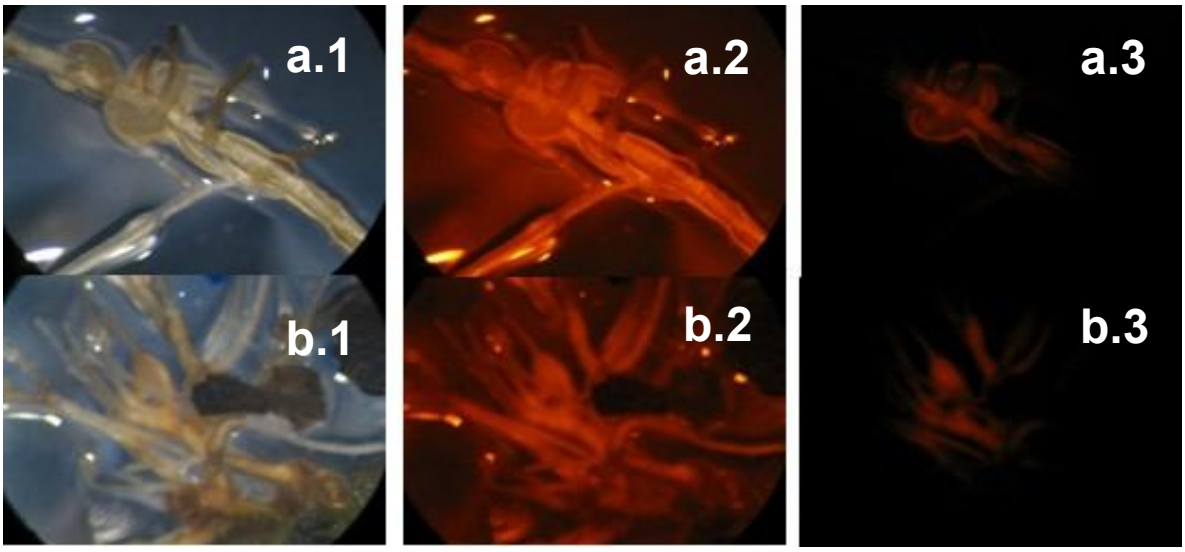

Figure 3: Red fluorescent hairy roots emerged from internodal segments of *Hyptis suaveolens*. Two hairy root lines (a and b) under different illumination conditions: **a.1** and **b.1** (left) micrographs tacked under white light; **a.2** and **b.2** (center) are micrographs of the same hairy roots tacked under white light, but using a red filter; **a.3** and **b.3** (right) are micrographs of the same hairy roots, but illuminated with green light and using the red filter. Under the last condition only hairy roots expressing the red fluorescent protein are fluorescent.

The other group of selected root lines was expressing the green fluorescent protein and the Gus gene. In order to show clearly this expression, histological preparations were obtained, and observed under the microscope. The preparations were stained with toluidine blue **(Fig 4a and 4b)**, this colorant is usually cited in staining of roots. The expression of the Gus-gene is also observed as a blue color in the organ, but in this case there is a specific expression in the vascular tissue. So, it was important to show both cuts as stained **(Fig 4a and 4b),** and without staining **(Fig 4c and 4d)**, it allows to corroborate that blue coloration corresponds to Gus reaction instead of the blue toluidine staining.

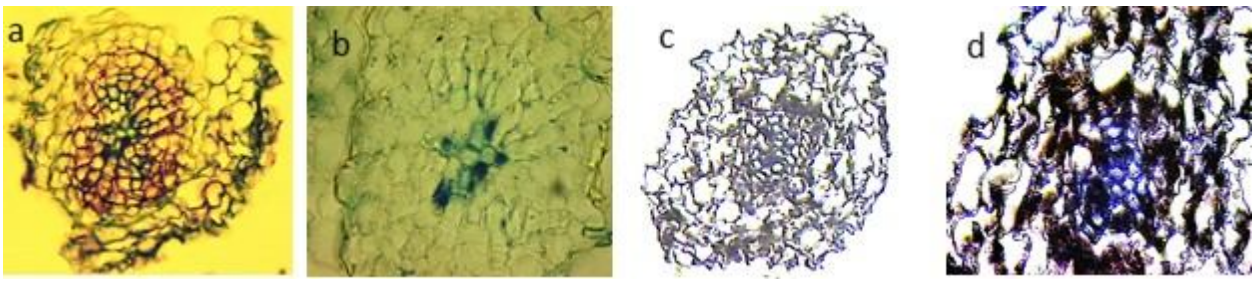

Figure 4: Histological preparations of hairy roots showing the Gus gene expression. **a** and **b**) Histological cuts stained with toluidine blue observed at 20X and at 40X (respectively), **c** and **d**) Histological cuttings without stain (20 X and 40 X, respectively), the blue color correspond to the Gus reaction.

Identification of PTOX and analogs from selected hairy roots lines of *H. suaveolens*. High performance liquid chromatography (HPLC) was used to identify and to quantify PTOX in the chloroformic extracts of four selected lines generated by infection with ATCC15834 wild type strain, on either leaves or internodal explants **(Figure 5)**. Surprisingly, the lines obtained by infection with *A. rhizogenes* ATCC15834 pTDT modified strain did not produce PTOX, but produced 6-MPTOX. This observation was corroborated by LC/MS **(Figure 6).**

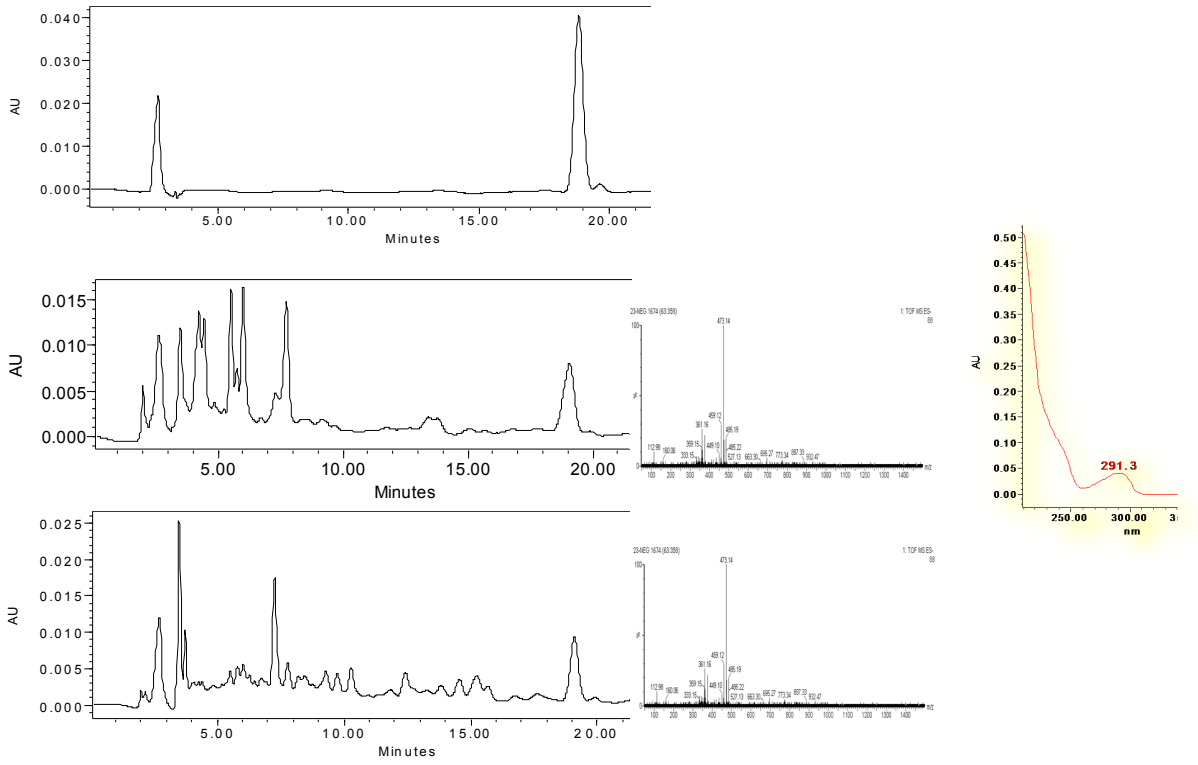

Figure 5: HPLC chromatograms showing a peak with retention time of 18.82 min corresponding to PTOX. The isocratic mobile phase was acetonitrile-water-acetic acid (33:67:0.2, v/v). Top left) PTOX (control SIGMA®); Right) Maximum absorption spectrum (291.3 nm); Middle left) Chromatogram of biomass chloroformic extract, showing one peak with an alike retention time of PTOX, beside m/z negative value. Bottom left) Chromatogram of the supernatant chloroformic extract, showing one peak with a similar PTOX retention time, beside m/z negative value.

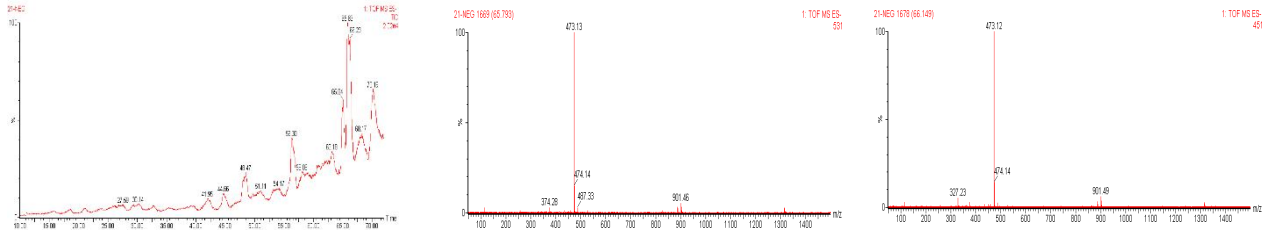

Figure 6: LC/MS chromatogram of the biomass chloroformic extract, showing two peaks at 65.7 and 66.1 min which correspond to the 6-metoxipodophyllotoxin isomer and β-peltatin, respectively (left). Molecular ion on negative mode of a 6-MPTOX isomer, possibly the β-peltatin (middle and right).

However, in order to obtain a PTOX producer root line, the plant was infected with *A. rhizogenes* strain (K599+pGus-GFP$^+$). In this case, the obtained roots showed both green fluorescence as well as the GUS gene expression, the yield of PTOX in this case has been preliminarily established by HPTLC **(Figure 7).**

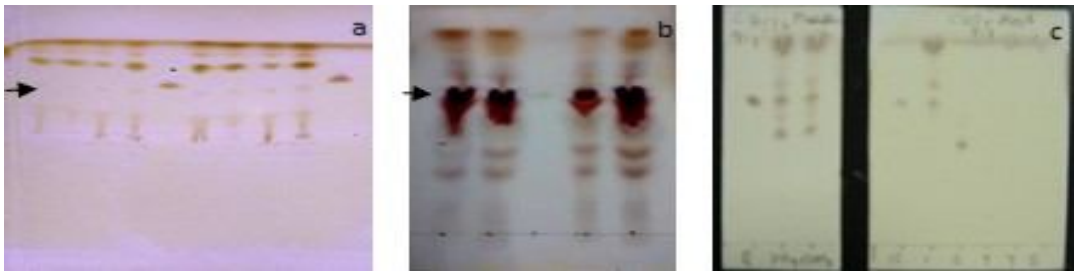

Figure 7: Preliminary **i**dentification of PTOX by TLC and HPTLC plates in the extracts of hairy roots induced with the K599+pGus-GFP$^+$ strain. The arrow indicates the RF of PTOX, a) HPTLC glass plate developed with dichloromethane-methanol-formic acid (9.5:0.0:0.5, v/v/v), each lane corresponds to the extract of a selected line (by duplicate), and the control (PTOX) in the middle and the right; b) TLC aluminum plate developed with chloroform-methanol (80-20); abd c) TLC plate later from an extract fractioned by chromatographic column.

Quantification of PTOX. The highest yield of PTOX (biomass +culture medium) of the L1 (selected transformed line) was 1.01% DW **(Table 1)**. This value is higher than the 0.01 % DW found in wild roots. Another advantage of this selected line is that almost half of the PTOX was excreted into the culture medium. In this sense, it represents the opportunity for obtaining PTOX without losing biomass. The yield of PTOX in this hairy root line is also higher than that reported for *Hyptis verticillata* (0.25 % DW)[23].

Table 1: Quantification of PTOX (% DW) in the hairy root line L1 of *H. suaveolens*.

Line	mg/ mL	mg/ g DW	% DW
L 1	0.0855	5.341	0.53
Medium L1	0.0766	4.790	0.48

Cytotoxic evaluation. The results are shown in two parts. The first one shows the results related to the biomass of root lines producing PTOX; in this case it is noticeable that in all cases the cytotoxic activity against KB and HF6 cancer cell lines was higher than that reported for the wild root extract (12.3 ± 0.7, and 12.7 ± 0.7, respectively) [20], **Table 2.**

Table 2: Cytotoxic evaluation of chloroformic extracts from *H. suaveolens* hairy roots lines producing PTOX.

Hairy Roots Lines	Cancer Cell Lines CI_{50} (µg/mL)			
	KB	MCF 7	HF 6	PC 3
L 1	2.94	3.16	2.47	1.48
L 2	2.98	6.75	3.11	4.17

L 3	2.26	6.25	3.13	5.73
L 4	3.28	7.19	5.92	2.24
Medium Line 1	3.77	>20	7.96	11.86
PTOX	$3.6X10^{-5}$	$1.2X10^{-4}$	$5.3X10^{-4}$	0.035
CPT	0. 0215	0.0528	0. 042	0. 1679

Carcinoma cell lines: nasopharyngeal (KB), breast (MCF 7), HF 6 (colon), PC 3 (prostate). Positive controls: PTOX = Podophyllotoxin, and CPT = Camptothecin.

The second part of this study include the cytotoxic evaluation of extracts obtained from the culture medium of root lines that did not produce PTOX, but could synthesized its analogs (β-peltatine and 6-MPTOX) or other non-identified compounds **(Table 3).** Here is important to remark that the extract from culture medium of line 8 shows the highest cytotoxic activity, and that in this specific extract none PTOX analog was identified.

Table 3: Cytotoxic evaluation of chloroformic extracts from culture media of *H. suaveolens* hairy root lines not producing PTOX.

Hairy Root Medium Culture	Cancer Cell Lines CI_{50} (µg/mL)			
	KB	MCF 7	HF 6	PC 3
L 5	7.11	>20	7.91	12.6
L 6	14.3	15.07	10.85	17.15
L 7	9.38	>20	6.99	14.49
L 8	0.61	1.18	0.30	0.74
L 9	2.17	4.91	1.96	4.39
L 10	1.12	4.03	2.75	3.31
PTOX (+)	1.5X10-4	2.1X10-5	3.5X10-3	0.042
CPT (+)	1.18x10-4	5.1x10-3	2.1x10-3	0.033

L Selected hairy root line.

DISCUSSION

Some biological activities that have been reported for the genera *Hyptis* include analgesic, cardiovascular, genotoxic, insecticide, and cytotoxic activities; however, there are a big number of reports related with this cytotoxic action. Three active species (*H. pectinata* (L) Poit, *H. verticillata* Jacq and *H. suaveolens* (L) Poit have been studied; nevertheless, the yield of podophyllotoxin (PTOX) or possible analogs, are documented in the last two species[20,24,25]. *H. verticillata* produces nine PTOX-like lignans, five of them with high cytotoxic activity[24]; in the other hand, Lautie *et al.* reported that *H. suaveolens* produced PTOX (0.01% DW) and traces of PTOX-like lignans which were found in the chloroformic extracts of the roots of this species[20]. The cytotoxic values (IC_{50}) of the chloroformic extracts from wild roots, were lower (on nasopharyngeal [KB 12.3 µg/ mL], and colon [HF6 12.7 µg/ mL] carcinomas) than the cytotoxicity obtained from the extracts of hairy roots (Table 2); but similar (on breast carcinoma [MCF-7 2.8 µg/ mL]) to that obtained with the chloroformic extracts from hairy roots. Velóz *et al.* reported that this species produces three times more PTOX in its roots than in the

leaves or stems[21]. The purpose of the present study was to establish hairy roots lines highly producing PTOX or its analogs. The use of plant growth regulators (PGR) was necessary to obtain hairy roots from leaves-explants. However, those PGR could interact with the yield of PTOX. In this way, it was better to change the type of explants to avoid the use of PGR. Furthermore, an important factor for root induction was to use phytagel at 2.5 g/L, since higher concentrations limited the development not only of the plantlets, which serve as source of explants, but also of hairy roots. *H. suaveolens* produces a big quantity of phenols in its roots. Due to oxidation, these phenols complicated the establishment of root cultures, and in order to diminish this action it was necessary to increase the dose of polyvinylpyrrolidone (2 to 3 g/L).

In this work, three *Agrobacterium* strains were tested. It was possible to establish a relationship between the type of explant and the *Agrobacterium* strain, with the percentage of induced roots. The highest percentage of induced hairy roots (21%) was obtained with the strain generated by our group (ATCC15834 pTDT) infecting internodal explants; even tough, at the beginning the ATCC15834 strain was selected considering previous results. On this respect, Chandran and Potty[26] obtained a higher percentage of root induction using the last referred strain to infect intermodal explants of *Ipomea batatas*, while the lowest percentage was achieved with K599 pTDT strain (0.5%). This result differs with the results reported by Estrada-Navarrete *et al*., since they obtained between 75 to 90 %, although the species and type of explant, were different[27].

In respect to the production of podophyllotoxin (PTOX) or analog lignans, it is not possible to establish any relationship between the type of explant, and kind of lignan produced. Nevertheless, it is interesting that four of the selected roots produce a higher concentrations of PTOX (1.01% DW) than that produced by wild roots (0.01% DW) [20]. Other six selected root lines produced PTOX-like lignans. However, on those root lines producers of PTOX, neither 6-metoxipodophyllotoxin (6-MPTOX) nor β-peltatin, were identified. On the contrary, those root lines producers of 6-metoxipodophyllotoxin or betapeltatin, did not produce PTOX. Maqbool (2011) reported a similar relationship between plants of *Podophyllum peltatum*, collected in several sites along forests of USA, observing that those plants that produce PTOX do not produced β-peltatin, and contrariwise, those that produced β-peltatin do not produce PTOX[28].

The cytotoxicity of the extracts was of the same order, regardless the type of lignan produced. One related biosynthetic pathway in *Linum flavum*, suggested 6-MPTOX as a product of the conversion of β-peltatin; and deoxypodophyllotoxin as the precursor of either PTOX or β-peltatin[29]. The semi-synthesis of the drugs derived from PTOX (Etoposide, Teniposide, and Etopophos) includes the modification of PTOX to epipodophyllotoxin. On this respect, Vasilev *et al*. reported the bioconversion of deoxypodophyllotoxin to epipodophylotoxin[30], and by considering that the precursor of PTOX and β-peltatin is the same molecule (deoxipodophyllotoxin), it could be postulated that the main compound, used for the semi-synthesis of these drugs (epipodophyllotoxin), could be obtained either by semi-synthesis through PTOX or by bioconversion through deoxypodophyllotoxin.

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