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ORIGINAL ARTICLE

Cytotoxic activity of callus extract from *Vachellia farnesiana* (L) Wight & Arn.

José Alejandro Domínguez-Colín[1] · Leticia Buendía-González[2] · Carmen Hernández-Jaimes[2] · Francisco Cruz-Sosa[3] · Juan Orozco-Villafuerte[1]

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Abstract

The in vitro cultures of *Vachellia farnesiana* (L) Wight & Arn. have demonstrated cytotoxic activity through callus extract on the HeLa cell line. Explants excised from in vitro-grown seedlings from seeds of two different locations were inoculated on Murashige and Skoog (MS) culture media containing various concentrations of N-6 benzyladenine (BA) or kinetin with 2,4-dichlorophenoxyacetic acid (2,4-D). Optimal efficiency in friable callus induction (100%) was achieved in leaf explants cultured on MS media containing 2.32 µM BA + 13.57 µM 2,4-D. Plant tissues (callus and leaf) were extracted and subjected to quantitative phytochemical analysis, revealing the highest total alkaloid and phenolic content in leaf extracts from Queretaro adult specimens (339.5 ± 20.9 mg atropine equivalents (AE) per g dry extract (DE) and 158.4 ± 12.5 mg gallic acid equivalents (GAE) per g DE, respectively). In contrast, callus cultures exhibited significantly higher total triterpene content (356–381 mg ursolic acid equivalents (UAE) per g DE) compared to leaf extracts (208–243 mg UAE/g DE). Both leaf and callus extracts displayed cytotoxic activity against the HeLa cell line, with a significantly lower half-maximal inhibitory concentration (IC_{50}) for leaf extracts (28–32 µg/mL) compared to callus cultures (43–66 µg/mL), suggesting that alkaloids were primarily responsible for the cytotoxic activity. Furthermore, this study provides valuable insights into the controlled production of bioactive compounds with cytotoxic activity, with callus serving as a rich source.

Keywords Antineoplastic drugs · Cancer · Bioactive compounds · Alkaloids · Triterpenes · Phenols

Introduction

Cancer is a leading global health concern, with 19.3 million new cases and approximately 9.9 million deaths reported worldwide in 2020 alone (Ferlay et al. 2021). Current treatment strategies include chemotherapy, surgery, radiotherapy, and hormone therapy, with chemotherapy being the primary choice in most cases (Shynu et al. 2011). However, chemotherapy's destructive effects on rapidly dividing cells, including healthy ones, lead to debilitating side effects such as alopecia, peripheral neuropathy, and cardiotoxicity (Brianna and Lee 2023). This has driven many to seek alternative treatments, including traditional medicine based on plant extracts. Plants are a natural source of numerous commercially available antineoplastic drugs (Newman and Cragg 2020).

Plants have evolved complex defense mechanisms, including chemical defenses, to survive in diverse ecosystems. These secondary metabolites confer adaptive advantages and have been exploited for pharmaceutical

✉ Juan Orozco-Villafuerte
jorozcovi@uaemex.mx; jov202001@yahoo.com.mx

José Alejandro Domínguez-Colín
jdominguezc143@alumno.uaemex.mx

Leticia Buendía-González
lbuendiag@uaemex.mx

Carmen Hernández-Jaimes
mdhernandezj@uaemex.mx

Francisco Cruz-Sosa
cuhp@xanum.uam.mx

[1] Facultad de Química, Universidad Autónoma del Estado de México, Carretera Toluca-Ixtlahuaca Km 15.5, Piedras Blancas, 50200 Toluca de Lerdo, México

[2] Facultad de Ciencias, Universidad Autónoma del Estado de México, Carretera Toluca-Ixtlahuaca Km 15.5, Piedras Blancas, 50200 Toluca de Lerdo, México

[3] Departamento de Biotecnología, Universidad Autónoma Metropolitana-Iztapalapa, Av. Ferrocarril San Rafael Atlixco 186, Leyes de Reforma 1ª. Sección Iztapalapa, 09310 Ciudad de México, México

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applications (Noel et al. 2005). Among these metabolites, alkaloids (vincristine, vinblastine, taxol, camptothecin, etc.), terpenes (placitaxol, gigantol, batasin, erianin, etc.), and phenols (denbinobine, podophyllotoxin, etoposide, etc.) have demonstrated cytotoxic activity (Teoh 2016). However, low concentrations and cultivation challenges have led to exploring alternative production methods, such as plant tissue culture (Yue et al. 2016). The production of chemical compounds through in vitro culture provides an excellent opportunity to obtain secondary metabolites under controlled conditions, independent of environmental factors, both biotic and abiotic. This method also offers the possibility of significantly increasing the yield of specific metabolites and ensuring constant production throughout the year (Vanisree et al. 2004). Callus culture can facilitate the optimization of secondary metabolite production and subsequent isolation. Callus masses can sometimes yield a high amount of secondary metabolites (Wani et al. 2010).

Vachellia farnesiana (L) Wight & Arn., formerly known as *Acacia farnesiana,* and commonly referred to as "huizache" or "sweet acacia" has been utilized in Mexican traditional medicine for a variety of purposes, including the treatment of stomach ailments, dysentery, and skin inflammations (Popoca et al. 1998), used as toothbrushes, for sore throat, as well as antispasmodic, aphrodisiac, astringent, stimulant and demulcent, to treat diarrhea, fevers and rheumatism; the root infusions in the treatment of stomach cancer, similarly, it has been documented that the fillings made with the pulp decrease the severity of some tumors (Hartwell 1970). Its compounds, such as farnesol (terpene), geraniol (terpene), betulinic acid (terpene), and diosmetin (phenol), extracted from roots, flowers, or leaves, have shown cytotoxic activity against cancer cell lines (Lin et al. 2009; Wang et al. 2009; Qi et al. 2018; Qiu et al. 2020).

However, *V. farnesiana* is being over-exploited mainly for coal production at an alarming rate, with an estimated annual loss of 600 ha. As a result, conservation of these ecosystems is of paramount importance. The plant's sexual propagation through seeds is severely hindered by low germination rates under natural conditions, primarily due to water-impermeable pods and hard seed coats. To address this, micropropagation techniques have been successfully applied to *V. farnesiana*, leveraging organogenesis and somatic embryogenesis (Ortiz et al. 2000; Khalisi and Al-Joboury 2012; Morales-Domínguez et al. 2019; Álvarez et al. 2023; Xu et al. 2023). Despite callus generation during micropropagation, there are no reports, to our knowledge, on the establishment of callus cultures for producing compounds with cytotoxic activity. This study aimed to investigate the cytotoxic activity and quantify the total phenolic, alkaloid, and triterpene content of *Vachellia farnesiana* callus under controlled production conditions.

Methods

Plant materials

Seeds, leaves, and specimens with stems, leaves, and flowers were collected in San Juan del Rio, Queretaro, Mexico (20°12′–20°34′ N latitude, 99°49′–100°12′ W longitude, 1,920 m above sea level, semi-dry climate with summer rainfall, average annual temperature 17.2 °C, and average annual rainfall 780 mm). The specimens were pressed and submitted to the Medicinal Herbarium of the Instituto Mexicano del Seguro Social, Centro Medico Nacional Siglo XXI, for identification. The species was confirmed as *Vachellia farnesiana* (L.) Wight & Arn. and assigned voucher number 16997. Additionally, seeds and leaves were obtained from Huerta Amezquita nursery, Leon, Guanajuato (− 101°41′ W longitude, 21° N latitude, 1,798 m above sea level, temperate sub-humid climate with summer rainfall, average annual temperature 19.6 °C, and average annual rainfall 650 mm).

In vitro *germination of V. farnesiana seeds*

Surface disinfection of seeds from Queretaro and Guanajuato was performed using the methodology described by Morales-Domínguez et al. (2019). The seeds were scarified by immersion in concentrated H_2SO_4 with constant agitation for 1 h. Subsequently, they were washed five times with sterile distilled water, followed by a 30-s wash with 70% (v/v) ethanol. Then, the seeds were immersed in a 0.5% (v/v) sodium hypochlorite solution for 15 min, and the excess solution was removed with sterile double-distilled water. The disinfected seeds were inoculated into culture tubes (25 × 150 mm) containing half-strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 1% sucrose and 0.2% phytagel. The culture medium was sterilized by autoclaving at 121 °C for 18 min. Cultures were incubated in a growth chamber (ICP20, Lumistell, Mexico) under a 16-h photoperiod at 25 ± 2 °C. Cultures were monitored every third day for one month, and radicle emergence was recorded as an indicator of germination.

Callus induction

Explants from 15-day-old in vitro germinated seedlings (hypocotyl, cotyledon, root, and leaf) were inoculated into tubes containing MS culture medium supplemented with 3% sucrose and various concentrations of plant growth regulators (PGRs): 2,4-dichlorophenoxyacetic acid (2,4-D; 0.0, 9.04, and 13.57 µM) in combination with either kinetin (KIN) or 6-benzyladenine (BA; 0.0, 2.32, and 4.64 µM), and solidified with

0.2% phytagel. The culture medium was sterilized by autoclaving at 121 °C for 18 min. Cultures were incubated in a growth chamber (ICP20, Lumistell, Mexico) under a 16-h photoperiod at 25 ± 2 °C. Over 30 days, cultures were monitored, and the percentage of callus induction was recorded. Cultures exhibiting high induction percentages and friable callus were selected for proliferation through subculturing every 30 days on fresh medium with the same PGRs formulation.

Preparation of plant material for phytochemical analysis

Leaf biomass from adult specimens and callus leaf from both collection sites were weighed (FW) and then dried using lyophilization (Labconco, Kansas City, MO, USA) to determine their dry weight (DW). The dried biomass was subsequently pulverized using a mortar and stored in an amber flask within a desiccator until further use. The moisture content (percentage) of each sample was calculated using the following equation:

$$MC = \frac{FW - DW}{FW} \times 100 \quad (1)$$

where MC represents the moisture content (%), FW is the fresh weight, and DW is the dry weight.

Obtention of leaf and callus extracts

The dried biomass powder was macerated in ethanol (1:30, w/v) with constant agitation at 100 rpm for 24 h. Subsequently, the mixture was subjected to an ultrasonic bath (Ultrasonic cleaner, SK2219HP, Shanghai, China) at 40 °C and 53 kHz for 40 min. The supernatant was then recovered and underwent a degreasing process in a separation funnel using equal volumes of hexane, retaining the ethanolic extract. The extract was concentrated under reduced pressure (IKA RV 10 digital V, USA) and reconstituted in water. A second partition was performed in a separation funnel to remove polar compounds, using equal volumes of chloroform, and collecting the organic fraction. This process was repeated in triplicate, and the obtained extracts were pooled. The sample was concentrated under reduced pressure (IKA RV 10 digital V, USA). The organic extract underwent a third partition with a 1% NaCl solution to partially remove tannins. The extract was concentrated under reduced pressure (IKA RV 10 digital V, USA) until dryness and stored in amber bottles at 4 °C until use (Wall et al. 1996).

Determination of total alkaloids content in plant extracts

The total alkaloid content in the extracts was determined using the bromocresol green (BCG) reaction, as described by Shamsa et al. (2008). A standard curve was constructed using a stock solution of atropine (1 mg/mL). For the extracts, 800 µL of each extract was transferred to a separating funnel, followed by the addition of 5 mL of bromocresol green solution and 5 mL of phosphate buffer solution (pH 4.7). The mixture was vigorously shaken, and the resulting complex was extracted with chloroform. The samples were then recovered and adjusted to a final volume of 10 mL with chloroform. Absorbance was measured at 470 nm using a spectrophotometer (Genesis 10S UV–VIS, Thermo Scientific). The results were expressed as milligrams of atropine equivalents per gram of dry extract (mg AE/g DE).

Determination of total triterpenes content in plant extracts

A standard curve was created using ursolic acid (1–16 mg/L) as the reference standard. To prepare the samples, 1 mL of sulfuric acid and 400 µL of vanillin-glacial acetic acid reagent (5%) were added. The reaction mixture was then incubated at 70 °C for 35 min, followed by cooling to room temperature for 5 min. The mixture was subsequently diluted with glacial acetic acid to a total volume of 10 mL. Absorbance was measured at 547 nm using a spectrophotometer (Genesis 10S UV–VIS, Thermo Scientific) (Yin et al. 2010). The results were expressed as milligrams of ursolic acid equivalents per gram of dry extract (mg UAE/g DE).

Determination of total phenols content in plant extracts

The total phenolic content was determined using the Folin-Ciocalteu method, as described by Singleton and Rossi (1965), with gallic acid as the reference standard. To 200 µL of the extract, 100 µL of the Folin-Ciocalteu reagent was added, and the mixture was allowed to stand in the dark for 8 min. Then, 200 µL of anhydrous sodium carbonate (14% w/v) and 1500 µL of distilled water were added, and the mixture was thoroughly mixed. The sample was then allowed to stand at room temperature in the dark for 1 h. The absorbance was measured at 765 nm using a spectrophotometer (Genesis 10S UV–VIS, Thermo Scientific). The results were expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g DE).

Antioxidant activity of plant extracts: DPPH and ABTS free radical scavenging assays

The antioxidant capacity was assessed using two different techniques: (1) α, α-diphenyl-β-picrylhydrazyl (DPPH) radical method (Brand-Williams et al. 1995): The sample was reacted with the DPPH radical (0.025 g/L) and incubated for 30 min. Absorbance was then measured at 515 nm. (2)

2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) method (Fellegrini et al. 1999): 3 mL of ABTS solution was added to each sample, incubated for 10 min at room temperature in the dark, and then absorbance was measured at 734 nm. Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as the standard for both methods, with results expressed as the IC_{50} value, representing the concentration required to scavenge 50% of free radicals.

Cytotoxicity assay of plant extracts

Maintenance and proliferation of HeLa cell line

The HeLa cell line (ATCC CCL-2, lot 59,681,574) was donated by Laboratorio de Especialidades Inmunologicas S.A. de C.V. The cells were initially grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS) to verify sterility. Following a one-week quarantine period (Freshney 2010), no contamination was observed, and the cell line was cleared for expansion to establish a working bank. The HeLa cells were subsequently maintained in an EMEM medium supplemented with 10% FBS, 2 mM L-glutamine, and 1% non-essential amino acids under controlled conditions: 37 °C, 5% CO_2, and 90% relative humidity.

Cytotoxic bioassay against HeLa cells

The cell concentration of the cultures was determined, and a cell suspension with a concentration of 1×10^4 cells/mL was prepared. This suspension was then inoculated into the plate wells. Each plate was organized as follows: three blank wells (no cells), three negative control wells (cells without extract), three positive control wells (4.27 ng/mL of paclitaxel; Sigma-Aldrich), three solvent control wells (ethanol), and four wells with different concentrations of the extracts to be evaluated (1, 5, 10, 50, and 100 µg/mL). Unused wells were filled with a phosphate-buffered saline (PBS) solution to prevent evaporation and separate neighboring wells to avoid interference. The final volume in each well (150 µL) consisted of 120 µL of the cell suspension and 30 µL of the corresponding extract solution. The cell cultures were incubated for 24 h before adding the aliquots of controls and extracts. Two exposure times were evaluated: 48 and 72 h. After the exposure time, 15 µL of thiazolyl blue tetrazolium bromide (MTT) reagent (1:10; v/v), previously filtered (0.22 µm), was added to each well. The samples were shaken at 600 rpm for 1 min and then incubated for 4 h at 37 °C in a CO_2 incubator. After the incubation period, the culture medium was removed, and the formed crystals were dissolved with 150 µL of acidified isopropyl alcohol. The plate was then allowed to sit for 10 min, and spectrophotometric readings were taken at 570 nm using a microplate reader (Epoch, Biotek, USA). The percentage of viability for each extract was determined using the following expression:

$$Cellviability(\%) = \frac{ABS_{treatment} - ABS_{blank}}{ABS_{control} - ABS_{blank}} \times 100 \quad (2)$$

The half-maximal inhibitory concentration (IC_{50}) was determined using GraphPad Prism version 8.0.1.

Statistical analysis

The experiment was designed as a randomized factorial study, with callus induction involving five seeds per replication, conducted in batches. All experiments and measurements were performed in triplicate. The resulting data were subjected to statistical analysis using analysis of variance (ANOVA), followed by the Tukey test for mean comparison. Meanwhile, the viability percentage data for each concentration at two times points were analyzed using a *T*-test. The significance level for all statistical analyses was a set at $p \leq 0.05$. The statistical analyses were performed using the Statgraphics statistical software (Centurion XVI.II).

Results and discussion

Callus induction

Seeds germinated synchronously (> 85%) after chemical scarification (96% H_2SO_4, 1 h), and 15-day-old seedlings were used as explant sources for callus induction. All treatments with plant growth regulators (PGRs) induced callus formation in explants from both distribution sites after 30 days of culture. However, significant differences in induction percentage were observed among explant types, regardless of distribution site (Fig. 1A–B). Leaves and hypocotyls showed the highest induction percentages (100% and 70%, respectively), whereas roots exhibited the lowest (65.6%) (Leaf > Hypocotyl ≥ Cotyledon > Root). No callus formation occurred in the control treatment (without PGRs). MS medium supplemented with BA was the most effective for callus induction, outperforming KIN. Moreover, increasing concentrations of BA and KIN positively impacted callus induction. Explants from Guanajuato showed slightly lower induction values than those from Queretaro, possibly due to environmental variables influencing genetic variation, such as solar radiation, precipitation, and altitude (Chi et al. 2024). The more stressful environmental conditions at the Queretaro site may contribute to these differences.

Distinct morphological characteristics were observed in the formed calli. BA-treated cultures produced a higher amount of friable green callus with high hydration levels,

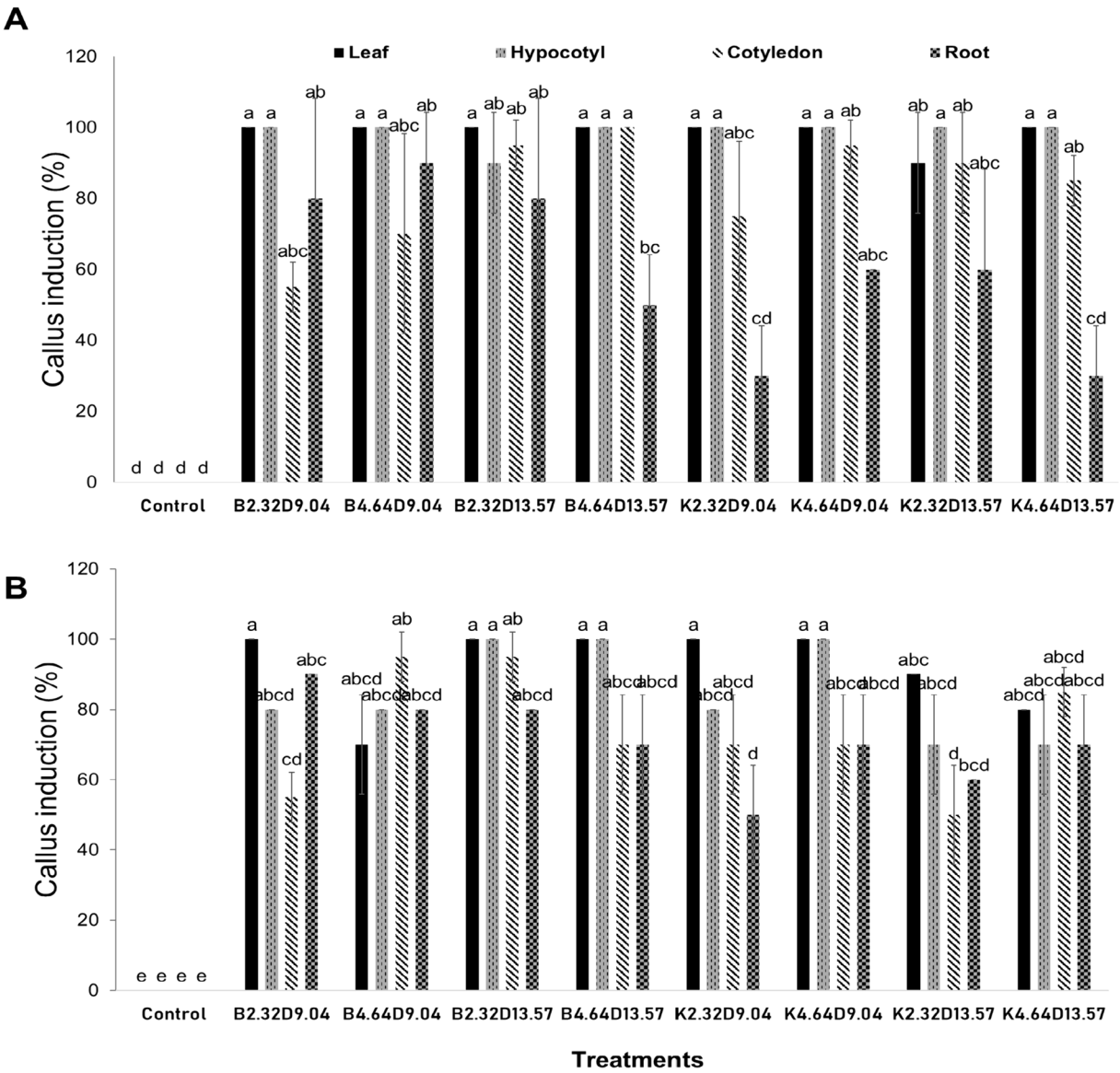

Fig. 1 Callus induction percentage in *Vachellia farnesiana* explants from in vitro-germinated seedlings: comparison of two distribution sites. **A** Guanajuato and **B** Queretaro. The figure shows the average callus induction percentage (±SD) in explants treated with various plant growth regulator combinations. Data represent the mean of three repetitions. Bars with different letters indicate significant differences ($p \leq 0.05$) according to the Tukey test

whereas KIN-treated cultures yielded heterogeneous calluses with whitish spongy, and compact brown sections. In contrast, root, cotyledon, and hypocotyl explants showed limited yellow-compact callus formation, mainly on the edges (Fig. 2). Notably, Guanajuato leaf explants exhibited callus formation across the entire surface, with the $B_{4.64}D_{13.57}$ treatment resulting in almost complete explant transformation into yellow-green, friable callus (Fig. 2d). Similarly, Queretaro leaf explants treated with $B_{2.32}D_{13.57}$ showed abundant yellow-green, friable callus formation (Fig. 2h). These results can be attributed to the higher dedifferentiation capacity of leaf tissue, which has a high metabolic rate (Takemori et al. 2010) and contains meristematic cells capable of dividing and generating callus (Yun et al. 2012). Therefore, leaf callus from both distribution sites was selected for proliferation. The callus cultures were subsequently harvested for extract obtention. The callus induction results are consistent with those reported for other *Acacia* or *Vachellia* species. Leaves of *Acacia* species have been shown to exhibit high induction percentages (Gantait et al. 2018). The efficiency of 2,4-D and BA PGRs in inducing callus in *A. sinuata* (Lour) Merr. and *A. nilotica* (L.) Willd. ex Delile have been reported (Vengadesan et al. 2002; Dhabhai and Batra 2010). Additionally, auxin 2,4-D

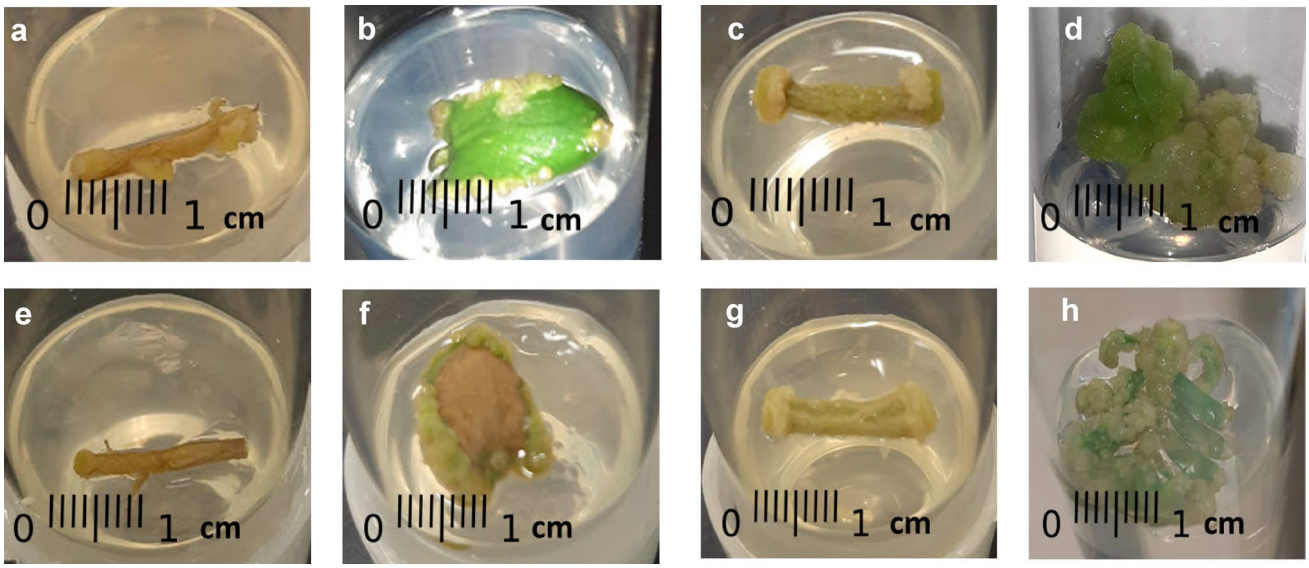

Fig. 2 Callus cultures in *Vachellia farnesiana* explants. Tissues: (**a**, **e**) Root, (**b**, **f**) Cotyledon, (**c**, **g**) Hypocotyl, (**d**, **h**) Leaf. Treatments: (**a**–**d**) Guanajuato: 4.64 µM BA + 13.57 µM 2,4-D, (**e**–**h**) Queretaro: 2.32 µM BA + 13.57 µM 2,4-D

has been reported to induce callogenesis in woody plants (Sharma 2017) and other plant species, such as *Rosa* spp. (Chettri et al. 2024), *Phoenix dactylifera* L. cv. Hayani (El-Dawayati et al. 2020), *Gerbera jamesonii* Bolus ex Hooker f. (Gantait and Mahanta 2021). Combinations of 2,4-D and KIN have also produced high callus induction percentages (90% and 81%) for immature zygotic embryos of *V. farnesiana* and *V. schaffneri* (S. Watson) Seiger & Ebinger, respectively (Ortiz et al. 2000).

Moisture content in leaf and callus tissues

Callus cultures exhibited significantly higher moisture content (92–93%) compared to wild leaves, regardless of distribution site (Table 1). This is noteworthy, as excessive water saturation in plant tissue culture can lead to physiological disorders (Ievinsh 2023). In woody plants, water typically constitutes over 50% of fresh weight, with 60–90% of total water content residing within cells and the remaining 10–40% primarily located in cell walls. The water in cell walls forms a continuum with specialized transport cells throughout the plant, facilitating solute transport and participating in metabolic activities (Giménez et al. 2013). Additionally, our results for the leaves are higher than those reported for species of the same genus, such as *Acacia modesta* Wall. (46.44% w/w) and *V. nilotica* (L.) P. J. H. Hurter & Mabb. (55.22% w/w) (Azim et al. 2011), which could be attributed to the season in which the collection was made, during the rainy period.

Table 1 Phytochemical composition and antioxidant activity of leaf and callus extracts from *Vachellia farnesiana*: A comparison of two distribution sites

Tissue	Distribution site	Alkaloids (mg AE/g)	Phenols (mg GAE/g)	Triterpenes (mg UAE/g)	Antioxidant activity		Moisture content (%)
					DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	
Leaf	Queretaro	339.5 ± 20.9^{a}	158.4 ± 12.5^{a}	243.6 ± 6.2^{b}	120.99 ± 2.7^{c}	66.81 ± 1.6^{b}	78.24 ± 3.4^{b}
	Guanajuato	258.7 ± 17.6^{b}	119.3 ± 7.3^{b}	208.2 ± 10.6^{b}	188.00 ± 3.9^{b}	77.42 ± 2.2^{b}	76.8 ± 1.2^{b}
Callus	Queretaro	139.0 ± 16.4^{c}	66.4 ± 4.9^{c}	356.9 ± 27.8^{a}	684.97 ± 76.4^{a}	415.24 ± 13.0^{a}	93.46 ± 1.7^{a}
	Guanajuato	187.3 ± 15.4^{c}	61.5 ± 2.5^{c}	381.4 ± 22.3^{a}	587.29 ± 88.8^{a}	347.37 ± 15.9^{a}	92.46 ± 2.3^{a}

The data represents the average of three replicates ± SD. Different letters in columns indicate significant differences ($p \leq 0.05$) as determined by the Tukey test

Phytochemical composition in leaf and callus extracts

Total alkaloid content

The leaf extracts from Queretaro exhibited significantly higher alkaloid content (339.6 ± 20.9 mg AE/g DE) compared to callus samples. Notably, the leaf tissue from adult specimens contained up to 2.4 times more alkaloids than callus cultures (139 ± 17.6 mg AE/g DE). However, no differences in alkaloid content were observed between distribution areas (Table 1). The higher alkaloid content in leaves may be attributed to the lack of cellular differentiation in in vitro cultures (Gantait et al. 2018). As cells differentiate, they acquire specialization for metabolite biosynthesis and accumulation, associated with specific organelles like vacuoles (Shitan and Yazaki 2007). Although in vitro biomass showed lower alkaloid content than wild-collected leaves, callus cultures retained the capacity for alkaloid synthesis. Elicitation techniques can be applied to increase alkaloid production in these cultures. Alkaloids are responsible for cytotoxic activity in various species, such as *Catharanthus roseus* (L.) G. Don (Mistry et al. 2022), *Camptotheca acuminata* Decne (Dayan 2023), and *Ochrosia elliptica* Labill. (El-shiekh et al. 2019). Our results are consistent with those reported by Sparzak et al. (2015), who found higher securinine alkaloid concentrations in leaf extracts than in shoot cultures of *Phyllanthus glaucus* Wall. ex Müll. Arg. Similarly, Robins et al. (1987) reported that *Cinchona pubescens* Vahl cell suspension cultures maintained quinine-alkaloid synthesis capacity, albeit at lower concentrations than in adult specimens. In contrast, Abd'quadri-Abojukoro et al. (2022) reported lower alkaloid content in *A. nilotica* leaf extracts than in our study, even in callus extracts. Other studies have shown higher metabolite content in in vitro cultures than in wild or cultivated biomass. For example, Namdeo et al. (2012) reported higher camptothecin content in in vitro whole plants than in ex vitro cultivated *Ophiorrhiza mungos* L. plants.

Total phenols content

Phenols have been reported to be responsible for the cytotoxic activity of *V. farnesiana* (Qiu et al. 2020). In this study, the phenolic content was determined, revealing that the leaves of wild plants from Queretaro have a significantly higher content (158.41 ± 12.5 mg GAE/g DE). In terms of tissue type, the leaves of wild plants showed the highest phenolic content, up to 2.39 times higher than that found in callus cultures. Regarding distribution areas, significant differences in phenolic content were observed in the leaves of wild plants, whereas no differences were found in callus cultures (Table 1). The higher total phenol content in leaf extracts from wild plants compared to callus culture extracts can be attributed to the complex interactions between plants and their environment. Wild plants are exposed to various environmental stress factors, such as competition, changes in water and sunlight availability, and herbivores and pathogens, which induce a higher production of phenols as a defense mechanism. Additionally, phenolic compounds in the cell wall provide structural support and form barriers to prevent moisture loss and pathogen invasion (Lewis 1993). The variability in phenolic content among distribution areas in adult specimens is due to environmental and genetic factors. Secondary compounds are produced in response to adverse environmental conditions, such as intense sunlight, extreme temperatures, water availability, and pathogen attacks, which can vary by location and season. Furthermore, genetic variability within a species population influences the chemical composition of plants, with some individuals having a greater capacity to produce phenols in response to environmental factors (Josipović et al. 2016). This variability is reduced in tissue culture, but the concentration of secondary metabolites may be lower. To increase their concentration, strategies such as controlled abiotic stress, modifying the culture medium composition, adding precursors or inducers of phenolic synthesis, biotic elicitors, and genetic manipulation can be applied (Bajwa et al. 2021).

However, the total phenolic content in leaf extracts found in this study is comparable to that reported by Gabr et al. (2018) for leaves and pods extracts of various *Acacia* species, including *A. farnesiana* leaf extract (63.2–247.9 mg GAE/g DE), which were collected across different seasons. In contrast, Ramli et al. (2011) reported a higher total phenolic content in *A. farnesiana* leaf extracts (209.78 ± 3 mg GAE/g DE).

Total triterpene content

The quantitative determination revealed that the callus derived from Guanajuato had the significantly highest triterpene content (381.46 ± 22.3 mg UAE/g DE). In terms of tissue type, the leaf from adult specimens showed a lower triterpene content, up to 1.83 times lower than that found in callus cultures. However, no differences in triterpene content were observed among distribution areas (Table 1). The higher triterpene content in callus cultures compared to leaves is due to these compounds being part of cellular processes independent of tissue specialization (Haralampidis et al. 2002) and being membrane-bound molecules. Additionally, the higher rate of cell multiplication in callus cultures leads to increased synthesis of these compounds to meet cellular needs (Noushahi et al. 2022). Notably, triterpenes have been reported to be responsible for the cytotoxic activity of *V. farnesiana* (Lin et al. 2009). Therefore,

the higher triterpene content in callus cultures than in wild plant leaves is promising for controlled production through in vitro cultures. However, extract fractionation is necessary to identify the specific bioactive molecules versus those synthesized in callus cultures.

On the other hand, our results for total triterpene content were similar to those reported by Taniguchi et al. (2002) for *Eriobotrya japonica* (Thunb.) Lindl. callus (50 mg/g DW). Notably, they found that the content of ursolic acid and oleanolic acid was lower in callus tissues than in wild plant leaves, whereas the content of triterpenes like corosolic acid and maslinic acid was higher in callus cultures. Similarly, Srivastava and Chaturvedi (2010) reported that callus cultures of *Lantana camara* L. yielded 3.1% betulinic acid, 1.88% oleanolic acid, and 4.12% ursolic acid, whereas the stock plant leaves showed no betulinic acid and only marginally higher amounts of oleanolic and ursolic acids compared to in vitro cultures.

Free radical scavenging activities by DPPH and ABTS

The DPPH radical scavenging assay demonstrated that the leaf and callus extracts possess the ability to eliminate both DPPH and ABTS radicals, indicating its capacity to donate protons to free radicals. This suggests potential antioxidant properties, as the antioxidant effect is attributed to the ability to donate hydrogen to free radicals (Idamokoro et al. 2017). The scavenging activity was expressed as the IC_{50} value, representing the concentration required to scavenge 50% of free radicals. Notably, leaf extracts exhibited IC_{50} values below 80 µg/mL using the ABTS method, whereas callus extracts showed higher IC_{50} values (347–415 µg/mL), using the same method. This disparity may be attributed to the fact that antioxidant compounds are produced in response to environmental stimuli, which is lacking in in vitro-generated biomass. The obtained IC_{50} values were higher than those reported for *A. farnesiana* leaves (L.) Willd. by Ramli et al. (2011), who achieved an IC_{50} of 40.4 µg/mL. This discrepancy can be attributed to variations in compound composition and extraction methods, as an ethanolic extract was employed in this assay, suggesting a higher yield of compounds.

In summary, the distribution site significantly impacted phytochemical contents in wild plant leaves, with Queretaro showed higher total alkaloid, phenol, and triterpene contents, as well as lower IC*50* values for antioxidant activity, likely due to its semi-dry climate, in contrast to Guanajuato's sub-humid temperate climate. In callus cultures, despite non-significant differences in phytochemical compound contents, a trend of elevated values was apparent in Guanajuato's callus cultures, correlating with a twofold increase in BA content (4.64 µM *vs*. 2.32 µM). Plants synthesized a diverse array of secondary metabolites with complex chemical compositions in response to abiotic and biotic stresses, as well as to perform physiological tasks such as attracting pollinators, establishing symbiosis, and providing structural components to lignified cell walls (Ncube and Van Staden 2015). Therefore, callus culture presented a viable mechanism for producing and studying secondary metabolism of *V. farnesiana.* Although plant cells are totipotent and can express genes associated with secondary compound synthesis, in vitro cultures exhibited lower metabolite synthesis. However, various in vitro strategies, including suspension culture and elicitation, have been effectively used to enhance secondary metabolite production (Fazili et al. 2022).

Cytotoxicity bioassay of leaf and callus extracts

HeLa cells exposed to varying concentrations of leaf and callus extracts from two distribution areas exhibited decreased cell viability at 48 and 72 h, compared to control cultures (without extract), as determined by the MTT assay (Fig. 3A–D). The lowest viability was observed at 72 h, likely due to the dose-dependent nature of cytotoxic activity, which is influenced by exposure time and bioactive compound concentration. Paclitaxel (4.27 ng/mL) served as a positive control, showing $57.83 \pm 3.77\%$ and $46.15 \pm 5.16\%$ cell viability at 48 and 72 h, respectively, indicating lower toxicity compared to leaf and callus extracts, warranting further investigation. Notably, callus extracts increased cell viability at 48 h (109.5–120.03%) at the lowest evaluated concentration (5 µg/mL), regardless of distribution area, compared to control treatment (Fig. 3B–D). This phenomenon can be attributed to hormesis, an adaptive response to moderate stress (Mattson 2008). In this study, a dose-dependent response was observed for evaluated concentrations (10–100 µg/mL) at both exposure times, resulting in viability inversely proportional to extract concentration (Fig. 3A–D). Furthermore, leaf extracts exhibited lower cell proliferation compared to callus extracts, regardless of tissue type. Queretaro tissues (leaf and callus) showed the lowest cell viability among distribution areas.

The half-maximal inhibitory concentration (IC_{50}) values were calculated for various extracts using cell viability data, yielding IC_{50} values of ≤ 150 and ≤ 66 µg/mL for 48 and 72 h, respectively. Notably, the leaf extract from Queretaro wild plants exhibited significantly lower IC_{50} values (88.47 and 28.83 µg/mL) regardless of exposure time. Significant differences were observed between tissue types, with leaf extracts showing lower IC_{50} values (28–32 µg/mL). Additionally, Queretaro exhibited lower IC_{50} values (28–43 µg/mL) among distribution areas. Furthermore, IC_{50} values at 72 h were significantly lower, up to 3.35 times lower than those determined for 48 h (Table 2). Phytochemical analyses revealed a correlation between high alkaloid and total phenol content and the cytotoxicity of leaf extracts, where significantly lower IC_{50} values were found. However, it is essential

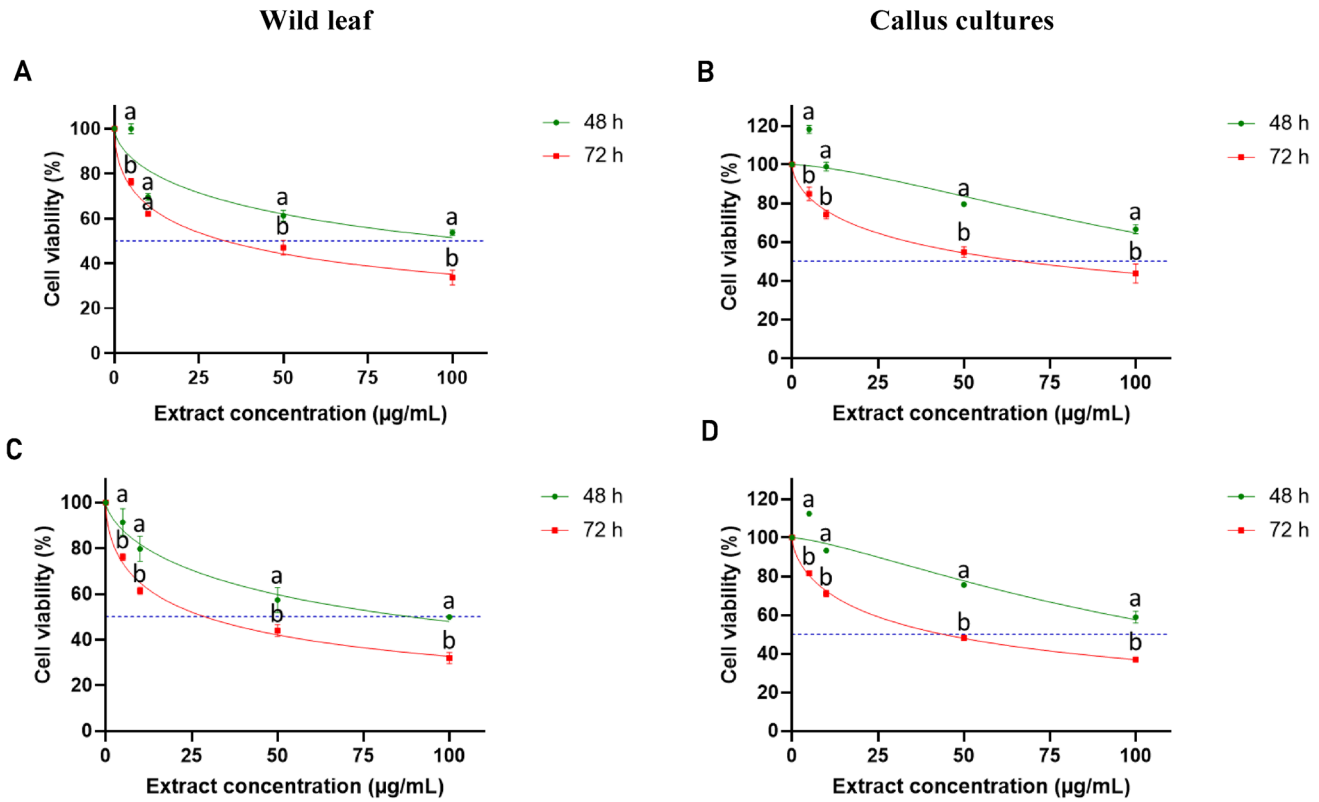

Fig. 3 Cell viability response curve: HeLa cell line exposed to *Vachellia farnesiana* extracts. **A** Guanajuato—Wild Leaf, **B** Guanajuato—Callus Cultures, **C** Queretaro—Wild Leaf, **D** Queretaro—Callus Cultures. Data represent the mean of three repetitions ± SD. Data points denoted by different letters indicate significant differences ($p \leq 0.05$) as determined by the T-test

Table 2 IC_{50} values of *Vachellia farnesiana* leaf and callus chloroform extracts: distribution sites and exposure times

Tissue	Distribution site	IC_{50} 48 h (µg/mL)	IC_{50} 72 h (µg/mL)
Leaf	Queretaro	88.47 ± 1.23^{d}	28.83 ± 1.49^{c}
	Guanajuato	109.7 ± 0.86^{c}	32.74 ± 1.40^{c}
Callus	Queretaro	125.1 ± 1.11^{b}	43.97 ± 1.88^{b}
	Guanajuato	150.4 ± 0.86^{a}	66.35 ± 1.41^{a}

The data represents the average of three replicates ± SD. Different letters in columns indicate significant differences ($p \leq 0.05$) as determined by the Tukey test

to consider that the extracts have a complex composition requiring further study. Moreover, callus cultures can produce compounds do not present in the natural gene pool of wild plant tissue due to continuous cell division and in vitro stress, leading to genetic and epigenetic variation, affecting a wide range of traits including biochemical characteristics, known as somaclonal variation (Gulzar et al. 2020).

Conversely, the American National Cancer Institute (NCI) considers IC_{50} values below 30 µg/mL for plant extracts to be promising for the purification and development of new chemotherapeutic agents (Boik 2001). Similarly, Ayoub et al. (2014) suggest that extract concentrations up to 100 µg/mL can be used to identify new cytotoxins through fractionation and bioactivity analysis. Our results align with those reported by Twilley et al. (2017), who found an IC_{50} of 54.40 ± 2.0 µg/mL for *A. mellifera* (Vahl) Benth. leaf extracts against the HeLa cell line at 72 h. Notably, betulinic acid, a triterpene isolated from *V. farnesiana* root extracts, exhibited an IC_{50} of 1.70 ± 0.04 µg/mL against the A549 cell line, demonstrating that isolated compounds typically have lower IC_{50} values.

In this study, the methodology for extract obtention was based on the method reported by Wall et al. (1996), a modification of Statz and Coon (1976), designed for obtaining plant extracts for antitumor screening at the National Cancer Institute. Subsequently, tannins were removed from the obtained plant extracts, a crucial step since tannins can yield false positive results in certain assays, such as sarcoma assays, due to their reactivity with proteins and enzymes (Wall et al. 1996). Therefore, the cytotoxic compounds present in the plant extracts are likely to be phenols, alkaloids, or terpenes.

Conclusions

Callus cultures were established with friable and abundant growth using leaves from in vitro-germinated seedlings treated with 2.32 µM BA or 4.64 µM BA + 13.57 µM 2,4-D for both distribution areas. Although the callus cultures exhibited lower total alkaloid and phenolic content compared to wild plant leaves, they showed significantly higher total triterpene content. Notably, all extracts from leaves and callus cultures displayed cytotoxicity, with a significantly greater reduction in cell viability observed in extracts from wild plant leaves for both distribution areas, categorizing them as cytotoxic according to NCI criteria. These results suggest that *V. farnesiana* callus cultures may produce compounds with potential activity against certain types of human cancer.

Author contribution All authors contributed to the conception and design of the study. JA Domínguez-Colín produced and maintained in *vitro* cultures. L Buendía-González performed phytochemical analyses and contributed to the writing and correction of the manuscript. C Hernández-Jaimes helped with experimental design. Data collection and analysis were performed by F Cruz-Sosa. J Orozco-Villafuerte supervised the whole study and wrote the manuscript. All authors read and approved the final manuscript.

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Data availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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