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Friable Calluses of a Brazilian Peanut Cultivar Increased Cytotoxic Activity against K562 Human Leukemia Cells

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HIGHLIGHTS

- *Trans*-resveratrol was detected in all extracts from *in vivo* plants.
- Other compounds displayed UV spectrum compatible with phenolic compounds.
- Cultivar IAC886 calluses extract showed highest cytotoxic activity by MTT assay.
- Brazilian peanut cultivars tested varied in composition and cytotoxic activities.

Abstract: Cancer is considered the leading cause of death worldwide, and the number of new cases is expected to rise over the next few years. In this context, plant materials are increasingly studied in the search for substances to prevent and/or treat this disease. In this work, the cytotoxic activity of extracts from plants and friable calluses of five Brazilian peanut cultivars (IAC 886, IAC Caiapó, IAC Tatu ST, IAC 8112 and BR-1) against a leukemic cell line (K562) was compared. Inhibition of K562 cells viability (79.8%) was significantly higher in response to extracts from calluses of cultivar IAC 886 as compared to extracts of aerial parts of *in vivo* and *in vitro* plants from the same cultivar. Callus extracts displayed low toxicity on non-tumor cells (NIH-3T3 fibroblasts and peripheral blood mononuclear cells). *Trans*-resveratrol was found in extracts from aerial

parts of cultivar IAC Tatu ST and from calluses of cultivar IAC 886. In addition, three compounds with UV spectrum compatible with phenolic compounds were detected in the samples. Calluses from cultivar IAC 886 displayed higher relative contents of these compounds, which can be contributing to their cytotoxic activity.

Keywords: resveratrol; peanut; plant tissue culture; cytotoxic activity; tumor cell lines; MTT assay.

INTRODUCTION

Cancer encompasses a set of more than 100 pathologies, with several causes, such as poor diet, sedentary lifestyle, smoking, alcoholism, radiation and heredity. The World Health Organization estimates that by the year 2030 the number of people afflicted by some form of cancer in the world can reach more than 20 million, an increase of 51% in comparison with 2010 [1]. Among the different types of cancer, leukemia ranks the second place in the leading causes of death of children and juveniles in the United States, and the first place in Asia, Central America, South America, Northwest Africa, and the Middle East [2].

Common cancer treatment modalities include chemotherapy, radiotherapy, iodine therapy, surgery and bone marrow transplantation, which can cause different adverse effects [3]. Therefore, the search for natural products of plant origin with protective or therapeutic activities represents a very important research area of science throughout the world [4]. In the last decade, the anticancer activity of extracts and compounds isolated from plants, including alkaloids, coumarins, saponins, and polyphenols has been extensively investigated [5]. Among these, the polyphenol group, comprising flavonoids, tannins, lignans, some caffeic acid derivatives and stilbenes, has been the most investigated, due to its capacity of modulating enzymatic activity and inhibiting oncogenes expression [6, 7].

Peanut is the fourth most consumed oleaginous in the world and is also considered as nutraceutical or functional food [8, 9]. Regular peanut consumption has been associated with reduced incidence of prostate, breast, bowel, and neuroblastoma cancers [10, 11]. A number of phenolic acids, flavonoids and stilbenes, such as trans-resveratrol (3,5,4'-trihydroxy-trans-stilbene), has been reported in the cultivated peanut (*Arachis hypogaea* L.). These compounds are phytoalexins, induced by abiotic and biotic stress [12]. The antiproliferative potential of resveratrol and other compounds found in peanuts has been investigated over the past few decades [13, 14], and its anti-leukemic effects and mechanisms include growth inhibition, induction of apoptosis and autophagy, and cell cycle arrest [15].

Since phytochemical composition and biological potential of peanut seeds and plants may vary significantly among genotypes, plant organs, developmental stages and environmental conditions [16, 17], resveratrol content and resveratrol synthase gene expression have been evaluated in different peanut materials [17, 18-20]. In addition, several biotechnological approaches, such as callus and hairy root cultures, have also been developed aiming at the large-scale production of resveratrol and other bioactive phenolic compounds [12, 21].

In Brazil, several peanut cultivars have been developed by the Instituto Agronômico de Campinas (IAC) and Empresa Brasileira de Pesquisa Agropecuária (Embrapa), in order to produce plants suitable for different climate and soil conditions [22, 23]. Phytochemical analyses of some of these Brazilian cultivars have been carried out in the last decade, indicating the presence of resveratrol in pods, kernels and leaves [24, 25]. Thus, this work was undertaken in order to investigate the *in vitro* cytotoxic activity of ethanolic extracts from aerial parts (leaves and stems) of five Brazilian peanut cultivars, cultured both *in vivo* and *in vitro*, as well as from friable calluses obtained from leaf segments. The presence of resveratrol and other phenolic compounds in these materials was also investigated.

MATERIAL AND METHODS

Reagents

Solvents were HPLC grade (Tedia®, Brazil). RPMI 1640 medium, Penicillin G potassium and streptomycin sulfate were acquired from Invitrogen®, USA. Bovine Fetal Serum (SFB) was acquired from Vitrocell (Brazil). Annexin-V-FITC and propidium iodide (PI) kit was purchased from Thermo-Fisher Scientific®, USA. *Trans*-resveratrol, trypsin, 3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyl-tetrazole bromide (MTT), sodium dodecyl sulfate (SDS), phytohemagglutinin, L-glutamine, and Ficoll-Hypaque were purchased from Sigma Chemical Co.®, USA.

Plant material

Seeds from five Brazilian peanut cultivars (IAC 886, IAC Caiapó, IAC Tatu ST, IAC 8112 and BR-1) were provided by Instituto Agronômico de Campinas (IAC), São Paulo, Brazil. Friable calluses, as well as aerial parts derived from plants maintained both under natural and *in vitro* conditions, were used for extract preparation.

In vitro plants were obtained from embryonic axes cultured for 30 days on hormone-free MS medium [26], and maintained at 25°C ± 2°C, under a photoperiod of 16 h, with mean irradiance of 46 $\mu mol.m^{-2}.s^{-1}$, provided by cold white fluorescent lamps. For callus induction, leaf explants excised from *in vitro* plants were cultured on MS medium supplemented with 1.25 µM picloram, and maintained under the same conditions for 30 days [27]. Greenhouse plants were used after 60 days of seed inoculation in Plantmax® HA under light intensity on a clear day as high as 1600 $\mu E/(m^2/s)$.

Extract preparation

Plant materials were dried in an oven at 45 ºC until constant weight, grounded into powder and weighed. Initially, a defatting step was performed with *n*-hexane to remove non-polar substances, which was repeated until exhaustion. The plant materials were then macerated until exhaustion in ethanol (30 mL/g dry material) at room temperature, in the absence of light. After filtration with filter paper and concentration with vacuum rotary evaporator at 45°C, the extracts were weighed and stored at -4°C until use.

Evaluation of cytotoxic activity

Cell lines and culture conditions

Human chronic myeloid leukemia (K562) cell line was used to evaluate the cytotoxic activity of extracts. A non-tumor fibroblast cell line (NIH-3T3) and peripheral blood mononuclear cells (PBMNC) were also used to screen specificity on tumor cells. K562 and NIH-3T3 cell lines were purchased from the Bank of Cells of Rio de Janeiro and were maintained in liquid nitrogen. After thawing, cells were cultured in RPMI 1640 medium containing 10% Fetal Bovine Serum (FBS), 70 mg/L penicillin G potassium and 100 mg/L streptomycin (complete medium) at 37°C in humidified atmosphere with 5% CO_2, and sub-cultured three times per week for a maximum period of 10 weeks. Adherent cells were detached from the bottles by treatment with 0.25% trypsin added by 0.53 mM ethylene-diamine tetra-acetic acid (EDTA), followed by trypsin neutralization with medium containing 10% FBS, centrifugation (400 x g, 5 min) and suspension in complete medium. Cells were maintained until the 20^{th} sub-culturing.

Isolation and culture of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from human blood by density gradient centrifugation using Ficoll-Hypaque solution. Blood was collected from healthy volunteers with heparin-containing syringes, and centrifuged for 20 min at 400 x g at 18-20°C. After discarding the plasma, the leukocyte-rich layer above the red blood cells (buffy coat) was transferred to a falcon tube. The buffy coat cell volume was homogenized slowly and diluted four times in RPMI 1640 medium, previously diluted twice with 0.9% NaCl containing 2 mM EDTA. A volume of 5 ml of the cell suspension obtained was slowly transferred through the wall into a tube containing 3 mL of Ficoll-Hypaque solution. After centrifugation (400 x g, 18-20°C for 30 min), the PBMC-containing layer was transferred to another tube. An aliquot of the cell suspension was diluted for counting viable cells by Trypan Blue exclusion staining assay. Cells were washed (resuspended in RPMI-EDTA and centrifuged at 400 x g for 3 min), the supernatant removed, and the precipitate suspended in RPMI complete supplemented with glutamine (2 mM) and 2-OH-mercaptoethanol (50 µM). These cells were cultured (1 x 106/mL) for 72 h at 37°C in humidified atmosphere with 5% CO_2 in supplemented RPMI, with and without phytohemagglutinin (PHA) 2.5 µg/mL, in the presence or absence of different sample concentrations (final volume of 200 µL/ well, 96 well plate).

MTT assay

Samples were solubilized in 100% dimethylsulfoxide (DMSO) at 100 mg/mL (stock solution kept at -20°C), and then diluted to 100 µg/mL with complete RPMI cell culture medium. Cells were incubated in 96-well plates (100 µL final volume) with different samples for screening cytotoxic activity by the MTT assay [28]. The control culture received only RPMI medium with DMSO. The most active sample was tested at different concentrations (1-200 µg/mL) for IC_{50} (concentration that inhibits 50% of cell viability) determination. The IC_{50}

values were determined by linear regression from individual experiments using GraphPad Prism 5.0 (GraphPad Software Inc., CA, USA). After 70 h of incubation, 10 µL of stock solution of MTT, prepared at 5 mg/L in phosphate buffered saline (PBS), pH 7.4, was added to each well, and the plates were further incubated at 37°C and 5% CO_2 for another two hours. After that, 100 µL of 10% sodium dodecyl sulfate containing 0.01 N (v/v) HCl was added to each well and the plates were incubated again under the same conditions for 24 h. The absorbance was then measured at 570 nm in a plate spectrophotometer (µQuant, Bio-Tek Instruments Inc., USA). Three independent experiments were done in triplicate. The results were expressed as the relative cell viability, considering the control culture as 100% viable. The selective index (SI) of the most active sample for tumor cells was determined as SI = IC_{50} on normal cells/ IC_{50} on tumor cells.

Qualitative HPLC-DAD-UV analysis

The qualitative profile of samples was performed on a High Performance Liquid Chromatography system coupled to a diode-array detector (HPLC-DAD-UV), using a Shimadzu® Class VP equipped with SCL-10A VP controller, DGU-14A degasser, LC-10AD VP binary pump, CTO-10 ASVP oven, detection system by DAD SPD-M10AVP and automatic injector SIL10-AF. The chromatograms were processed by the software Schimadzu® Class VP version 6.1. All samples were dissolved in methanol at a final concentration of 10 mg/mL and filtered through a 0.45 µm Milipore filter. Analyses were performed on a Thermo ScientificTM C18 column (250 mm x 4.6 mm i.d. x 5 µm particle size), at a flow rate of 1.0 mL/min and oven temperature at 50°C. The injected volume for all samples was 20 µL. Solvent system consisted of ultrapure aqueous acetic acid solution, pH 3, (solvent A) and acetonitrile (solvent B), with gradient elution starting at 95% of A and 5% of B to 5% of A and 95% of B in 80 min, and more 10 min in initial condition in order to system re-equilibration. A fresh standard solution of 100 µg/mL of *trans*-resveratrol in methanol was prepared for comparison in each analysis. The detection of *trans*-resveratrol in the samples was carried out at 305 nm, based on its UV spectrum (λ_{max} at 220 nm and 304-305 nm). The retention time (tR) of the *trans*-resveratrol standard was registered at 26.4 min (Figure 1). The percentage values of area were obtained by integrating the peaks using Class-VP software (Shimadzu®). The relative percentage of each substance is related to the sum of the area of all the chromatogram peaks at a given wavelength, representing 100%. The value of an area represents the relative percentage of a given substance [29-34].

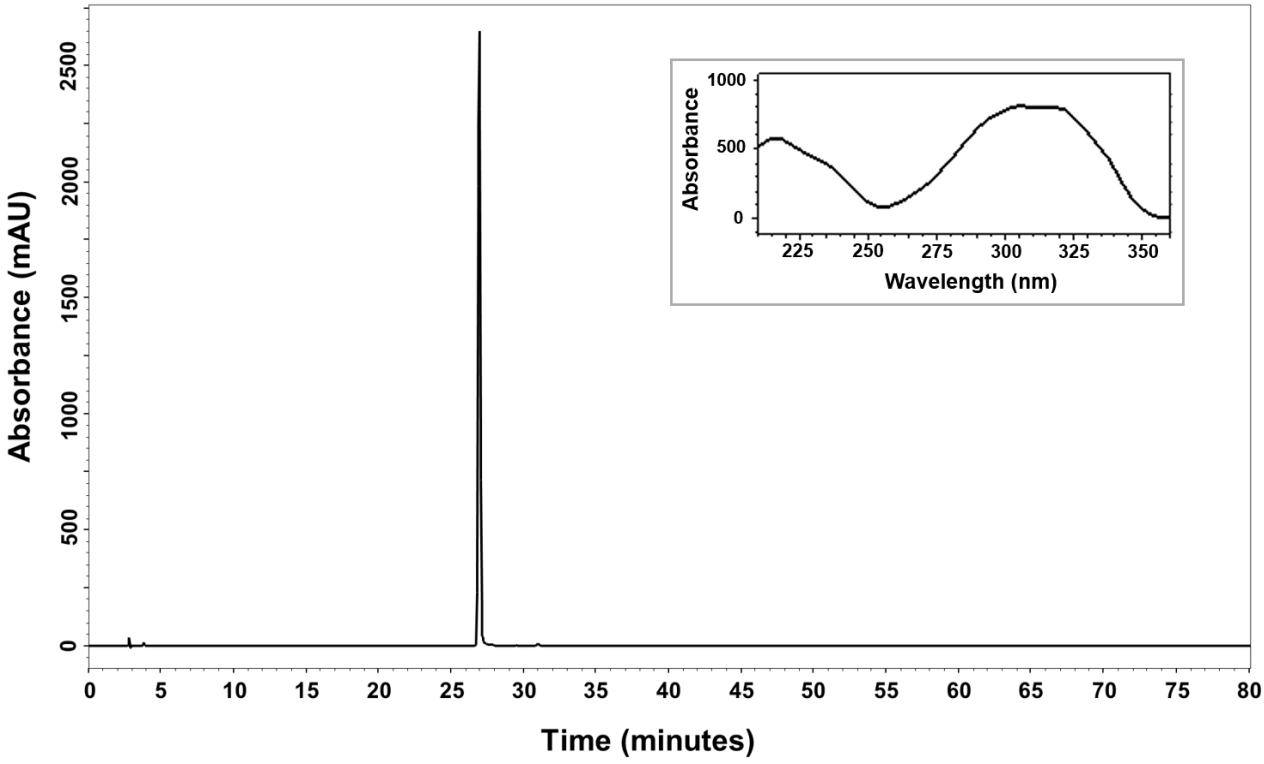

Figure 1. HPLC-DAD-UV chromatogram and UV spectrum of the *trans*-resveratrol standard (100 µg/mL). Analyses were performed at 304 nm, on Thermo Scientific™ C18 column (250 mm x 4.6 mm i.d. x 5 µm particle size), at a flow rate of 1 mL/ min, temperature at 50°C and injection volume of 20 µL.

Statistical analysis

Data were compared by one-way analysis of variance (ANOVA). Significant differences between groups were evaluated by the Dunnet or Tukey post-tests at p≤0.05, using the GraphPad Prism® software (GraphPad Software Inc., San Diego, CA).

RESULTS AND DISCUSSION

In vitro cytotoxic activity

The response of K562 leukemic cells varied significantly according to the materials tested (Figure 2). Similar cytotoxic effects were observed with extracts of aerial parts from both *in vivo* and *in vitro* plants, except for cultivars IAC 8112 and BR-1, which caused 1.76 to 51.09% cell viability inhibition (Figure 2).

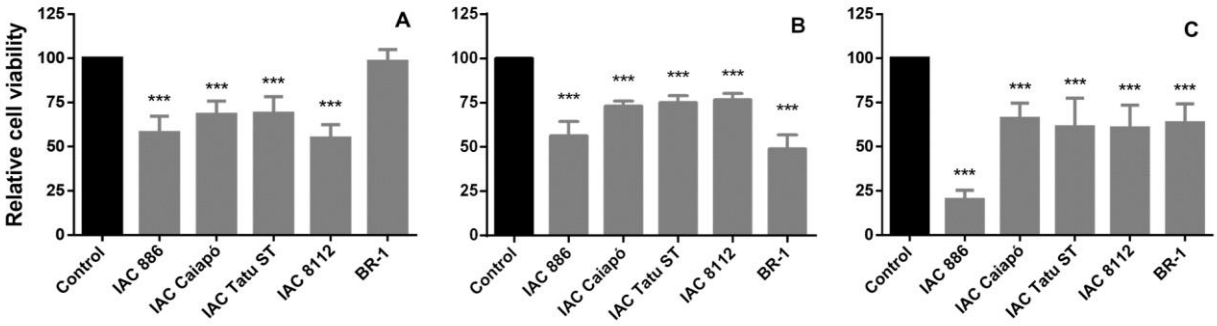

Figure 2. Relative cell viability of human leukemia cell line (K562) treated with ethanolic extracts from aerial parts of plants maintained both (A) *in vivo* and (B) *in vitro* and (C) calluses from five Brazilian peanut cultivars (*Arachis hypogaea* L. cvs. IAC 886, IAC Caiapó, IAC Tatu ST, IAC 8112 and BR-1). Cells (5 x 104 / mL) were incubated in the absence or presence of samples (100 µg/mL) for 72 h at 37 °C, 5 % CO_2 and humidified atmosphere. Data represent mean ± standard deviation (SD) of three independent experiments. The absorbance of the control culture of each experiment was considered as 100%. *** p ≤ 0.001, relative to the control culture (without sample) by One-way ANOVA, followed by the Dunnet test.

On the other hand, a significantly higher inhibition of K562 cell viability was observed with the extract of friable calluses from cultivar IAC 886 (79.8%). Therefore, this extract was evaluated at concentrations from 1 to 200 µg/mL, showing IC_{50} 41.65 µg/mL. In order to screen specific cytotoxic activity against this cell line, callus extract from cultivar IAC 886 was also tested against a non-tumor fibroblast cell line (NIH-3T3) and peripheral blood mononuclear cells (PBMNC), showing IC_{50} values of 143.8 µg/mL and 108.4 µg/mL, respectively. Selective indexes (SI) for these extracts were of 3.45 and 2.60 on NIH-3T3 and PBMC cells, respectively (data not shown). These results indicated that extracts from friable calluses of cultivar IAC 886 have a selective cytotoxic effect for leukemic cells, with low effect on non-tumor cells, as expected for suitable anticancer agents [35, 36].

Qualitative analysis of stilbenes

Considering that the presence of phenolic acids and stilbenes has been previously described in extracts from seeds, hypocotyls, roots, leaves, and *in vitro*-derived materials of *A. hypogaea* [37-43], a qualitative analysis of *trans*-resveratrol and other phenolic compounds was carried out in the extracts from the peanut cultivars studied in this work. *Trans*-resveratrol was detected in extracts of aerial parts of *in vivo* plants, but could not be identified in the extracts of aerial parts of *in vitro* plants or calluses.

Resveratrol can be found in several plant species, including those from *Eucalyptus*, *Pinus*, *Vitis* and *Arachis* genus. It is found in two isoforms, *trans*-resveratrol and *cis*-resveratrol. Although the *trans* isomer is converted in the *cis* photostable form, the *trans* isomer is more biologically active [44]. Due to its capacity to remove free radicals, induce apoptosis and inhibit important enzymes, such as cyclo-oxygenase (COX-1 and COX-2), lipoxygenase and protein C-kinase, the presence of *trans*-resveratrol has been associated with antioxidant, anti-inflammatory, anticarcinogenic and cardioprotective potential [45-49]. Moreover, resveratrol is also able to inhibit protein NF Kappa β, which regulates cell proliferation, avoiding tumor proliferation [15].

In addition to *trans*-resveratrol, other two compounds were detected in the peanut samples tested here. Compounds 1 (tR = 18.9 min) and 2 (tR = 20.6 min) showed UV absorption spectra compatible with phenolic compounds (λ_{max} ~ 220 nm and 304 nm) (Figure 3). These compounds were detected in extracts of aerial

parts from both *in vivo* and *in vitro* plants from all cultivars, and in extracts of callus from the cultivars IAC886 and BR-1.

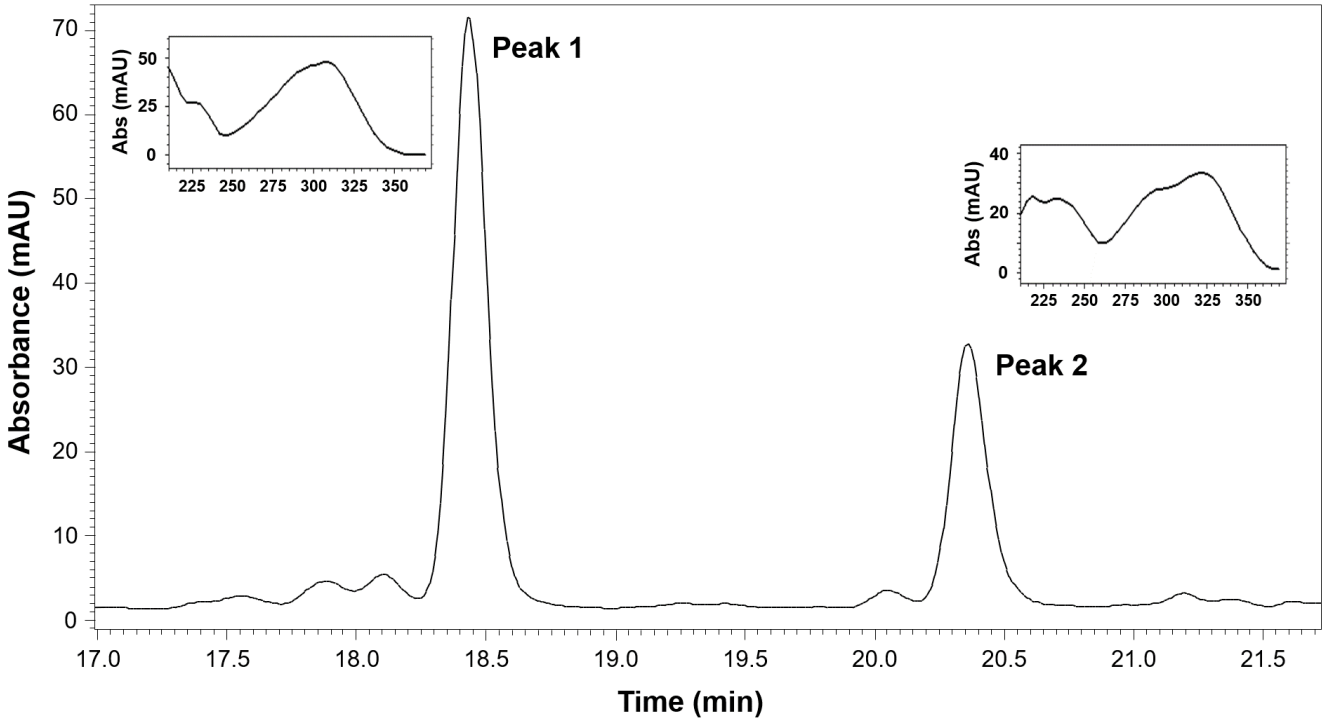

Figure 3. Chromatographic profile of the extract from aerial parts of *in vivo* plants of the Brazilian peanut (*Arachis hypogaea* L.) cv. IAC 886, considering the presence of peaks 1 (tR = 18.9 min) and 2 (tR = 20.6 min), with UV absorption spectra compatible with phenolic compounds (λ_{max} ~ 220 nm and 304 nm). Analyses were performed at 304 nm, on Thermo Scientific™ C18 column (250 mm x 4.6 mm i.d. x 5 µm particle size), at a flow rate of 1 mL/ min, temperature at 50 °C and injection volume of 20 µL.

Taken together, these results suggest that the anti-leukemic activity demonstrated by the extracts from aerial parts and calluses of Brazilian cultivars of peanut might be related to the presence of phenolic compounds other than *trans*-resveratrol. In the last years, several authors have studied the anticancer potential of stilbenes, which has been correlated to the oxidation of potentially carcinogenic molecules and alteration of gene expression [50-52]. Stilbenes isolated from different tissues of *Arachis* plants, such as leaves, roots and seeds, as well as from its derivatives, have also demonstrated antitumor properties [12, 53-57]. Among these compounds, *trans*-resveratrol, arachidin-1 and arachidin-3 purified from hairy roots of *A. hypogaea* were cytotoxic on two tumor cell lines *in vitro* (RAW 264.7 and HeLa) [53]. When evaluating the effects of stilbenes on leukemic cells, Huang and coauthors showed that arachidin-1 isolated from *A. hypogaea* seeds induced damages in cell membrane and activation of caspases, resulting in *in vitro* cell death [54]. In addition, Tolomeo and coauthors reported that 3'-hydroxypterostilbene were 50-97 times more potent than *trans*-resveratrol in inducing apoptosis of leukemic cells [58].

This is the first report investigating the anti-leukemic activity and phenolic compounds of IAC 886, IAC Caiapó, IAC Tatu ST, IAC 8112 and BR-1 Brazilian peanut cultivars. Considering the great genetic and phytochemical variation among cultivars developed around the world, this research contributes to deepening the knowledge on peanut species and their biological potential. The results demonstrated that the cultivars have significant variation in anti-leukemic activity and phenolic composition, including *trans*-resveratrol. In addition, it was demonstrated that extracts from friable calluses derived from leaf segments of cultivar IAC 886 have greater cytotoxicity on K562 leukemia cells when compared to the extracts from aerial parts of *in vivo* or *in vitro*-grown plants, highlighting the relevance of biotechnological methods to modulate the production of useful secondary metabolites. Taken together, our results suggest that callus cultures can be an useful source of stilbenoids from *Arachis* species. These systems could be used to establish cell suspension cultures aiming at scaling up the production of these compounds by modulating culture conditions and using elicitors supplementation.

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Conflicts of Interest: The authors declare no conflict of interest.

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