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In vitro cytotoxic activity of forage peanut (*Arachis pintoi* Krapov. & W.C. Greg.) oil against leukemic and tumor cell lines

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***Arachis pintoi* Krapov. and W.C. Greg. is widely used as forage and displays high seed productivity. Its seed oil contains high levels of phytosterols, mainly β-sitosterol, which has been associated with several biological properties, including cytotoxic activity against different types of cancer. In this study, we investigated the cytotoxic activity of its seed oil against five cancer cell lines using the MTT assay. The most representative fraction (F41) of the oil displayed higher cytotoxic potential than the crude seed extract, reducing K562 cell viability by 73% (IC^{50} = 85 µg/mL). Four fatty acids were detected in this fraction: 13-cis-octadecanoic, linoleic, hexanoic, and palmitic acids, whose antiproliferative activity against cancer cell lines has already been described. Therefore, this study might contribute to adding value to the forage peanut.**

Key words: *Arachis pintoi* seed extract, cytotoxic activity, F41 fraction, MTT assay, fatty acids.

INTRODUCTION

Arachis pintoi Krapov. and W.C. Greg. has gained increasing importance for forage production in tropical and subtropical areas due to its nutritional qualities and high persistence under heavy grazing. Similar to other *Arachis* species primarily cultivated for forage or ornamental purposes, few studies on the biological potential of A. *pintoi* are available in the literature, except for its allelopathic (Cunha et al., 2010), antihelminthic (Fernex et al., 2012), herbicidal (Thang et al., 2023), and antioxidant activities in leaves (Sang et al., 2014) and callus extracts (Sousa-Machado et al., 2018). This is understandable considering that the consumption of peanuts (*Arachis hypogaea* L.) has been correlated with the prevention of cardiovascular diseases, type II diabetes, Alzheimer's, and various types of cancer (Arya et al., 2016). Other reported activities include protection against insulin resistance (Liu et al., 2022), reduction of cholesterol and triglyceride levels, as well as weight loss through increased thermogenesis and satiety control (Alves et al., 2014). These effects are associated with the presence of phytosterols found in high concentrations in nuts, seeds, and vegetable oils, as well as with the high

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concentration of certain fatty acids present in the oil obtained from *Arachis* seeds (Çiftçi and Suna, 2022).

Phytosterols stabilize phospholipid membranes similarly to cholesterol and also act as hormones or hormonal precursors. Several biological activities have been associated with phytosterols, such as anti-atherosclerotic, antiulcerogenic, antifungal, antioxidant, angiogenic, chemopreventive, immunomodulatory, and anti-inflammatory effects (Bakrim et al., 2022). Studies on nutraceutical foods have shown that the ingestion of β-sitosterol, one of the main phytosterols found in the *Arachis* genus, inhibits intestinal cholesterol absorption, prevents cardiovascular disease (Li et al., 2022), and reduces the risk of lung, breast, stomach, esophageal, prostate, colon, rectum, endometrial, and ovarian cancers (Bin Sayeed and Ameen, 2015). In addition to prevention, cytotoxic activity against colon (Baskar et al., 2010), prostate (Awad et al., 2000), and breast (Awad et al., 2007) tumors has been demonstrated. In *Arachis* seeds, the types and content of phytosterols vary according to the species and genotypes (Awad et al., 2000; Grosso et al., 2000). In A. *pintoi*, β-sitosterol corresponds to 59.7% of the phytosterols present in the seed oil (Grosso et al., 2000).

Arachis seeds are also rich in fatty acids, which are the main constituents of triglycerides, phospholipids, and other complex lipids, accounting for approximately 50% of the seed composition (Chamberlin et al., 2014). They are important features determining seed quality in *Arachis*, including health benefits, flavor, and the durability of derived products. The main fatty acids found in peanut oil are oleic and linoleic acids, which account for approximately 80% of the oil, in addition to palmitic, stearic, arachidic, gadoleic, behenic, and lignoceric acids found in smaller amounts (Wang et al., 2015). Some unsaturated and polyunsaturated fatty acids have already been studied for their role in preventing cardiovascular diseases and cancers, regulating cholesterol and blood pressure, and other beneficial health effects (Calder, 2015).

Since *A. pintoi* has become widely cultivated worldwide, with the development of various cultivars with high seed productivity, the goal of this work was to evaluate the antiproliferative activity of the seed oil against two leukemic and three tumor cell lines. Additionally, the qualitative profile of one representative fraction was determined by GC-MS.

MATERIALS AND METHODS

Reagents

Solvents were HPLC grade (Tedia®, Brazil). RPMI 1640 medium was acquired from Invitrogen®, USA. Bovine Fetal Serum (SFB) was acquired from Vitrocell (Brazil). Silica gel, trypsin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazole bromide (MTT) e sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co.®, USA.

Plant material and extracts preparation

Fruits of *A. pintoi 'Amarillo* MG-100*'* was purchased from BRSEEDS Produção e Comércio de Sementes LTDA. Approximately 1.5 kg of dehulled seeds were dried at 40°C for 48 h and ground into powder. Seed oil was obtained by maceration with *n*-hexane at room temperature, repeated until exhaustion. Extracts were combined and evaporated to dryness at 40°C, using a rotatory vacuum evaporator (Marconi – M120).

The extract (100 g) was then resuspended in *n*-hexane and fractionated by column chromatography (5 cm diameter, 100 cm height) packed with silica gel, and eluted with *n*-hexane, dichloromethane, ethyl acetate and methanol in an increasing polarity gradient, according to Table 1. Fractions were collected and subjected to thin layer chromatography (TLC), using different solvent systems, and grouped according to the similarities in the chromatographic profile. All TLC plates were revealed using a 10% sulfuric acid solution in ethanol, followed by heating at 100°C.

GC-MS analysis

For the esterification of the most representative fraction (F41), 20 mL of a solution containing 0.9 mL of sodium methoxide in methanol, 9.1 mL of methanol and 10 mL of ethyl ether were added to 400 mg of the extract. Then, 60 mL of 10% aqueous sodium chloride solution was added to the mixture, followed by sonication for 15 min, before the addition of 12 mL of dichloromethane and a new sonication cycle of 10 min to solubilize the esters. The dichloromethane lower phase was collected and filtered with anhydrous sodium sulfate. The solvent was evaporated and the sample was resuspended in 1 mL of dichloromethane before the analysis.

GC-MS analysis was undertaken in Scion 456-GC-TQ-MS Brucker equipment, using a 5%-phenyl-methyl polysiloxane BR-5MS (30 m x 0.25 mm, 0.1 µm) Brucker column. A sample volume of 1.0 µL was injected at 250°C with a split ratio of 1:10, using helium at 0.5 mL/min as the carrier gas. The oven temperature was initially set to 60°C, increasing at 3°C/min to 270°C. The temperatures of the detector and transfer line were 300°C and 240°C, respectively. The source of ions operated at a potential of 70 eV. The compounds were identified using the NIST mass spectra library.

Evaluation of *in vitro* cytotoxic activity

Two human leukemic cell lines, chronic myeloid leukemia (K562) and acute T lymphocytic leukemia (Jurkat), as well as three human tumor cell lines – breast adenocarcinoma (MCF7), prostate adenocarcinoma (PC3) and basal alveolar adenocarcinoma (A549), were purchased from the Rio de Janeiro Cell Bank (Rio de Janeiro, Brazil) and used to evaluate the cytotoxic activity of the extracts. Cell lines cultivation and cytotoxic activity evaluation were performed as previously described (Casimiro et al., 2023). Briefly, *n*-hexane extract and the selected fraction F41 were dissolved in 100% dimethylsulfoxide (DMSO) at 100 mg/mL and diluted to 100 µg/mL with supplemented RPMI-1640 medium. Concentrations of 0 to 100 µg/mL were tested for IC_{50} (concentration that inhibits 50% of cell viability) calculation. Cell lines were incubated with extracts in 96-well plates (5×10^{-4} cells/ml per well) for 70 h at 37°C in a 5% CO_2 humidified atmosphere. The negative control consisted of the cells incubated without extracts. After incubation, 10 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, Sigma®, USA) at 5 mg/mL in phosphate-buffered saline (PBS, pH 7.4) were added to each well. After further incubation for 2 h, sodium dodecyl sulfate (SDS, Sigma®, USA) 10% solution (100 µl/well) was added for formazan solubilization. The absorbance was measured after 24 h

Table 1. Solvent elution and corresponding fractions from the *n*-hexane extract of Arachis pintoi seeds by silica gel column chromatography.

Solvents (fractionation)	Fractions
100% *n*-hexane	1 - 7
n-hexane + dichloromethane (9:1)	8 - 11
n-hexane + dichloromethane (3:1)	12 - 18
n-hexane + dichloromethane (1:1)	19 - 27
n-hexane + dichloromethane (1:3)	28 - 31
100% dichloromethane	32 - 36
dichloromethane + ethyl acetate (8:2)	37 - 43
dichloromethane + ethyl acetate (1:1)	44 - 47
dichloromethane + ethyl acetate (2:8)	48 - 49
100% ethyl acetate	50 - 52
ethyl acetate + methanol (1:1)	53 - 56
100% methanol	57 - 63

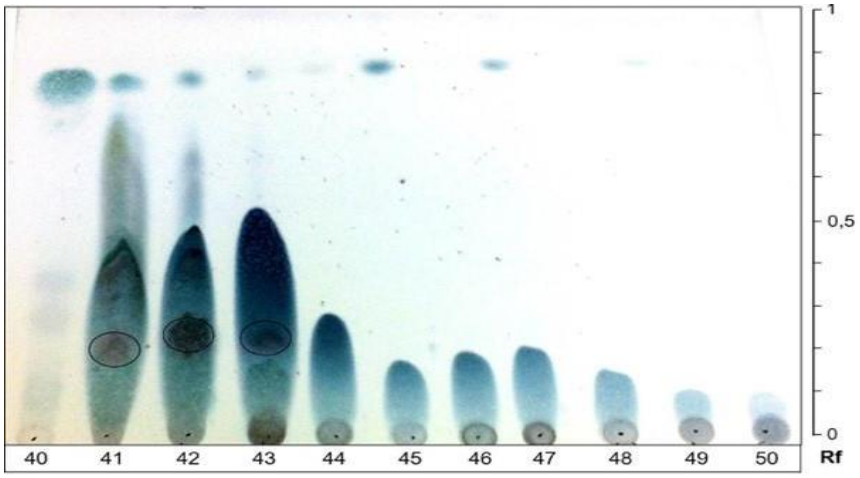

Figure 1. Thin layer chromatography of fractions 40 to 50 from *n*-hexane extract of forage peanut seeds with darker spots in fractions 41, 42 and 43 (circles), using 100% dichloromethane as mobile phase.

at 570 nm in a plate spectrophotometer (μQuant, Bio-Tek Instruments, Inc., USA). Cytotoxicity was expressed as percentages of the control cultures absorbance. Experiments were carried out in triplicates.

Statistical analysis

Data variance was evaluated by One-way ANOVA and the significance of sample differences in relation to control cells was assessed by Student's t-test using the GraphPad Prism 5® (GraphPad Company, San Diego, CA, USA) software, adopting a confidence interval of 95% ($p \leq 0.05$).

RESULTS AND DISCUSSION

Fractions preparation and phytochemical analysis

The fractionation of *n*-hexane extract by silica-gel column chromatography resulted in 63 fractions. Collected fractions were then analyzed by TLC and grouped into 16 combined fractions according to the similarity among their profiles. Fractions containing isolated or slightly mixed substances were not grouped.

The most representative fraction (F41) was selected for further assays, considering that it displayed a large blue spot (R_f = 0.85) that was also present in fractions F34-40 and F42-47, in addition to other spots detected in fractions F42-50 (R_f = 0.2), F42-43 (R_f = 0.25) and F42(R_f = 0.7), as shown in Figure 1. GC-MS analysis of F41 revealed four fatty acids (Figures 2 and 3): one unsaturated, 13-cis-octadecanoic acid (18:1); one polyunsaturated, linoleic acid (18:2); and two saturated, hexanoic (6:0) and palmitic acids (16:0). These compounds were also found by Grosso et al. (2000), who reported the presence of eight fatty acids in seeds of different *Arachis* species, including the *A. pintoi* in

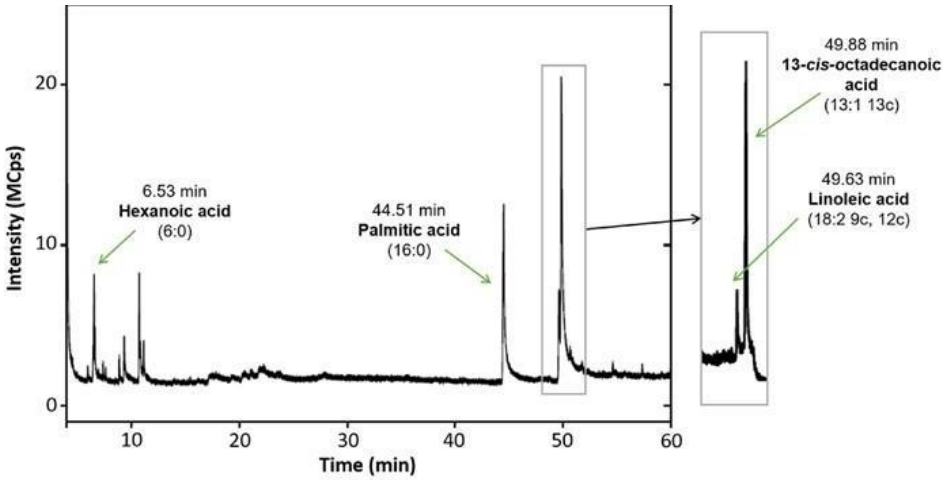

Figure 2. Total ion chromatography of the fraction F41 from *n*-hexane extract of forage peanut seeds by GC-MS.

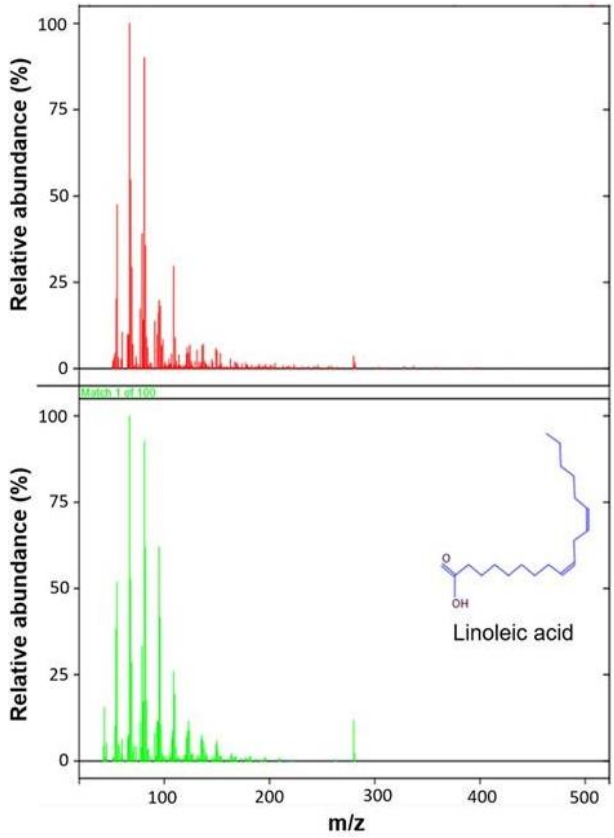

Figure 3. Mass spectra of one compound detected in fraction F41 of the *n*-hexane extract of forage peanut seeds and their likely correspondent from the mass spectrum library by GC-MS.

extracts obtained with petroleum ether. However, the short-chain saturated hexanoic acid (6:0), known as caproic acid, and the long-chain unsaturated 13-cis-octadecanoic acid (18:1) present in the *n*-hexane extract described here, were not previously detected in genus *Arachis*.

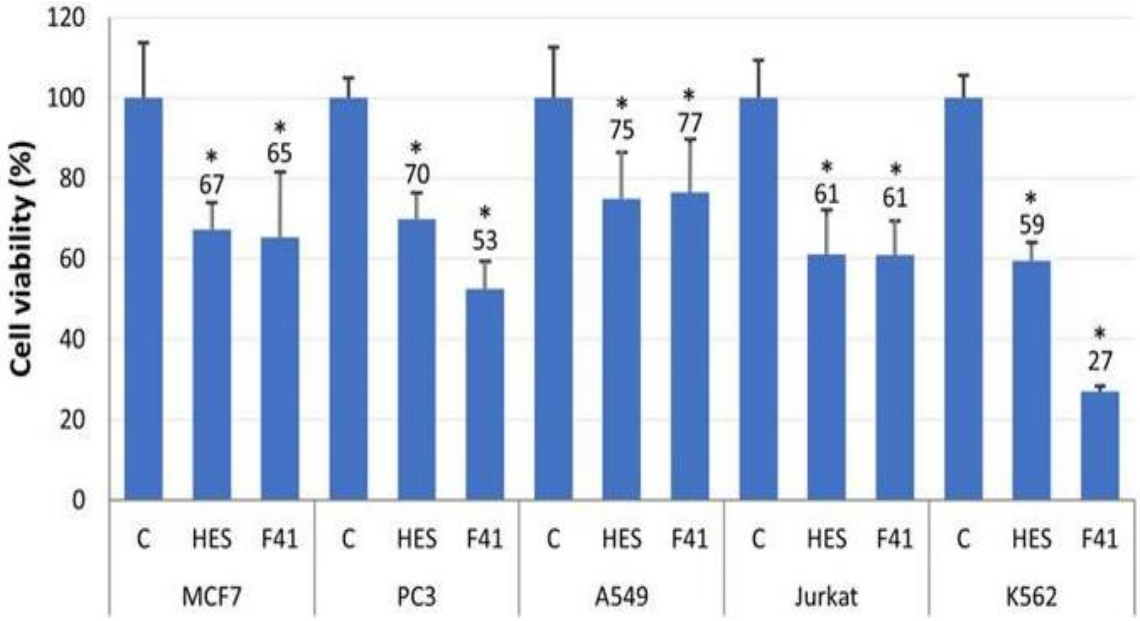

Figure 4. Cytotoxic activity of *n*-hexane extract of seeds (HES) and fraction 41 (F41) at 100 µg/mL against tumor and leukemic human cell lines in comparison with the control (C) without extracts, after 72 h, by MTT assay. The results were expressed as a percentage of cell viability of the cell lines. Data represent mean ± SD of three independent experiments in triplicate. Values of $p \leq 0.05$ were considered significant. *represents a statistical difference of $p < 0.001$ related to control by Student's t-test.

Oleic acid (an omega-9) and linoleic acid (an omega-6) correspond approximately to 80% of the total fatty acids of *A. pintoi* (Grosso et al., 2000), which is equivalent to the percentage reported for *A. hypogaea* (Chamberlin et al., 2014). Both fatty acids are essential for human metabolism and were associated with the reduction of the risk of cardiovascular diseases. Oleic acid is also related to lowering blood pressure and bad cholesterol (LDL) and has a beneficial effect on type II diabetes and obesity-associated inflammatory processes (Vassiliou et al., 2009). Moreover, conjugated linoleic acid, which is a mixture of isomers, was found to be beneficial for glucose homeostasis regulation for reducing oxidative stress, hepatic steatosis, and milk fat depression, and has also shown antitumor potential (Kim et al., 2016).

Assessment of cytotoxic activity

In this work, the crude seed oil and the representative fraction (F41) extracted from the forage peanut caused a reduction in the mitochondrial activity of the leukemic and tumor cell lines. In general, F41 showed greater cytotoxic potential than the crude *n*-hexane extract. In addition, the leukemic cell line K562 was more sensitive to this fraction (IC_{50} of 85 µg/mL) when compared with the other leukemic and tumor cell lines tested (Figure 4).

These results can be related to the high concentration of fatty acids in F41, especially palmitic, linoleic and octadecanoic acids, which have been associated with antiproliferative properties. Palmitic acid found in seaweed extracts (*Amphiroa zonata* Yendo) showed selective toxicity against human leukemic cells *in vitro* and *in vivo* (Harada et al., 2002). The toxicity of palmitic acid to different tumor lines via apoptosis through the mitochondrial pathway, facilitated by the promotion of intracellular reactive oxygen species (ROS) generation, was demonstrated by Wang et al. (2023). The effect of linoleic acid in reducing inflammation and tumor proliferation has also been studied *in vivo* and *in vitro* (Lausson et al., 2023).

The cytotoxic potential of seed oils has been studied in different species. For example, the oil from *Nigella sativa* L., whose main active compounds are thymoquinones, acts by different mechanisms, such as enhancement of the natural killer cell activity, tumor cell death, and inhibition of proliferation (Randhawa and Alghamdi, 2011). The oil of *Coix lacryma-jobi* L. contains four types of fatty acids forming triglycerides which appear to have a synergy with components that inhibit fatty acid synthase in tumor cells (Yu et al., 2008). Furthermore, antitumor activity has been reported in *A. hypogaea* seeds (Sobolev et al., 2011), stems (Chen et al., 2017) and hairy root cultures (Abbott et al., 2010), as well as in oil nanoemulsions (Fazelifar et al., 2020). This effect is usually associated with the action of proteins (Ahmad et al, 2020), phytosterols (Awad et al., 2000) and phenolic compounds, including resveratrol (Abbott et al., 2010;

Sobolev et al., 2011).

Conclusion

In this work, we demonstrated the cytotoxic activity of the n-hexane extract from seeds of A. píntoi. The oil most representative fraction (F41) showed a significantly higher activity against K562 leukemia cell line when compared with the crude extract. The analysis of F41 by GC-MS revealed the presence of four fatty acids, including hexanoic and 13-cis-octadecanoic acids, which have not been previously reported in the genus. Although the anticancer activity of the common peanut has been previously attributed to phytosterols, these compounds were not detected in the F41 oil fraction from the forage peanut. These results suggest that the cytotoxic activities of *Arachis* seeds could also be associated with the presence of fatty acids. This is the first report on the cytotoxic potential of A. *pintoi* seed oil. Taken together, the results contribute to adding value to the forage peanut, especially considering the availability of cultivars with high seed production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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