ARTICLE

ANTIPROLIFERATIVE ACTIVITY OF *ANNONA SQUAMOSA* L. FRACTIONS ON HUMAN TUMOR CELL LINES

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Abstract: *Annona squamosa* Linn.(Annonaceae) is a rich source of bioactive compounds, such as alkaloids, tannin, phenolic compounds, aromatic plyketides, steroids, diterpenes and mineral components. Several parts of this plant are traditionally used in the treatment of diabetes and cancer. The current assay evaluates antiproliferative activity of fractions of sugar-apple (*A. squamosa*) peel and seeds in nine human tumor cell lines (K-562, HT-29, PC-3, NCI-H460, MCF-7, NCI-ADR/RES, UACC-62, U251 e 786-0) and in a non-tumor cell line (HaCat), with doxorubicin as positive control. Cell differentiation was determined by spectrophometric quantification. Seed and peel fractions showed cytostatic and cytocidal effects on cell lines at different concentrations, except for K562. All fractions were selective for the NCI-H460 strain. However, other fractions had an antiproliferative effect on the other strains, especially on the methanol fraction of seeds, which showed the highest cytocidal percentage (up to 100%). Results indicate the potential of these fractions as sources of new chemotherapeutic and/or chemo-preventive agents to be isolated and identified.

Keywords: Antitumoral, Cytocidal, Cytostatic, Pericarp, Seeds.

Atividade Antiproliferativa de frações de *Annona squamosa* L. em células tumorais humanas

Resumo: *Annona squamosa* Linn.(Annonaceae) representa uma rica fonte de compostos bioativos, como alcaloides, taninos, compostos fenólicos, acetogeninas, esteroides, diterpenos e componentes minerais. Diversas partes dessa planta são empregadas tradicionalmente no tratamento de diabetes e câncer. Para avaliação da atividade antiproliferativa de frações de cascas e sementes dos frutos de *A. squamosa*, foram empregadas nove linhagens tumorais humanas (K-562, HT-29, PC-3, NCI-H460, MCF-7, NCI-ADR/RES, UACC-62, U251 e 786-0) e a linhagem não tumoral HaCat, utilizando como controle positivo a doxorrubicina. A proliferação celular foi determinada por quantificação espectrofotométrica. Frações de sementes e cascas da planta mostraram efeitos citostático e citocida para as linhagens em diferentes concentrações, com exceção da linhagem K-562. Todas as frações ensaiadas apresentaram seletividade para a linhagem NCI-H460. No entanto, as demais linhagens também sofreram efeito antiproliferativo das frações, em especial da fração metanólica de sementes, que apresentou maior percentual citocida (até 100%). Esses resultados apontam o potencial dessas frações como fontes de novos agentes quimioterápicos e/ou quimiopreventivos a serem isolados e identificados.

Palavras-chave: Antitumoral, Citocida, Citostático, Pericarpo, Sementes.

Introduction

The disorderly growth of cells is one of the defining characteristics of cancer – a term that encompasses more than 100 types of malignant diseases that can invade adjacent tissues or more distant organs, which can even spread to various regions of the body (Inca, 2022).

Cancer places a large burden on public health. It accounts for 3.5 million deaths per year worldwide (Prakash et al., 2013). It is the second leading cause of death in the world, reaching 70% of occurrence in low- and middle-income countries. It is estimated, for these countries, an increase of 81% of new cases for the next two decades (Shah et al., 2019).

Epidemiological context points to a worrying future. However, with the expansion of scientific knowledge and the advancement of biotechnology, the landscape of disease care and control may soon be transformed (Sturdy, 2017). As part of this, knowledge of tumor biology, the use of biomarkers and specific oncological therapies (precision medicine), collaborate to determine effective treatments (Blanchard, 2016; Iriart, 2019).

The modern therapies developed are often limited in their efficacy due to their associated side effects and high cost (Sabir et al., 2012). In that regard, plant extracts have been used as medicinal for decades (Chen et al., 2012). They have attracted special attention in the last 30 years owing to their potential as effective new therapeutic and anti-cancer agents (Newman et al., 2002; Newman, 2008; Costa & Cavalcante, 2018), given that plant-derived compounds can act as inhibitors of tumorigenesis (Solowey et al., 2014) and have shown antiproliferative effect in human tumor cell cultures (Guidoti et al., 2019). Indeed, ethnopharmacological studies are important in the construction of medicinal plant databanks with antineoplastic activity (Jacobo-Herrera et al., 2016).

Annona squamosa L. has been reported in the literature as a source of alkaloids, tanins, phenolic compounds (Pandey & Parve, 2011; Biba et al., 2013), acetogenins, steroids, diterpenes (Pontes et al., 2004) and mineral components (Bhardwaj et al., 2014) in different parts. This plant, with high antioxidant potential, has been used in popular medicine to treat a number of diseases such as diabetes, fertility disorders, in addition to cancer (Pillay et al., 2008).

Around 60% of medicaments currently used to treat cancer derive from natural sources (Gordaliza, 2007). Among antitumor drugs available between 1940 and 2010, 41% are natural products or derivatives and 22% are synthetic based on natural molecules (Newman & Cragg, 2012). Vegetables have been the most important source of these compounds (Solowey et al., 2014). These include vinblastine and vincristine obtained from *Catharanthus roseus* (Apocynaceae), and paclitaxel, a drug obtained from Taxus brevifolia (Taxaceae) (Srivastava et al., 2005) and a lactone from *A. squamosa* that is more active than the chemotherapeutic drugs cisplatin and fluorouracil in inhibiting the cell growth of hepatocellular carcinoma in rats (Zhang, 2006). Sugar-apple (*A. squamosa*) se-

eds and peel also contain compounds with antitumoral action, such as fatty acids (Fujimoto et al., 1990; Chandrababu et al., 2012), diterpenes (Joy & Remani 2008) and acetogenins (Ndob et al., 2009).

The antiproliferative activity of *A. squamosa* has been described for different extracts and parts of the plant, showing significant results against human tumor cell lines (Zhang, 2006; Joy & Remani, 2008; Anaya-Esparza et al., 2020; Guidoti et al., 2021). The antiproliferative potential of extracts and isolates can be confirmed in animal models. However, a preliminary analysis can be performed using in vivo tests with tumor cell lines to screen new antitumor agents.

The culture of cell lines derived from cancer cells, compared to their non-tumor counterparts, is commonly used to assess the properties of phytochemicals and medicinal plant extracts (Fernando & Rupasinghe 2013). Thus, these assays help determine the possible activities attributed or not to numerous plant species, in addition to being an important tool in the search for bioactive substances (Pilatova et al., 2010; Zakaria et al., 2011), allowing simultaneous assays with different types of tumor cell lines (Holbeck, 2004).

Although many bioative compounds obtained from plants are assessed for their antitumoral activity, it is known that the positive effects of plants are due to the complex interaction between compounds present in the entire plant, which may exhibit additive, synergic or antagonistic effects that do not occur with isolated constituents (Liu, 2003; Karna et al., 2012). Thus, the aim of this study was to determine the antiproliferaive effect of extracts from sugar-apple seeds and peel in tumor and non-tumor cell lines to assess the use of this plant against cancer and help in the selection of potentially bioactive extracts for future studies of anticancer molecules.

Material and methods

Plant Material

Annona squamosa L. (Annonaceae) ripe fruits were collected in the summer (January) of 2013 at Estância Peluma, Fazenda Jagora in the municipality of Fernandópolis, São Paulo, Brazil (20°25'47.0"S 50°19'50.1"W, altitude = 403 meters). A voucher specimen was identified and deposited in the herbarium of Maringa State University, Maringá, Paraná, Brazil under protocol HUEM 29903.

Chemical Extraction

Frozen sugar-apple (*A. squamosa*) peel and seeds (8 kg and 2.3 kg, respectively) were ground in a domestic blender and submitted to exhaustive extraction with ethyl acetate (EtOAc), at ambient temperature (28 ºC) for 14 days, followed by filtering. Both extracts were concentrated, separately, in a rotating evaporator at 50 ºC, obtaining 59.5 g and 173 g of crude extract (EtOAc), respectively. The extracts were partitioned separately in chloroform (400 mL) and distilled water (50 mL x 3), to obtain the CHCl3 phase and aqueous extract. The CHCl3 phases of each fraction were concentrated and partitioned separately between n-hexane (400 mL) and methanol (50 mL x 3), yielding 12.8 g and 23.1 g of methanolic fraction of peel and seeds, respectively. After preparation, the *A. squamosa* L. extracts obtained were the ethyl acetate fraction of peel and seed and the methanolic fraction of the peel and seed.

In vitro analysis of antiproliferative activity

Antiproliferative activity was assessed in nine human tumor cell lines, from INCA - National Cancer Institute (Frederick, MA, USA): K-562 (leukemia), NCI-H460 (lung, non-small cells), MCF-7 (breast); U251 (glioma); PC-3 (prostate); UACC-62 (melanoma); HT29 (colon); NCI-ADR/RES (ovary with phenotype resistant to multiple drugs); 786-0 (kidney) and HaCat (immortalized keratinocytes, non-tumor cell line).

The cell lines were cultivated in 5 mL of Gibco BRL - RPMI 1640 medium, supplemented with 5% fetal bovine serum and gentamicin (50 $mg.mL^{-1}$). The cells were seeded in 96-well plates (10 µL cells/well) and exposed to concentrations of the sample dissolved in DMSO/RPMI (0.25; 2.5; 25 and 250 $\mu g.mL^{-1}$), at 37 ºC with 5% atmospheric CO_2, for 48 hours. The final concentration of DMSO was assessed in a previous assay and did not affect cell viability (data not shown). The drug doxorubicin was used as positive control.

The cells were fixed with 50% trichloroacetic acid and cell proliferation was determined according to the Monks et al. (1991), calculated by spectrophotometric quantification (570 nm) of cell protein content, using the sulforhodamine B assay.

If T > C, there is cell growth stimulation. If T ≥ T0, but > C, there is cytostatic activity and the formula used will be 100 x [(T-T0)/(C-T0)]. If T < T0, there is cytocidal activity and the formula used will be 100 x [(T-T0)/T0], where: T = mean number of treated cells, C = cell control and T0 = cell control on the day samples were added. The results are presented as TGI (total growth inhibition) values, calculated by the response curve of each cell line, using linear regression analysis and ORIGIN 7.5 software, with values >50 $\mu g.mL^{-1}$ considered inactive (Fouche et al., 2008).

Results and discussion

The ethyl acetate and methanolic fractions of *A. squamosa* peels and seeds were sub-

mitted to antiproliferative activity assessment in cultures of human tumor cells and HaCat non-tumor human cell lines (immortalized keratinocytes), using doxorubicin as positive control (Fig. 1). The concentration values necessary to promote total cell growth inhibition (TGI) are shown in Tab. 1.

Ethyl acetate fraction of peel exhibited dose-dependent selectivity for the NCI-H460 cell line (TGI 11.4 µg.mL-1), with a cytocidal effect of around 75% (Fig. 2). The cytostatic effect was also observed for MCF-7 and PC-3 cell lines at a concentration of 2.5 µg.mL-1. At a concentration of 25 µg.mL-1, the fraction acted cytostatically for the remaining lines, except for UACC-82 and 786-0. The cytocidal effect occurred at the highest concentration (250 µg.mL-1) for all the cell lines, except the leukemia line (K-562), being the most significant for the UACC-62 strain (82%).

The methanolic fraction of peels also demonstrated dose-dependent selectivity (Fig. 3)

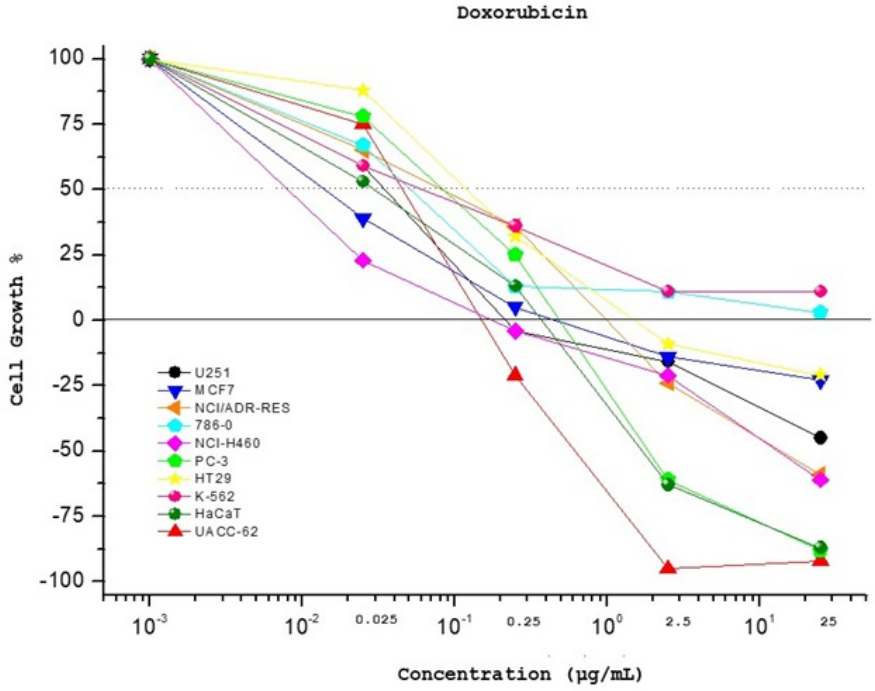

Fig. 1. TPercent cell growth of human tumor and normal cell lines at different concentrations (0.025; 0.25; 2.5 and 25 µg.mL^{-1}) of doxorubicin, after 48 hours of exposure.

Tab. 1. Tab. 1. Concentration necessary for total cell growth inhibition (TGI) of tumor and non-tumor cell lines treated with different fraction concentrations of *Anonna. squamosa* peel and seeds.

Treatments	TGI (µg mL^{-1})									
	U251	UACC-62	MCF-7	NCI-ADR/RES	786-0	NCI-H460	PC-3	HT-29	K-562	HaCat
Doxorubicin	0.65	0.16	0.93	1.0	13.5	0.19	0.45	3.2	>25	0.28
EAFP	27.3	69.5	36.4	23.8	79.7	11.4	39.8	70.6	>250	44.4
MFP	15.5	59.9	27.4	47.2	164.0	10.7	37.3	50.6	>250	43.6
EAFS	45.9	66.1	34.1	66.9	120.2	6.4	74.3	124.7	>250	48.4
MFS	4.2	5.7	4.5	6.6	8.7	0.72	5.7	8.9	>250	4.6

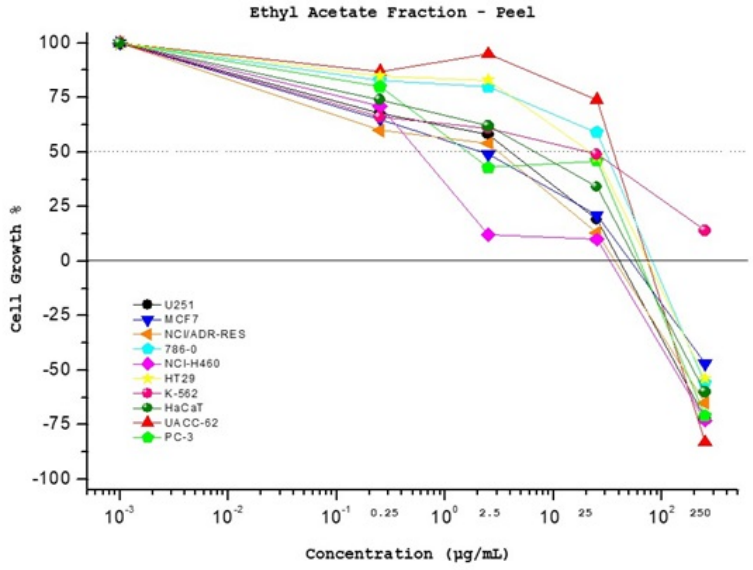

Fig. 2. . Percent cell growth of human tumor and normal lines at different concentrations (0.25; 2.5; 25 and 250 µg.mL^{-1}) of the ethyl acetate fraction of *Anonna. squamosa* peel, after 48 hours of

for NCI-H460 (TGI 10.7 µg.mL-1). At the lowest concentration (2.5 µg.mL^{-1}), the fraction showed a cytostatic effect on U251 and MCF-7 cell lines. For the remaining cell lines, except UACC-62, the cytostatic effect occurred at a concentration of 25 µg.mL^{-1}. At the highest concentration (250 µg.mL^{-1}), except for the K-562 cell line, a cytocidal effect was observed, with around 90% inhibition for the U251 cell line.

Several bioactive components have been identified in the fruit peel, such as catechins, caffeine and gallic acid in ten varieties of *A. squamosa* (Manochai et al., 2018). Analysis by GC-MS (Gas Chromatography coupled to a Mass

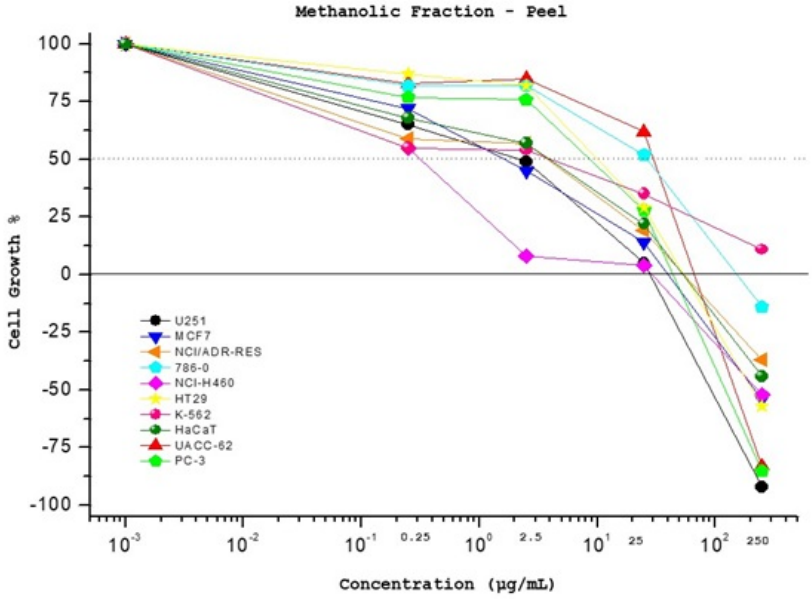

Fig. 3. .Percent cell growth of human tumor and non-tumor cell lines at different concentrations (0.25; 2.5; 25 and 250 µg mL-1) of the methanolic fraction of *Anonna. squamosa* peel, after 48 hours of exposure.

Spectrometer), performed by Adesanwo et al. (2020) in the fruit, revealed the presence of monoterpenes, diterpenes, sesquiterpenes and their derivatives, in addition to fats and esters. Altaee et al. (2020) showed that fruit peel is also rich in nutrients, vitamins and minerals, constituting an excellent source of potassium, fiber, folic acid, manganese, phosphorus, zinc, copper, iron and calcium. The phytochemical screening performed by Oliveira et al. (2022) indicated the presence of phenolic compounds, alkaloids, carbohydrates, steroids, triterpenes, flavonoids, quinones, saponins, tannins and terpenes, indicating that the use of this part of the plant is promising, since its constituents can have numerous biological activities, including the antitumor, according to Srivastava et al. (2005), Fan et al. (2006) and Leung et al. (2009). The content of secondary metabolites in the fruit peel was also analyzed by Ponce-Sánchez et al. (2023), which revealed the presence of quercetin, ascorbic acid, citric acid, malic acid, tartaric acid, gallic acid, glucose and fructose.

According to Abdulsalami et al. (2016), most of the anticancer activities described for *A. squamosa* are correlated with the methanolic extract. Guidoti et al. (2019) isolated kaurenoic acid from the methanolic fraction of the pericarp of *A. squamosa*, which demonstrated cytostatic activity for all strains tested and cytocidal, except for K-562 and 786-0 strains. The observed antiproliferative effect was approximately 88% inhibition. Antiproliferative activity was assessed by the authors in nine human tumor cell lines, obtained from the National Cancer Institute (Frederick, MA, USA): U251 (glioma); UACC-62 (melanoma); MCF-7 (breast); NCI-ADR/RES (ovary with phenotype resistant to multiple drugs); 786-0 (kidney); NCI-H460 (lung, non-small cells); PC-3 (prostate); HT29 (colon); K562 (leukemia). The cell line HaCat (immortalized keratinocytes, non-tumor cell line) was used as control.

The antitumor activity of this metabolite has also been described by other authors, especially against breast, colon and leukemia cancer cell lines (Mongelli et al., 2002; Ambrosio et al., 2004; Lizarte-Neto et al., 2013).

Through the MTT assay, the cytotoxic effect of the methanolic extract of the peel of *A. squamosa* was demonstrated by Altaee et al. (2020) in melanoma and lymphoma cell lines. Compared to the aqueous and ethanolic extracts, the methanolic extract showed a more significant effect on cytotoxicity for these strains. Furthermore, Joy & Remani (2008) showed that the chloroform fraction of the pericarp had a dose-dependent cytotoxic activity on HeLa and DLA cell lines, suggesting their potential chemotherapeutic use. The behavior of the fraction was attributed to kauranic diterpenes, subsequently isolated and identified.

As observed in Fig. 4, the ethyl acetate fraction of seeds also showed selectivity for the NCI-H460 cell line (TGI 6.4 μg.mL^{-1}), with cytostatic effect at concentrations 0.25, 2.5 and 25 μg.mL-1 and cytocidal at the highest concentration (250 μg.mL^{-1}). The fraction also demonstrated a cytostatic effect for MCF-7 and

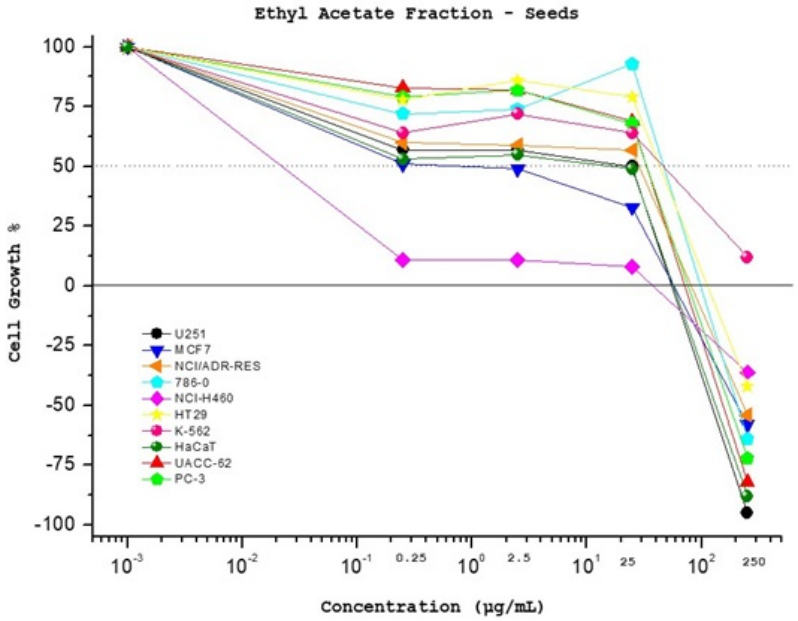

Fig. 4. .Percent cell growth in human tumor and non-tumor cell lines at different concentrations (0.25; 2.5; 25 and 250 μg.mL^{-1}) of the ethyl acetate fraction of *Anonna. squamosa* seeds, after 48 hours of exposure.

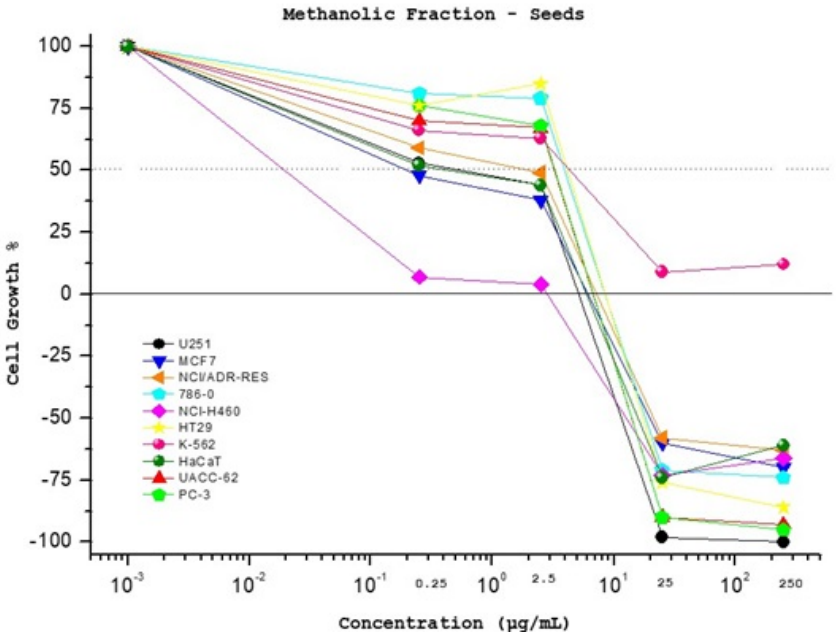

Fig. 5. .Percent cell growth of human tumor and non-tumor cell lines at different concentrations (0.25; 2.5; 25 and 250 µg.mL^{-1}) of the methanolic fraction of *Anonna. squamosa* seeds, after 48 hours of exposure, 48 hours after exposure.

U251 cell lines at a concentration of 25 µg.mL^{-1}. It also showed a cytocidal effect for all strains, except for K-562, at a concentration of 250 µg.mL^{-1}, with the most potent antiproliferative effect for the U251 strain (100%).

The methanolic fraction of seeds was effective for all tumor cell lines tested (TGI between 0.72 to 8.9 µg.mL^{-1}), except K-562 cell line (TGI >250 µg.mL^{-1}), as shown in Table 1. The highest selectivity was observed for the NCI-H460 cell line (Fig. 5). In this fraction, the cytocidal effect was found at a concentration of 25 µg.mL^{-1} for all the cell lines except K-562, with an antiproliferative effect between 60% and 100%.

All the fractions tested showed higher selectivity for the NCI-H460 cell line (lung, non-small cells). According to Carretero et al. (2004), this line exhibits a nonsense mutation in the LKB1 gene, which leads to the absence of its protein product (LKB1), the primary regulator, under energy stress, of the AMP kinase enzyme, which in turn, modulates cell growth and metabolism in conditions of intracellular ATP reduction (Inoki et al., 2003; Hardie et al., 2012). Thus, components of the fractions tested may be acting in a similar way to that described by Hirsch et al. (2009), whereby in LKB1-deficient tumor cells substances may inhibit the mitochondrial complex I in this line, inducing the emergence of reactive oxygen species and consequent apoptosis, due to the inability to effectively neutralize this species.

Despite the higher selectivity observed for the NCI-H460 cell line, the fractions in general showed a cytostatic and cytocidal effect for the remaining cell lines under study (except for K-562). The antiproliferative activity can be explained, in part, by the presence of phytochemicals in the peel and seeds of the *A. squamosa* fruit, as demonstrated by Zhang (2006), Joy & Remani (2008), Anaya-Esparza et al. (2020) and Guidoti et al. (2019).

Fujimoto et al. (1990) isolated and identified a fatty acid in the seeds and peel of the fruit, which exhibits chemical similarity with bullatacin acetogenin, an acetogenin with potent antitumoral activity, with selectivity for human PC-3 cell line (Hoop et al., 1996). In the present study, this line was also influenced (cytostatic and cytocidal) by the peel and seed fractions.

Seeds, on the other hand, are widely studied, especially owing to the presence of acetogenins, secondary metabolites derived from long-chain fatty acids and obtained through acetic acids (Alali, 1999). These are found in different parts of *A. squamosa*, mainly in seeds, demonstrating antitumoral activity by inhibiting the NADH of the electron transport chain and the NADH oxidase of the tumor cell plasma membrane (Lu et al., 1995).

Due to their ability in blocking ATP production, acetogenins can selectively inhibit the growth of specific types of tumor cells, such as those resistant to multiple drugs (Alali et al., 1999). In the present work, the methanol fraction of seeds showed potential antiproliferative activity (TGI of 0.72 µg.mL^{-1}) for the lung tumor

cell line (NCI-H460). This same fraction was used for the isolation of acetogenins by Chang et al. (1999) in Annona atemoya.

Chen et al. (2012) showed that seed extracts have also demonstrated an efficient antitumor effect in vivo in rats transplanted with hepatoma cells (H22), exhibiting tumor inhibition of 69.55% compared to cyclophosphamide and no side effects in animals. Two main acetogenins were identified: squamostatin-A and bullatacin. The latter, an acetogenin known for its potent inhibitory effect of complex I from the electron transport chain, was 300 times more efficient than taxol drug when essayed in vivo (McLaughlin, 2008; Liaw et al., 2010).

In addition to the presence of acetogenins in seeds, other classes of bioactive compounds have also demonstrated an antiproliferative effect in vitro, as reported by Chandrababu et al. (2012). These authors isolated and identified two fatty acids (propanoic acid and hexadecanoic acid) of *A. squamosa*, the latter found for the first time in this plant. Propanoic acid exhibits potent cytotoxic activity for MCF-7 and HeLa cell lines, similar to that demonstrated by the methanolic fraction of seeds in the present study for the MCF-7 cell line (TGI 4.5 $\mu g.mL^{-1}$).

The methanolic fraction of seeds had the lowest TG1 values for all the cell lines tested (Tab. 1). This indicated its potential use in chemical isolation studies of compounds with antiproliferative activity. On the other hand, concentrations above 250 $\mu g.mL^{-1}$ are needed in the K562 line in order to obtain total growth inhibition. According to Tsiftsoglou et al. (2003), the K562 cell line exhibits a high degree of plasticity that allows its differentiation when submitted to different chemical agents, which could partially explain its behavior in the present study.

Shehata et al. (2021) demonstrated that the methanolic extract of *A. squamosa* seed had a greater anticancer effect against four cell lines: Caco-2 - colorectal adenocarcinoma, HepG-2 - liver cancer, MCF-7 - breast and PC-3 - prostate. Morphological analysis showed that the extract led to cell shrinkage in the test strains, which was confirmed by apoptosis assay.

Pinto et al. (2017) demonstrated the cytotoxicity of the methanolic extract of the *A. squamosa* seed against human tumor strains, with the most expressive effect against the MCF-7 cell line. Furthermore, the extract led to reduced clonogenic survival of $HCT^{-1}16$ - colorectal carcinoma and MCF-7 cell lines. Six alkaloids were identified in the extract: anonaine, asimilobine, corypalmine, liriodenine, nornuciferine and reticuline, which may be associated with the observed effect.

It is also worth noting that the ethyl acetate fractions of the peel and seed and the methanolic fraction of the peel exhibited an average TGI of 45.5 $\mu g.mL^{-1}$ for the normal human strain HaCat, proving to be less toxic when compared to doxorubicin (TGI 0.28 5 $\mu g.mL^{-1}$). Doxorubicin is a chemotherapeutic widely used in the treatment of different types of cancer, blocking advancement of some tumors. However, it has negayive effects such as causing tissue damage and bubble formation during intravenous administration (Pessina et al., 2001). Therefore, these fractions, in addition to being potential sources of antitumor molecules, have less toxicity for normal cells, with the exception of the methanolic fraction of seeds that exhibited a TGI of 4.6 $\mu g.mL^{-1}$.

Conclusion

With respect to treatments with tumor cell lines, it was evident that fractions of *A. squamosa* seeds and peel exerted a considerable antiproliferative effect for all cell lines tested at different concentrations. In particular, it was observed selectivity of all fractions for the cell line NCI-H460, which establishes new precedents for the use of cell-specific substances.

The chemodiversity present in *A. squamosa* is reflected in the different pharmacological activities described for this plant, including antitumoral. Therefore, the results of this study emphasize literature data in terms of the traditional use of *A. squamosa* in combating and/or preventing physiological disorders and collaborate in the selection of fractions for future chemical isolation and identification of bioactive compounds with antiproliferative potential that can act as new anticancer agents.

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