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Kaurenoic acid from *Annona squamosa* L. exhibits antiproliferative effect on human tumor cell lines and induces apoptosis in *Aspergillus nidulans*

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Abstract

Annona squamosa is a source of bioactive compounds, with several pharmacological activities. However, tests are needed to confirm the safety of these compounds in terms of cytogenotoxic potential and their possible medicinal activity. Thus, the aims of the present study were to isolate kaurenoic acid from sugar apple peel and determine its antiproliferative activity in nine human tumor cell lines and mutagenic activity in germinating *Aspergillus nidulans* conidia. Chemical extraction resulted in the isolation of kaurenoic acid, a terpene that demonstrated a cytostatic effect at concentrations of 25 and 250 µg mL^{-1} and antiproliferative at a concentration 250 µg mL^{-1}. The germination test showed that this compound can activate apoptosis, since there was an increase in dead conidia and a decrease in malformed individuals in all treatments. These results indicate its antiproliferative effect and apoptosis activation, representing a useful tool in the development of new chemotherapy drugs.

Keywords: Diterpene. Cytostatic Activity. Cytocidal Effect. Sugar Apple.

Introduction

Plants are a source of substances with pharmacological potential[1], so-called bioactive compounds, produced in the form of secondary metabolites capable of triggering pharmacological or toxological activities in humans and animals[2].

Pharmacological activities include antioxidant, antimutagenic, anticarcinogenic, antiinflammatory and antimicrobial effects[1]. These metabolites can therefore act in preventing diseases and maintaining genetic material stability[3]. In this respect, antitumor activity has been reported for compounds of plant origin since 1950[4]. Since then, numerous antitumor agents obtained from plants have been used to treat cancer[5].

Annona squamosa L. (Annonaceae) is a plant that bears edible fruit with commercial and medicinal potential[6]. Traditionally, different parts of *A. squamosa* have been used in popular medicine, such as the roots[7], leaves, powder from the green fruit and the seeds[8].

Several pharmacological activities have been described for this plant, including pro-apoptotic, antiproliferative[9,10], antidiabetic[11], hepatoprotector[12], antigenotoxic[13], antimicrobial[14], antioxidant[15] and anti-HIV[16]. The diverse bioactivity of the different structures of this plant reflects its wide range of nutrients and different classes of bioactive compounds[17].

Phytochemical studies with *A. squamosal* fruit peel (exocarp) extracts have demonstrated its rich bioactive compound content, such as alkaloids, proteins, flavonoids, saponins, tanins, steroids[18,19], cumarins and terpenes[18], which explains its wide range of biological activities.

Given its nutraceutic and therapeutic properties, *A. squamosa* has been the object of studies searching for new compounds that could serve as the basis for developing drugs, especially those with antitumr activity.

Despite the extensive research that demonstrates the medicianl potantial of different parts of this plant, tests should be conducted to determine the possible toxicity of its isolates, contributing to future formulations of herbal medicines and/or phytopharmacological drugs. Furthermore, the absence of cytogenotoxicity studies with secondary metabolites isolated from the exocarp of this plant is relevant to the investigation of the possible mutagenic or antimutagenic effects of these compounds.

Thus, the aims of the present study were to isolate kaurenoic acid from the fruit peel of *A. squamosa*, determine its antiproliferative activity in nine human tumor cell lines and genotoxicity in the germination of conidia from the *biA1methG1* line of *Aspergillus nidulans*, thereby helping to elucidate the bioactivity of these compounds, and develop new chemotherapy treatments.

Material and Methods

Plant Material

Ripe *Annona squamosa* L. (Annonaceae) fruit, obtained from Estância Peluma, Fazenda Jagora, Fernandópolis, São Paulo, Brazil (20°25'47.0"S 50°19'50.1"W, altitude 403 meters), were collected in January 2013. The climate in the region is tropical wet (Köppen-Geiger climate classification: Aw). The voucher specimen was identified and deposited in the herbarium of the State University of Maringá, Maringá, Paraná, Brazil, registered under HUEM 29903.

Extraction and Chemical Isolation

Frozen *A. squamosa* fruit peel (8 kg) was ground in a domestic blender and submitted to extraction with eight liters of ethyl acetate (EtOAc), at room ambient (≈ 28 ºC) for 14 days, followed by filtering, obtaining

E1. The residue was once again submitted to extraction, under the same conditions, generating E2. Both extracts (E1 and E2) were concentrated separately in a rotating evaporator at 50 ºC, obtaining 40.0 and 19.5 g of crude extract, respectively. E1 and E2 were partitioned separately in chloroform to obtain the organic phase and aqueous extract. The $CHCl_3$ phase of both extracts was concentrated and partitioned separately between *n*-hexane and methanol. The methanolic phase (F1), from E1 produced 2.86 g (7%), while that obtained from E2 (F2), generated 9.22 g (47%).

The methanolic extracts (F1 and F2) were submitted separately to column chromatography (Ø 20 mm) silica gel 60 (26.0 g), with 70-230 mesh, eluted with *n*-hexane, chloroform, chloroform/methanol gradient mixtures (in proportions of 99:1, 19:1, 9:1 and 4:1) and methanol, according to Chang et al.[20], resulting in 36 flasks in each fraction. Thin-layer chromatographies (TLC) were perfomed in aluminum plates covered with 0.20 mm-thick silica-gel 60 UV254 nm, obtained from Merck, as stationary phase, using *n*-hexane/EtOAc (2:1) as mobile phase. The flasks of each fraction (F1 and F2) were combined separately, according to the chromatographic profile. Only the 10th and 11th fractions obtained from F1 MeOH column chromatography and the 7th fraction obtained from F2 MeOH column chromatography were submitted to ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) analysis, since they were homogeneous in appearance in TLC.

NMR spectrophotometry

The NMR spectra of ^{1}H were obtained in a Varian Gemini-300P spectrophotometer (300.06 MHz). Chemical displacements were recorded in δ, using tetramethylsilane (TMS, δ 0.00) or $CDCl_3$ (δ 7.27) as internal reference standards. The solvents used were all deuterated. The multiplicity of the signals obtained characterized according to AUREMN (Association of Nuclear Magnetic Resonance Users) recommendations. The NMR spectra of ^{13}C were obtained in a Varian Gemini-300P spectrophotometer (75.45 MHz). Chemical displacements were recorded in δ, using tetramethylsilane (TMS, δ 0.00) or $CDCl_3$ (δ 77.00) as internal reference standards. The number of hydrogens bound to carbon atoms was determined using NMR spectra of ^{13}C, with the help of ^{13}C/DEPT NMR techniques (90 and 135, where CH_3/CH = positive signal, CH_2 = negative signal and C_0 = absent).

***In vitro* analysis of antiproliferative activity**

Antiproliferative activity was assessed 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 *in vitro* antiproliferative assay was performed as described by Monks et al.[21].

The cell lines were cultivated in 5 mL of RPMI 1640 medium (Gibco BRL), 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 µg 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). Doxorubicin was used as positive control. The cells were then fixed with 50% trichloroacetic acid and cell proliferation was determined 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, determined by the response curve of each cell line, using linear regression analysis and ORIGIN 7.5 software, with values > 50 µg mL^{-1} considered inactive[22].

Genotoxicity analysis

Strain and culture media

The strain used to analyze germination was *biA1methG1,* from green conidia of *A. nidulans* with normal growth and sporulation, obtained from the University of Glasgow, Scotland. This strain requires biotin and methionine in order to develop and was grown on complete solid medium, prepared according to Pontecorvo et al.[23,24], incubated at 37ºC for five days.

Concentrations

The isolated compound was diluted in DMSO to obtain concentrations of 2.5, 25 and 250 µg mL^{-1}. Standardization of concentrations was determined using survival assays of the *bia1methG1* strain from *Aspergillus nidulans* and based on the concentrations used in the antiproliferative activity in human tumor and non-tumor cell lines adopted by the Pluridisciplinary Center for Chemical, Biological and Agricultural Studies of Campinas University, São Paulo (CPQBA-UNICAMP).

Spore and control suspension

Conidia were collected from five-day colonies grown on solid medium (SM) at 37 °C in Tween 80 (0.01%) and filtered in glass wool. Half the volume filtered was submitted to irradiation with UV light for ten seconds (0.24 mJ/cm^2). Distilled water was used as negative control, and the positive control, also with distilled water was irradiated with UV light.

Germination assay: analysis of dead and malformed conidia

Irradiated and non-irradiated conidial suspensions (≈500 x 10^4 spores mL^{-1}) were inoculated in liquid SM in the presence and absence of each sample concentration (2.5; 25 and 250 µg mL^{-1}). Next, 100 µL of suspension from each condition was transferred to microscope slides in a humid chamber and incubated at 37 ºC for seven hours.

After the incubation period, three slides from each condition were analyzed under optical microscope, with a final increase of 200 x, by image capture (Canon EOS Rebel 3TI), using an SLR/DSLR NDPL-2 (2x) adaptor for a binocular microscope. At each reading, 200 conidia were randomly analyzed and the percentages of germinated, dead and malformed conidia were calculated, according to Berti et al.[25] and Reis and Rocha[26].

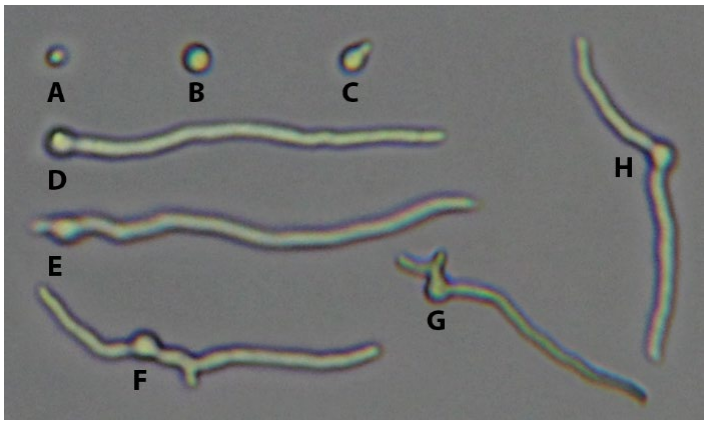
Statistical analysis

The average results of the germination assay were compared, using the standard error of the mean (SEM) as reference, according to Gravetter and Wallnau[27], and analyzed by ANOVA (one-way).

Interpretation of results

In the germination assay, estimate survival considered conidial buds and germinated conidia as alive, and only dormant and embedded conidia as dead. The estimate of malformed individuals considered conidia with abnormal morphology and growth in the second germination tube, as shown in **FIGURE 1**.

FIGURE 1: Germination phases of *A. nidulans* conidia and morphology of malformed individuals. 100x increase with SLR/DSLR NDPL-2 (2x) adaptor. **A**. Dormant; **B**. Embedded; **C**. Bud; **D** and **E**. Germinated; **F**, **G** and **H**. Malformed.



Fonte: Photo - archive of authors.

Interpretation of germination analysis results of *A. nidulans* conidia considered two situations: (1) analysis in the absence of UV irradiation for spontaneous mutation and (2) analysis of the presence of UV irradiation for induced mutation. The results can be interpreted according to the possibilities illustrated in **TABLE 1**.

TABLE 1: Interpretation of the results of *A. nidulans* conidia germination.

Result Observed	Indication
↑ nº. of dead and nº. of malformed unchanged	Cytotoxic substance
↑ nº. of dead and ↑ nº. of malformed	Cytogenotoxic substance
↑ nº of dead and ↓ nº. of malformed	Pro-apoptotic substance
↓ nº. of dead and ↑ nº. of malformed	Anti-apoptotic substance
↓ nº. of dead and ↓ nº. of malformed	Pro-repair substance

Legend: ↑ = increase; ↓ = decrease.

Non-irradiated conidia were analyzed by comparing the treated groups with the negative control. The results will indicate cytotoxicity of the test-substance when the survival values of the treatment are lower than those of the control and average malformed values are equal, and genotoxicity, if the values of the malformed are higher than those of the control. If there is a concomitant increase in survival and malformed conidia, the results will indicate that the substance is anti-apoptotic, which is, hindering the death of malformed individuals.

Analysis of irradiated conidia result in the same conclusions with respect to cytotoxicity and genotoxicity as previously described, provided that the irradiated treated group is compared with the positive control, also irradiated.

Protection of the test substance is exhibited can be demonstrated in two ways: (1) in the non-irradiated group compared with the negative control, showing pro-repair or pro-apoptotic protection in relation to spontaneous mutation; (2) in the irradiated group compared with the positive control, indicating pro-repair or pro-apoptotic protection in relation to UV-induced mutation.

Thus, higher average treatment survival values than those of the control and lower malformed values will indicate pro-repair protection where as lower treatment survival and malformed values than those of the control would suggest pro-apoptotic protection.

Results and Discussion

Chemical Extraction and NMR data

Chromatographic separation of the fractions F1 MeOH and F2 MeOH from the exocarp of *A. squamosa,* using a number of eluent systems, yielded 1.5 and 5.0 g, respectively, of the kaurane diterpene, kaur-16-en-19-oic acid (kaurenoic acid) (**FIGURE 2)**, based on NMR data of ^{1}H and ^{13}C (**TABLE 2**), in line with Batista et al. [28].

FIGURE 2: Molecular structure of kaurenoic acid.

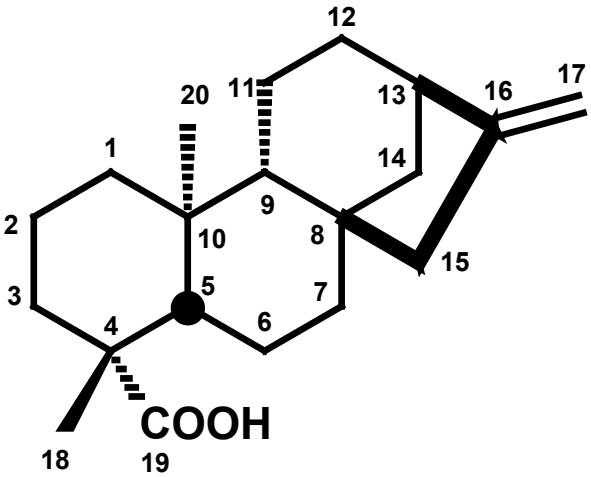


TABLE 2: NMR data of ^{1}H (300.06 MHz) and ^{13}C (75.45 MHz), in $CDCl_3$, from kaurenoic acid of *A. squamosa* L. pericarp.

N°	δ_C /δ_H (multiplicity)	N°	δ_C /δ_H (multiplicity)
1	40.7	11	18.4
2	19.1	12	33.1
3	37.7	13	43.8 / 2.62 (m)
4	43.7	14	39.6
5	57.0	15	48.9 / 2.03 (m)
6	21.8	16	155.9
7	41.2	17	103.0 / 4.79 (bs) and 4.76 (bs)
8	44.2	18	28.9 / 1.22 (s)
9	55.0	19	184.5

10	39.7	20	15.6 / 0.93 (s)

Kaurenoic acid is a white solid[29], as observed in the present study, with melting point of 181-182 ºC. It is one of the intermediary compounds involved in the biosynthesis of several kauran diterpenes, including the gibberellins, which encompass a large family of tetracyclic diterpenoid carboxylic acids[30]. According to Cavalcanti et al.[30], kaurane diterpenes are commonly found in *Copaifera, Mikania, Xylopia*, and *Annona*.

Annona squamosa, a plant with edible fruits that exhibit medicinal and commercial potential[6], is a rich source of bioactive compounds such as alkaloids, tanins, phenolic compounds[6,31], acetogenins, steroids and diterpenes[32].

Kaurenoic acid was isolated from the exocarp of this plant for the first time by Joy and Remani[33], through successive extraction with *n*-hexane, chloroform and methanol. The *n*-hexane and chloroform extracts were combined and submitted to column chromatography, resulting in two crystalline compounds identified by infrared and NMR spectra (^{1}H and ^{13}C): kaurenoic acid and dihydroxi-kaurenoic acid.

Kaurenoic acid and a number of related kaurans have exhibited a wide spectrum of biological activities, such as anti-inflammatory[34], cytotoxic, embryotoxic[35] and antitumor[36,37], against breast, colon and leukemia cancer cell lines[38].

Antiproliferative activity

Kaurenoic acid was submitted to antiproliferative activity assessment in a culture of human tumor cells and a HaCat non-tumor cell lines (immortalized keratinocytes), using doxorubicin as positive control (**FIGURE 3**). The concentration values needed for total growth inhibition (TGI) are shown in **TABLE 3**.

FIGURE 3: Percent cell growth of tumor and normal human cell lines at different concentrations (0.025; 0.25; 2.5 and 25 µg mL^{-1}) of doxorubicin (positive control) after 48 hours of exposure.

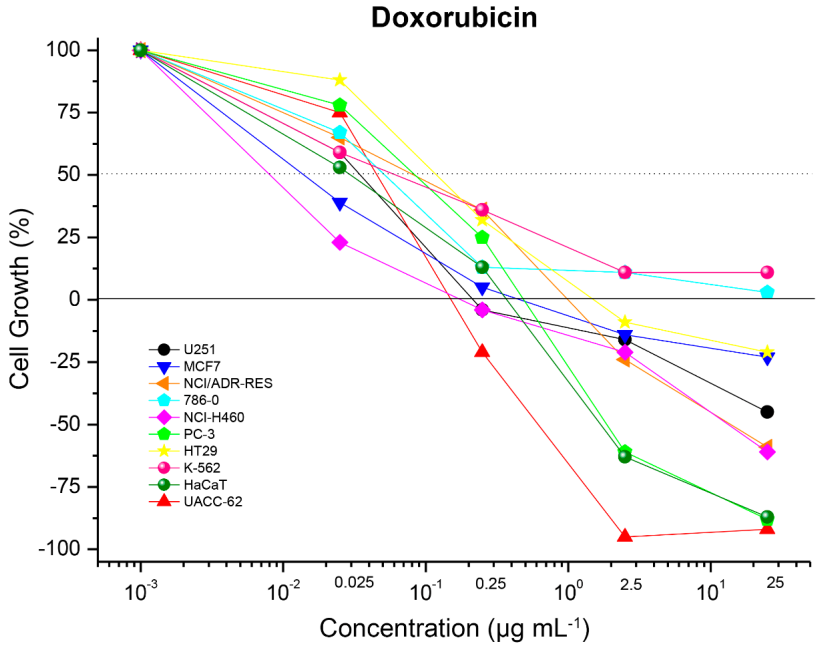


FIGURE 4: Percent cell growth of tumor and normal human cell lines at different concentrations (0.025; 0.25; 2.5 and 25 µg mL^{-1}) of kaurenoic acid (positive control) after 48 hours of exposure.

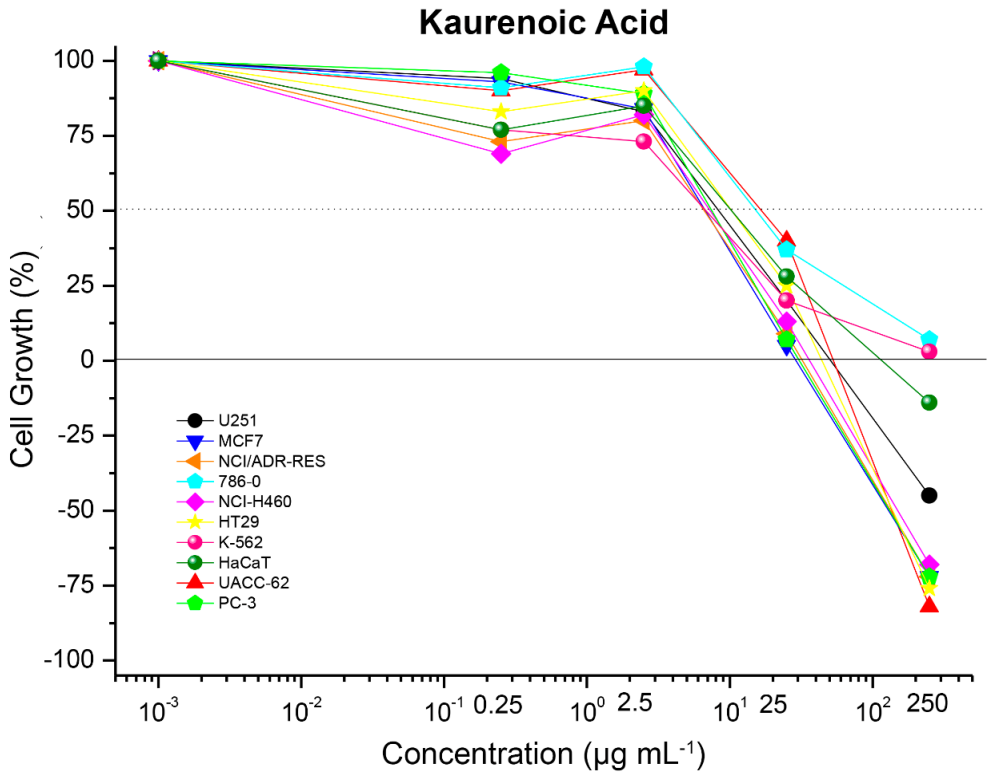


TABLE 3 - Concentration needed for total growth inhibition (TGI) of tumor and normal human cell lines treated with different concentrations of kaurenoic acid.

	TGI (µg mL^{-1})									
Treatments	**U251**	**UACC -62**	**MCF-7**	**NCI-ADR/RES**	**786-0**	**NCI-H460**	**PC-3**	**HT29**	**K562**	**HaCat**
Doxorubicin	0.65	0.16	0.93	1.0	13.5	0.19	0.45	3.2	>25	0.28
Kaurenoic acid	53.0	46.3	29.3	29.9	>250	33.5	31.0	39.8	>250	123.9

Legend: Human tumor cell lines: U251 (gliome); UACC-62 (melanoma); MCF-7 (breast); NCI-ADR/RES (ovary with phenotypic resistance to multiple drugs); 786-0 (kidney); NCI-H460 (lung, non-small cells); PC-3 (prostate); HT29 (colon); K562 (leukemia); HaCat (immortalized keratinocytes: human non-tumor cell line).

Kaurenoic acid was cytostatic in all the cell lines tested (**FIGURE 4**) and cytocidal, except for lines K-562 (leukemia) and 786-0 (kidney), both with TGI > 250 µg mL^{-1}. Considering the TGI values according to Fouche et al.[22], kaurenoic acid was inactive for tumor lines 786-0, K-562 and U251 (gliome) and the HaCat non-tumor line. However, for the remaining cell lines, kaurenoic acid exhibited an antiproliferative effect with approximately 88% inhibition at a concentration of 250 µg mL^{-1}, with no selectivity between the lines.

Plant-derived compounds are known to exhibit curative potential against many types of physiological disorders[37]. Kauran diterpenes, in particular, have been considered bioactive compounds that can help in the development of new and efficient antitumor agents, given their cytotoxic properties related to apoptosis induction by NF-kB inhibition (transcription factor) and caspase-8 activation[36,39,40].

In a bioguided study, Joy and Remani[33] assessed the cytotoxicity of the fraction containing the isolated compounds (kaurenoic acid and dihydroxy kauranoic acid) from the pericarp of *A. squamosa*, in Dalton's

lymphoma acites (DLA) and cervical (HeLa) tumor cell lines using the Trypan blue exclusion test. In both lines, even after 48 and 72 hours of exposure to the fraction, a dose-dependent cytotoxic effect was observed, similar behavior to that obtained in the present study.

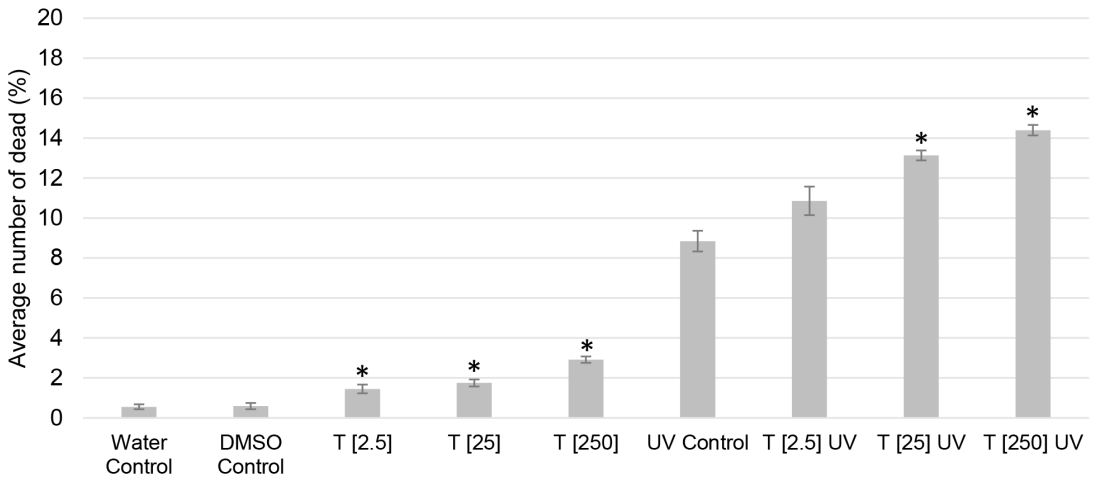
Similarly, the kaurenoic acid isolated from the roots of *Xylopia sericeae* showed no selectivity between the MDA-MB435 (melanoma), SF295 (glioblastoma), HL60 and K562 (leukemias) cell lines, but exhibited a moderate cytotoxic effect on these lines in an MTT assay[30].

In addition to cytotoxic and antiproliferative activity, Cavalcanti et al.[41] assessed the genotoxicity of kaurenoic acid in non-cancerous V79 cell lines (Chinese hamster lung fibroblasts). According to the authors, kaurenoic acid did not significantly induce the frequency of DNA and micronucleus damage at concentrations of 2.5, 5 and 10 µg mL^{-1}. However, at higher concentrations (30 and 60 µg mL^{-1}) there was a significant increase in cell damage in the lines, indicating its potential genotoxic effect on non-cancerous cells.

Germination analysis

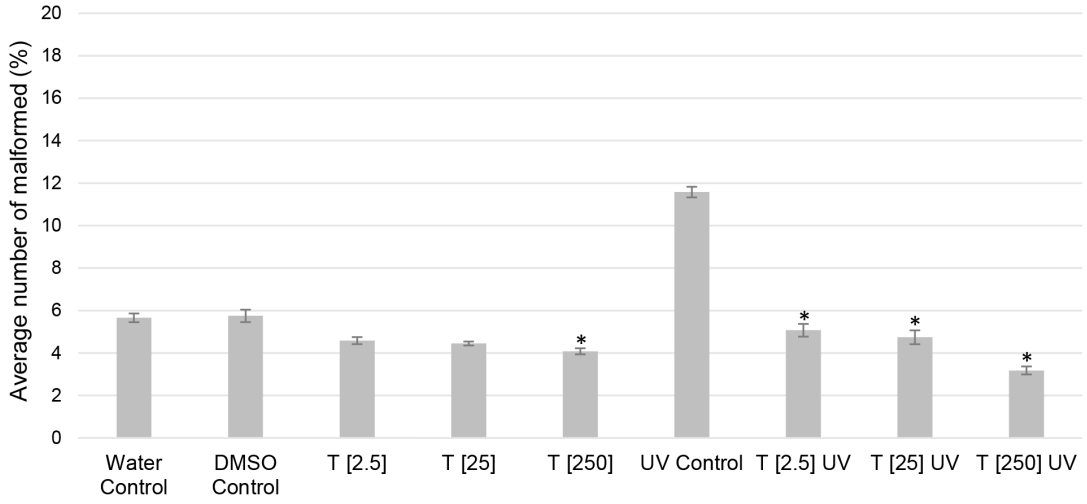
After seven hours of exposure to kaurenoic acid, the non-irradiated group exhibited a statistically significant dose-dependent increase in dead conidia for all the concentrations. This tendency persisted in the irradiated group, with a statistically significant increase in dead conidia, except for 2.5 µg mL^{-1} (**FIGURE 5**).

FIGURE 5: Percent of non-irradiated and UV-irradiated dead *A. nidulans* conidia, following treatment with different concentrations of kaurenoic acid. *Significantly different from the respective controls (one-way ANOVA, $p<0.05$).



With respect to the percent of malformed conidia, there was a slight decline in the non-irradiated group, with a statistically significant value only for 250 µg mL^{-1}. In the irradiated group, this decrease was evident and statistically significant for all the concentrations (**FIGURE 6**).

FIGURE 6: Percent of non-irradiated and UV-irradiated malformed *A. nidulans* conidia, following treatment with different concentrations of kaurenoic acid. *Significantly different from the respective controls (one-way ANOVA, p<0.05).



Analysis of dead and malformed conidia from the non-irradiated and irradiated groups makes it possible to obtain a perspective of death/survival and the occurrence of/protection against damage influenced by the test substance. According to D'Enfert[42], germination is regulated by a large number of gene cascades, similar to what occurs in the embryogenesis of more complex organisms, marked by a set of morphogenetic events that reflect the expression of groups of genes and the type of influence that the environment can exert in this phase. Furthermore, assays with *A. nidulans* are able to identify the risks of genetic effects induced by environmental agents, such as a chemical agent or heterogeneous mixture[43-49].

According to **TABLE 1**, the increase in dead conidia in the two groups and the concomitant decline in malformed individuals indicate the possible mode of action of kaurenoic acid: apoptosis induction. This evidence had already been reported by Joy and Remani[33], who assessed the extract (bioguided) containing kaurenoic acid. Ethidium bromide and acridine orange staining showed that the extract induced apoptosis.

In a later study, Cavalcanti et al.[30] showed that kaurenoic acid could interact or intersperse with DNA, in addition to exhibiting the activity of topoisomerase I, and lead the cell to apoptosis. The literature reports that some diterpenic compounds inhibit cell proliferation through topoisomerase inactivation[50,51]. The effect of kaurenoic acid, also assessed in the comet test by Cavalcanti et al.[30], revealed that 30 and 60 µg mL^{-1} doses of this compound exhibited class 3 and 4 tail, with behavior similar to that of doxorubicin. It was also found that in addition to apoptosis, kaurenoic acid induces necrosis in cells treated with higher concentrations (30 and 60 µg mL^{-1}).

Lizarte-Neto et al.[37] recently demonstrated that kaurenoic acid induced apoptosis in U87 cell lines (glioblastoma) by suppressing antiapoptotic genes (*c-FLIP* and *miR-21*) and apoptotic gene expression (*Fas*, *caspase-3* and *caspase-8*) by real-time CPR analysis. Thus, kaurenoic acid is a powerful tool for restoring tumor cell susceptibility to apoptosis, given that most chemotherapy agents exhibit only a pro-apoptotic effect. This behavior can be observed, for example, in the spontaneously immortalized HaCat cell line used in the present study, with high proliferative power that does not develop tumors *in vivo*[52]. Kaurenoic acid induced death, including in this line, at a concentration of 250 µg mL^{-1}.

According to Rossi and Gaidano[53], apoptosis, a key step in tumorgenesis, plays a crucial role in inhibiting the development of cancer. Apoptosis pathway activation, a mechanism by which cytotoxic compounds kill tumor cells[53,54], is an important method in assessing the effectiveness of the clinical use of several antitumor substances and the selection of new compounds with this ability[55,56].

Conclusion

The results obtained in the present study reinforce the antiproliferative effect of kaurenoic acid, reported in the literature only for some tumor cell lines. However, this compound showed a cytostatic effect for all cell lines tested and was cytocidal for lines UACC-62, HT29, MCF-7, NCI-ADR/RES, PC-3, NCI-H460, U251, particularly UACC-62. Furthermore, the results of the germination assay for *A. nidulans* conidia indicated a decline in cell viability and possible activation of the apoptosis mechanism, previously described for cell tumors. Thus, kaurenoic acid is a useful tool in developing new chemotherapy compounds that could be used in the treatment of different types of cancer, acting primarily by activating the cell death mechanism programmed in these cells.

Acknowledgements

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