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Molecular Profile of Non-Small Cell Lung Cancer Cell Lines A549 and H460 Affects the Response against Cyclophosphamide and Acid Hydrolysis Extract of Agave Sisalana *(AHEAS)*

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Abstract

Although different driver mutations had already described in Non-Small Cell Lung Cancer (NSCLC), which are implicated with different therapeutic responses, KRAS is the most commonly mutated oncogene in NSCLC. Based on this, herein, we compared the therapeutic response of two NSCLC-derived cell lines, with different point mutations in KRAS (A549 - c.34G>A and H460 - c.183A>T) against Cyclophosphamide (CP, chemotherapeutic used for the NSCLC treatment) and hydrolysis Acid Extract of Agave Sisalana (AHEAS, a novel candidate for the NSCLC treatment). For this, we evaluated the susceptibility of these cell lines to these drugs using Annexin-FITC/PI assay, mitochondrial membrane potential ($\Delta\Psi$m), DCFH-DA, and γ-H2AX assays. Results showed that the A549 cell line is most susceptible to the cytotoxic effect of both CP and AHEAS. On the one hand, the AHEAS demonstrated an antioxidant potential, which was exclusively observed with the highest concentration of the extract, on the other hand these concentrations were highly cytotoxic for lung fibroblasts (MRC-5 cell line), indicating that the AHEAS has a non-specific cytotoxic effect, limiting its use for cancer treatment. Despite this, our data suggest that the CP is a useful chemotherapeutic for patients with c.34G>A.

Keywords: Agave sisalana; Lung cancer; Cytotoxicity; γ-H2AX assay; Medicinal plant.

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Introduction

Non-Small Cell Lung Cancer (NSCLC) is the most prevalent histological type of lung cancer [1]. Different driver mutations were already identified in NSCLC, and associated with the therapeutic response and, therefore, with the prognostic of the disease [1,2]. For this reason, the therapeutic approach for the NSCLC treatment has been based on the molecular profile of the NSCLC specimen [2]. In a currently published study, investigating the molecular profile of 17,095 NSCLC specimens using DNA Next-Generation Sequencing (NGS), Judd et al. [2] confirmed that the KRAS is the most commonly mutated oncogene in NSCLC.

Although the discovery of molecular profiles that can be targeted therapeutically has launched a new era for lung cancer therapy [3,4], novel mutations generated as a consequence of the genetic instability promoted by the replication fork stress [5], or mechanisms of acquired resistance in oncogene-driven malignancies can lead to drug resistance, negatively impacting the survival rate of the patients. Thus, to identify novel drugs able to selective act in cancer cells, particularly in those that develops acquired mechanism of drug resistance is crucial to improve the survival rate of patients. In this sense, medicinal plants emerges as a useful source of bio-active antineoplastic drugs, since about 60% of all chemotherapeutic drugs available on the market are derived from molecules extracted from plants [6].

Medicinal plants have been used in traditional medicine practices since prehistoric times and, currently, they still remain important as a primary healthcare mode for about 85% of the world's population [6-8]. Worldwide, 50,000-80,000 plant species are used for medicinal and therapeutic purposes. These plants also serve as a resource for drug discovery, with 80% of all synthetic drugs deriving from them [6-9].

In this context, Brazil is the country with the largest biodiversity in the world, accounting with 20-22% of all living plants species in the planet. Due to the chemical and biological diversity present in Brazil, the country is recognized as a universe of opportunities for bio-based innovation [9]. Notably, the Brazilian Northeast is recognized by its rich diversity, possessing 650 plant species belonging to 407 genera and 11 families with ethnopharmacology information [10].

Currently, the Brazilian Northeast is the world's largest producer of *Agave sisalana* Perrine for the supply of the sisal fiber [11]. *A. sisalana* (sisal) is a herbaceous plant native from Mexico and well adapted to the semi-arid region [11-13]. Nowadays, the state of Bahia is responsible for 95% of national production, making the sisal culture an important economic activity of semi-arid region [11]. However, only 4% of sisal leaves are used to fiber production [11]. Although the residual by-product (mucilage and sisal juice), that comprises 95% of sisal leaves, are commonly discarded in the soil, it is rich in saponins [11,12,14], flavones [15], polysaccharides [16], pectin, mannitol and succinic acid [17], making the sisal juice an unexplored source of biomolecules with pharmacological interest [18]. Moreover, numerous studies have demonstrated that extracts obtained from *A. Sisalana* juice exhibit different pharmacological properties, including: antiseptic [19], antioxidant [18], anti-inflammatory [14,20-24], analgesic [12], antimicrobial [11,25], anthelmintic [26], and antineoplastic [27-29].

A. Sisalana (sisal) extract can be obtained through the Acid Hydrolysis (AHEAS), extraction with Hexane (HE) or Alcohol (EE), or through the Dry Precipitation (DP) of sisal juice [18,30]. In previous study, we demonstrated that saponin-rich extracts from *A. Sisalana* (AHEAS) elicit cytotoxic, mutagenic and antioxidant effects in Vero cell line (cell line derived from African green monkey kidney), suggesting that AHEAS can be used as a natural chemotherapeutics [18]. Based on this, herein, we investigated the in vitro effects of AHEAS for the Non-Small Cell Lung Cancer (NSCLC), which corresponds to the most prevalent histological type of lung cancer, being verified in 85% of all cases of the disease [31,32].

Materials & methods

Ethical statement

Considering that the *Agave Sisalana* Perrine belongs to the Brazilian genetic heritage, the investigational use of the Acid Hydrolysis Extract of *A. Sisalana* (AHEAS) as a possible novel antineoplastic agent was registered in the National System for the Management of Genetic Heritage (SisGen, process number A177E74).

Obtaining the acid hydrolysis extract from *A. sisalana* Perrine

The acid hydrolysis extract from *Agave Sisalana* (AHEAS) was obtained as described by Araldi et al. [18] using fresh leaves collected on a sisal farm located in Valente city, in the state of Bahia (Brazil). The sisal juice obtained from the leaves was heated at 100ºC for ten times and then, hydrolyze with sulfuric acid for four hours, under agitation. The precipitate was separated from the acid solution by filtration at room temperature. In order to preserve the phytochemical properties, the AHEAS was lyophilized and kept protected from the light.

Cell lines

In order to investigate the antineoplastic potential of AHEAS for NSCLC, two cell lines were used: A549 (ATCC CCL-185) and H460 (ATCC HTB-177). These cell lines have the KRAS mutations c.34G>A (A549) and c.183A>T (H460), which represent the most prevalent mutations verified in patients with NSCLC [33,34], and also related to chemoresistance [35,36]. Fibroblasts derived from healthy lung tissue of a 14-week-old male fetus (MRC-5 cell line, ATCC CCL-171) were used to access whether the AHEAS has a cytotoxic and mutagenic potential specific for neoplastic cells (A549 and H460), or could promote cell death and damages for normal lung cells. MRC-5 cell line has been widely used as a negative control in studies for lung cancer [37-40]. Cell lines used in this study were cordially donated by Prof. Dr. Sergio Bydlowski (School of Medicine of the University of São Paulo, FM-USP, São Paulo-Brazil). The molecular identity of these cell lines was confirmed by Short Tandem Repeat (STR).

Cell lines A549 and H460 were cultivated in RPMI 1640 culture medium, supplemented with 10% bovine fetal serum, and 1% streptavidin/penicillin solution (all from Gibco, Carlsbad, USA). Cell line MRC-5 was cultivated in α-MEM culture medium, also supplemented with 10% fetal bovine serum, and 1% streptavidin/penicillin solution (all from Gibco, Carlsbad, USA). Cells were maintained at 37ºC, with 5% of CO2 atmosphere. Details about the cell lines and culture conditions are shown in Table 1.

Table 1: Cell lines employed to investigate the antineoplastic potential of AHEAS.

Cell lines	Tissue origin	ATCC reference	Mutations[1]	Basal medium[2]
MRC-5	Healthy lung	CCL-171	No mutations	α-MEM
A549	NSCLC	CCL-185	CDKN2A KRAS	RPMI 1640
H460	Metastatic NSCLC	HTB-177	CDKN2A ,PIK3CA, KRAS	RPMI 1640

[1]According to the American Type Culture Collection (ATCC)
[2]Complete medium was obtained through the supplementation of basal medium with 10% of fetal bovine serum and 1% of streptomycin/penicillin solution (Gibco, Carlsbad, USA).
NSCLC: Non-Small Cell Lung Cancer.

Treatment with AHEAS and cyclophosphamide

Cell lines were expanded in 6-well culture plates (Corning, USA) using 3 mL of complete growth medium (Table 1) at 37°C and 5% CO_2 atmosphere. When the cells reached 70% of confluence, the complete medium was replaced by a fresh complete culture medium containing the serial dilutions of AHEAS (400, 200, 100, 75, 50, and 25 µg/mL) or Cyclophosphamide (CP) at 100 µg/mL (Sigma-Aldrich, Germany). Cells were incubated with these drugs for 24 hours. Cells not incubated with these drugs were used as a negative control. The AHEAS concentrations were determined based on a previous study that showed that the AHEAS at 25, 50, and 100 µg/mL had cytotoxic activity for Vero cells [41]. Considering that, the cell lines employed in this study exhibit mechanisms of chemoresistance, higher concentrations of the extract were also investigated (200 and 400 µg/mL).

We also analyzed the cytotoxic potential of CP since this drug is an alkylating agent [42] widely employed alone or combined with other drugs for the treatment of metastasis of different malignancies, including NSCLC [43-46]. Moreover, CP is recognized as a positive control for mutagenesis assays [47].

Cytotoxicity analysis using Annexin V-FITC/PI assay

We determined the percentage of live, early and late apoptotic, and necrotic cells through the Annexin V-FITC/PI assay using the FITC Annexin V/Dead Cell Apoptosis Kit (Molecular Probes Inc., Eugene, USA). For this, 24 hours after the incubation with the drugs, culture medium containing death cells were transferred to 15 mL centrifugation tubes. Adherent cells were harvested and added to the 15 mL centrifugation tubes containing the culture medium previously collected. The material was centrifuged at 300 X g (RCF) for 5 minutes at room temperature, discarding the supernatants. The pellet was resuspended in 500 µL of PBS 1X and centrifuged as previously described. The cell pellet was homogenized in 100 µL of binding buffer and incubated with 5 µL of annexin V-FITC and 5 µL of Propidium Iodide (PI) for 15 minutes at 37°C, protected from light. The samples were centrifuged at 300 X g (RCF) for 5 minutes, discarding the supernatant. The cell pellet was homogenized in 300 µL of PBS 1X and immediately analyzed in the BD Accuri™ C6 plus flow cytometer (BD Biosciences, USA) using the channels FL1 (533 ± 15 nm, for Annexin V-FITC) and FL4 (675 ± 12.5 nm, for PI). Analyzes were performed in triplicate, being acquired 10,000 events per analysis. Results were analyzed using the density plot generated by the BD Accuri™ C6 software (BD Biosciences, USA). The data were analyzed using Analysis Of Variance (ANOVA) one way, followed by the Tukey post-hoc test; both performed using the GraphPad Prism 5 software (GraphPad Software Inc., USA) with a significance level of 5%.

Assessment of mitochondrial membrane potential (ΔΨm)

We investigated the mitochondrial membrane potential (ΔΨm) using the Mitotracker® Deep Red probe (Invitrogen, Carlsbad, USA). For this, 24 hours after the incubation with the AHEAS or CP, the culture medium was discarded, and cells were incubated for 40 minutes at 37°C with 2 mL of fresh medium containing 500 nM of MitoTracker® Deep Red probe. After this time, cells were harvested and transferred to 15 mL polypropylene tubes, which were centrifuged at 300 X g (RCF) for 5 minutes at room temperature, discarding the supernatants. The cell pellet was resuspended in 300 µL of PBS 1X and the samples were analyzed in the BD Accuri™ C6 plus flow cytometer (BD Biosciences, USA) in the FL4 channel (675 ± 12.5 nm). Analyzes were performed in triplicate, being acquired 10,000 events per analysis. Results were presented based on the number of probe-positive cells using FlowJo™ V10 software (TreeStar®, USA). Statistical analyses were achieved through the Kruskal-Wallis test, followed by Dunn post hoc test, both performed using GraphPad Prism 5 software (GraphPad Software Inc., USA) with a significance level of 5%.

Analysis of the production of reactive oxygen species (ROS) by the DCFH-DA assay

In order to confirm the depletion of the mitochondrial membrane potential and to evaluate the intracellular redox dynamic, the levels of Reactive Oxygen Species (ROS) were assessed by the DCFH-DA assay [48,49]. After 24 hours of the treatments, the culture medium was replaced by 1 mL of fresh complete medium, containing 100 µM of the probe DCFH-DA (Sigma-Aldrich, Germany). The cells were incubated for 40 minutes at 37°C and then the medium was aspirated and discarded. The cells were harvested and transferred to 1.5 mL polypropylene tubes. Then, the cells were centrifuged at 300 X g (RCF) for 5 minutes, discarding the supernatant. The pellet was homogenized with 300 µL of PBS (1X). The material was analyzed using the Accuri C6 flow cytometer (BD Bioscience, USA). A total of 10,000 events were evaluated per analysis using the FL1 filter (533 ± 15 nm). The results were analyzed using Accuri and FlowJo™ software version V10 (TreeStar, USA) using histograms based on the percentage of cells marked for the DCFH-DA probe. Statistical analyses were performed using the Kruskal-Wallis test, followed by Dunn post hoc test in GraphPad Prism 5 software (GraphPad Software Inc., USA) with significance level of 5%.

Analysis of clastogenicity by γ-H2AX assay

In order to assess the clastogenic potential of AHEAS, the cell lines treated with the AHEAS or CP (positive control) were subjected to the immunodetection of phosphorylated histone-2AX (γ-H2AX). The phosphorylation of this histone occurs in response to DNA double-strand breaks as part of the DNA repair mechanism [18,50,51]. For this reason, we immunodetection of γ-H2AX has been used as a biomarker to assess the clastogenic potential in replacement of comet assay [18,52]. For this assay, 24 hours after the treatments, the cells were harvested and transferred to 1.5 mL polypropylene tubes. The cells were fixed for

two hours at 4 °C using a 2% paraformaldehyde solution (diluted in PBS 1X). Next, the cells were washed twice in PBS 1X, and were permeabilized for 15 minutes at 4°C with 300 µL of 0.1% Tween 20 (Sigma-Aldrich, Saint Louis, USA). The cells were incubated for 40 minutes with a 5% BSA blocking solution (Sigma-Aldrich, Saint Louis, USA). After this step, the cells were incubated overnight at 4°C with primary anti-human-γH2AX antibody (Novus Biologicals, USA, and reference code NB100-384). The cells were washed twice with PBS (1X), and incubated for one hour at 4°C with a secondary anti-rabbit-IgG antibody conjugated to FITC (Sigma-Aldrich Inc., Germany). The cells were washed twice with PBS (1X) and the pellets were resuspended in 300 µL of PBS 1X. The cells were analyzed in the Accuri C6 flow cytometer (BD Bioscience, USA), using the FL1 filter (533 ± 15 nm). A total of 10,000 events were analyzed. The results were exported and analyzed using FlowJo ™ software version V10 (TreeStar, USA). As controls, we used cells not incubated with any drug, but incubated with the both primary and secondary antibodies, as well as cells only incubated with the secondary antibody (in order to verify the absence of non-specific labelling of the secondary antibody). Statistical analyses were performed based on the median fluorescence intensity (MFI) values of the treated and untreated cells (negative control), using the GraphPad Prism 5 software (GraphPad Software Inc., USA).

Results

A549 and H450 cell lines show different cytotoxic response to AHEAS and CP

In order to evaluate the cytotoxic potential of AHEAS, the cells treated with AHEAS or CP were subjected to Annexin V-FITC/PI assay. Results of one-way ANOVA, based on the number of live cells, showed significant statistical differences between the treatments with AHEAS and CP, as well as among the cell lines (Figure 1). Although the results suggest that the AHEAS exhibits a dose-response effect for the MRC-5 cell line, Tukey's test did not show statistical differences among the treatments with AHEAS 25, 50, and 75 µg/mL (Figure 2A). However, the post-hoc test confirmed a dose-response effect for the treatments with AHEAS 100, 200, and 400 µg/mL. Interestingly, necrosis was the predominant type of cell death verified in MRC-5 cells. The AHEAS 400 µg/mL showed the highest cytotoxic potential, causing 42.73% of cell death, of which 36.2% of these occurred through necrosis (Figure 2A). There were no statistical differences among the percentage of live cells treated with CP in relation to the cells not treated (control, Figure 2B). On the other hand, the neoplastic cell lines showed heterogeneous responses to the treatment with AHEAS and CP, as expected.

As verified in MRC-5 cells, the A549 cell line presented a dose-response effect to the treatment with AHEAS 100, 200, and 400 µg/mL, being the necrosis the predominant type of cell death (Figure 2B). The highest concentration of the extract (400 µg/mL) also exhibited the highest cytotoxicity, leading to the death of 96.65% A549 cells (Figure 2B). The results did not show statistical differences among the percentage of live cells treated with AHEAS 25, 50, and 75 µg/mL (Figure 2B).

The treatment with CP 100 µg/mL led to a significant reduction in the percentage of live cells in A549 (Figure 2B), but not in H460 cell line (Figure 2C). Interesting, the H460 cell line also showed a high resistance to the cytotoxic activity of AHEAS, being observed a statistical reduction in the number of live cells only in H460 cells treated with 200 and 400 µg/mL of AHEAS (Figure 2C).

AHEAS only reduces the mitochondrial membrane potential in A549 cell line

The analysis of mitochondrial membrane potential (ΔΨm) showed that the NSCLC-derived cell lines (A549 and H460) have a high ΔΨm in relation to the MRC-5 cell line (Figures 3 and 4). However, while the treatment with CP or AHEAS did not affect the ΔΨm of MRC-5 and H460 cell lines (Figures 4A and C, respectively), the treatment with 400 µg/mL of AHEAS statically reduced the number of MitoTracker-positive A549 cells (Figure 4B).

AHEAS exhibits antioxidant effect for MRC-5 and A549 cell lines, but not for H460 cells

Considering that saponins present in AHEAS elicit an antioxidative effect, as previously demonstrated by us [53], we evaluated the levels of ROS in cell lines treated with the AHEAS using the DCFH-DA assay (Figure 5). Results of this assay showed that the treatment with 25 µg/mL of AHEAS increased the ROS production in the MRC-5 cell line (Figure 6). However, the treatment with 200 and 400 µg/mL of AHEAS statistically reduced the ROS production in the MRC-5 cell line (Figure 6), as expected. Similar results were verified for the A549 cell line treated with 400 µg/mL of AHEAS (Figure 6). However, there is no observed statistical differences among the H460 treated with the different concentrations of AHEAS, neither to CP (Figure 6), confirming that this cell line exhibits natural mechanisms of resistance to the AHEAS.

AHEAS is clastogenic for MRC-5 and A549 cell line, but not for H460 cells

The histone γ-H2AX assay was performed in order to assess the genotoxic potential of AHEAS and CP. The results presented for this assay were quite distinct between all cell lines.

The normal cells MRC-5 showed low basal levels of clastogenicity (negative control), as expected for healthy (non-cancer) cells. On the other hand, the cells treated with CP and AHEAS showed an increased MIF (Figure 7), indicating that both treatments induce DNA double-strand breaks and, therefore, are genotoxic for health lung cells. By contrast, we observed that, for NSCLC-derived cells, the AHEAS only exhibited clastogenic potential for the A549 cells at a concentration of 50 µg/mL (Figure 7). However, no clastogenicity was verified in H460 cell line treated with AHEAS (Figure 7). But curiously, we verified that the treatment with 100 µg/mL of CP increased the MFI in H460 cells (Figure 7), suggesting that this cell line is most susceptible to the clastogenic effect of cyclophosphamide.

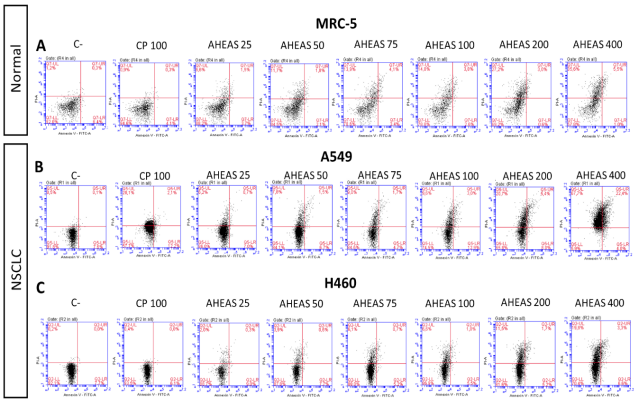

Figure 1: Dot plots showing the results of Annexin V-FITC/PI assay after 24 hours of treatment with CP or AHEAS in fibroblast from health lung (MRC-5, **A**) and NSCLC-derived cell lines A549 (**B**) and H460 (**C**). Dot plots show the percentage of live (Q-LL), early apoptotic (Q-LR), late apoptotic (Q-UR) and necrotic (Q-UL) cells.
C: negative control, with no treatment; CP: Cyclophosphamide. Concentrations represented in µg/mL. Total of 10,000 events analyzed in triplicate.

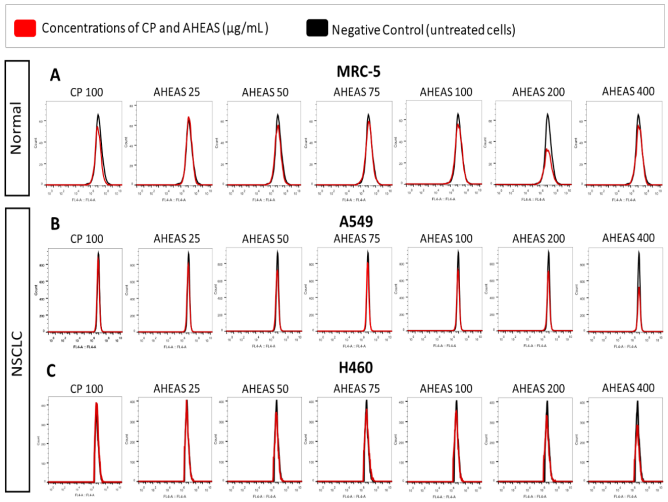

Figure 3: Results of the ΔΨm assay after 24 hours of treatment with CP or AHEAS in MRC-5 (**A**), A549 (**B**) and H460 cell lines (**C**). Histograms show the number of MitoTracker Deep Red-positive cells of a negative control (cells not treated with CP, neither AHEAS - black line) and treated cells (red line). CP: Cyclophosphamide. Total of 10,000 events analyzed in triplicate.

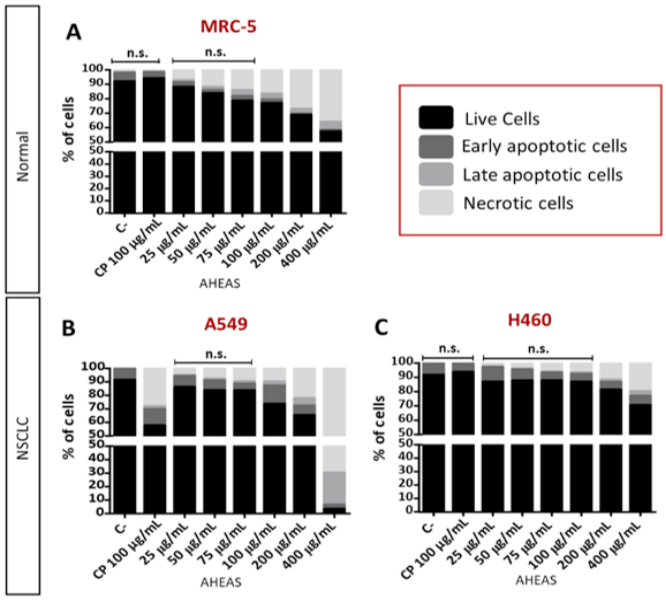

Figure 2: Histograms showing the percentage of living cells (black) and apoptotic or necrotic cells (grayscale). Results show a dose-dependent cytotoxic effect of AHEAS in A549 and MRC-5 cell lines (100, 200 and 400 µg/m). CP was cytotoxic only to A549 cell line. H460 cell line shows high resistance to both treatments (AHEAS and CP). Data were analyzed by one-way ANOVA, followed by the Tukey post-hoc test, both with a significance level of 5%. P-values > 0.05 are Non-Significative (n.s.). Total of 10,000 events analyzed in triplicate.

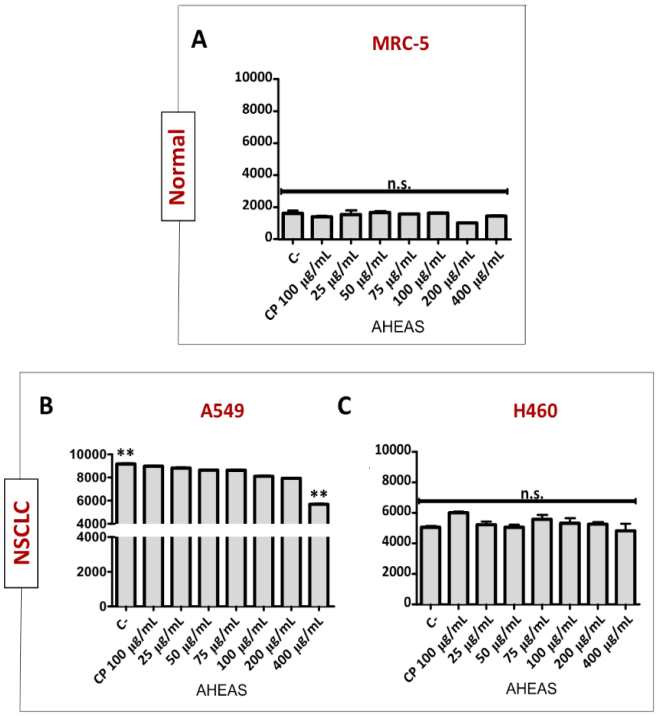

Figure 4: Histograms showing the number of MitoTracker Deep Red-positive cells after 24 hours of treatment with CP or AHEAS. Results show that cell lines MRC-5 (**A**) and H460 (**B**) did not show alterations on ΔΨm at any treatment. In contrast, the A549 cell line (**C**) showed a significant decrease in the number of MitoTracker Deep Red-positive cells after the treatment with 400 µg/mL. Statistical analyses were performed using the Kruskal-Wallis test, followed by the Dunn post-hoc test, both with a significance level of 5%. P-values > 0.05 are non-significative (n.s.). P-values < 0.001 (**). Total of 10,000 events analyzed in triplicate.

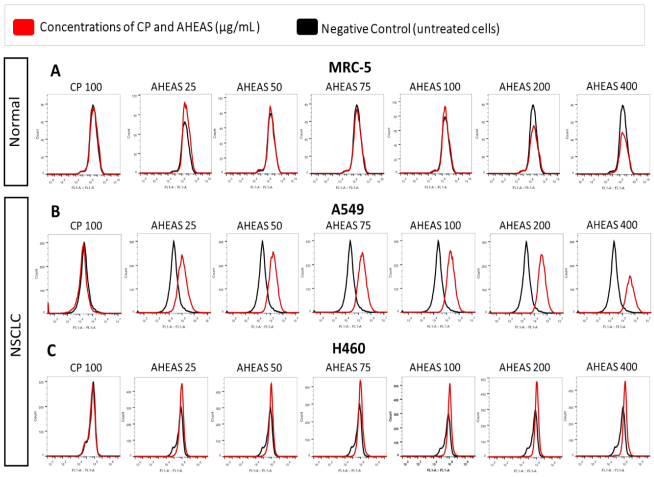

Figure 5: Results of the DCFH-DA assay showing the levels of ROS generation. Data show that the treatment with CP does not increase the ROS production, neither the number of ROS-positive cells in the three cell lines analyzed. However, the treatment with AHEAS increases the number of ROS-positive cells at a concentration of 25 µg/mL, but reduces the number of ROS-positive cells at a concentration of 200 and 400 µg/mL in MRC-5 cells (**A**). In contrast, the all concentration of AHEAS tested increased the ROS generation in A549 cells, without affecting the number of ROS-positive cells, excepted by the treatment with 400 µg/mL, which reduced the number of ROS-positive cells (**B**). Results also show that the AHEAS does not affect the ROS production, neither the number of ROS-positive H460 cells (**C**), suggesting that these cells are most resistant to the effects of this saponin-rich extract. Black line shows the negative control (cells not treated with CP, neither AHEAS), and red line, the cells treated with the drugs. Total of 10,000 events analyzed in triplicate. P-values > 0.05 are non-significative (n.s.).

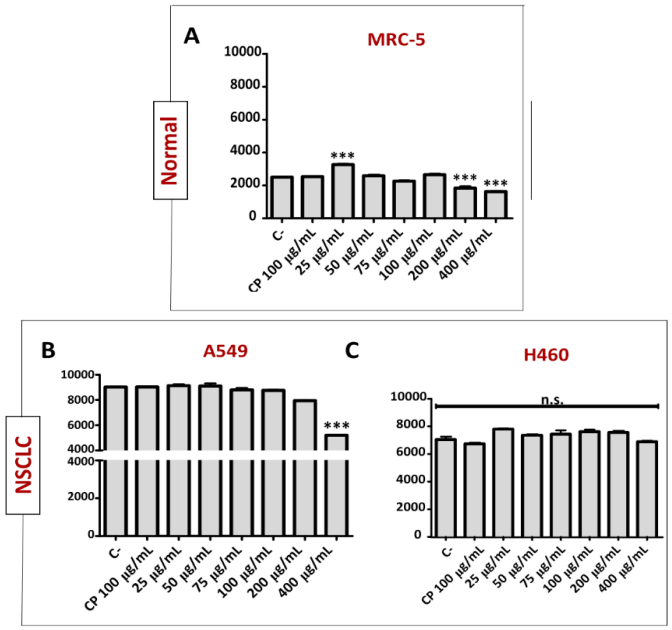

Figure 6: Histograms showing the number of ROS-positive cells of the three-lung cell lines (MRC-5, A549 and H460). The results show and increase in ROS-positive MRC-5 cells after 24 hours of treatment with 25 µg/mL of AHEAS, and a reduction in the number of ROS-positive cells after the treatment with 200 and 400 µg/mL of AHEAS (**A**). Similar effect was also observed in A549 cells that exhibit a reduced number of ROS-positive cells after 24 hours of treatment with 400 µg/mL of AHEAS (**B**). No effect was observed in H460 cell line (**C**). Data analyzed by one-way ANOVA, followed by the Tukey post-hoc test, both with a significance level of 5%. P-values > 0.05 are non-significative (n.s.). P-values < 0.0001 (***). Total of 10,000 events analyzed in triplicate.

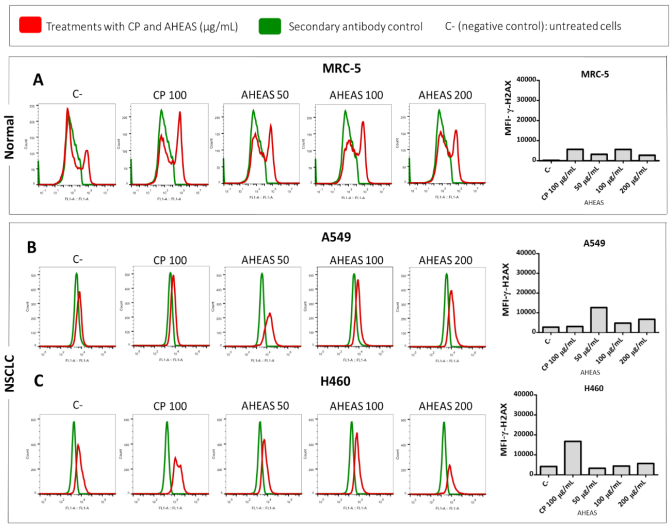

Figure 7: Results of the γ-H2AX-assay. Results show that the treatment with CP increased the number of DSBs in MRC-5 (**A**) and H450 cell line (**B**), but not promoted clastogenic effect for A549 cells (**C**). By contrast, the AHEAS increased the number of DSBs in MRC-5 cells (**A**), indicating that this extract has a clastogenic effect for lung fibroblasts. However, for cancer cells, the AHEAS only exhibited a clastogenic effect at a concentration of 50 µg/mL, and in A549 cells (**B**). Total of 10,000 events analyzed in triplicate. Green line - cells incubated with the secondary antibody (control), red line - cells immunolabelled with the human anti-γ-H2AX antibody.

Discussion

Different driver mutations were already identified as associated with the chemotherapeutic resistance in NSCLC and, therefore, as prognostic biomarkers of the disease [1,2]. Although these mutations have been extensively studied, and used as target for different drugs [3,4], novel mutations generated as a consequence of the genetic instability promoted by the replication fork stress [5], or mechanisms of acquired resistance in oncogene-driven malignancies can lead to drug resistance, negatively impacting the survival rate of the patients.

Thus, to identify novel drugs able to selective act in cancer cells, particularly in those that develops acquired mechanism of drug resistance is crucial to improve the survival rate of patients.

Based on this, herein we aimed to investigate the therapeutic potential (antineoplastic effect) of a saponin-rich extract obtained through the acid hydrolysis extract from *Agave sisalana* (AHEAS) in two cell NSCLC cell lines with different single point mutation in KRAS gene - A549 (c.35G>A) and H460 (c.183A>T). This is because, in previous studies, we demonstrated that this extract (AHEAS) show antioxidant [53] and cytotoxic effect [18] that, combined, could lead to the cancer cell death, without increase the oxidative stress in tumor microenvironment.

Initially, we assessed the cytotoxic potential of AHEAS in NSCLC-derived cell lines (A549 and H460) and non-cancer lung

fibroblasts (MRC-5 cell line). Interesting, we observed that the AHEAS exhibits a dose-dependent cytotoxic effect for MRC-5, whereas the CP, chemotherapeutic already used for the NSCLC treatment does not show cytotoxicity for non-cancer fibroblasts. These data suggest that the cytotoxicity of AHEAS is not selective for cancer cells. By contrast, we verified that the A549 cell line was most susceptible to the cytotoxic effect of AHEAS, when compared to the H460 cell line. Confirming these results, we also observed that the treatment of CP statistically reduced the number of live cells in A549 cell line, but not in H460 cells. These data are in accordance to the literature, since the H460 cell line exhibit distinct mechanisms of chemoresistance [54-57], which confers resistance against different drugs, including CP [57] and saponins (the main active component present in AHEAS) [15,58]. Thus, these data suggests that the cyclophosphamide may be prescribed by patients with the c.34G>A mutation, but not for patients with the molecular profile of H460 cell line (c.183A>T).

Regarding to cell death pathways, we observed that the necrosis was the main type of cell death observed in both cancer and non-cancer cell lines. This result suggest that the cytotoxicity induced by the AHEAS is caused by the saponins present in the extract that lead to cell membrane permeabilization, which is recognized as a primary marker of necrosis [59]. However, we also verified that the necrosis was the predominant type of cell death in A549 cell line treated with CP, suggesting a mechanism of evasion of cell death by apoptosis. In contrast, the lack of response observed in H460 cell line suggests the involvement of mechanisms of chemoresistance based on drug efflux, supposedly promoted by the overexpression of ABC transporters. However, novels studies are needed to proof this hypothesis.

The necrosis promoted by the AHEAS in the three cell lines is of great relevance since the intrinsic (or mitochondrial) pathway of apoptosis is identified as the main mechanism of death induced by extracts of *A. Sisalana* [15,60]. In this regard, our data on mitochondrial membrane potential (ΔΨm) indicates that the AHEAS can promote cell death without reducing the ΔΨm, suggesting a different mechanism of cell death induced by AHEAS.

Interestingly, the activation of extrinsic (non-mitochondrial) apoptosis pathways shares the same receptors and inducing factors of the necroptosis pathway (programmed necrosis), and may be initiated by the same stimuli. In cancer, the exploration of the necroptosis pathway and the investigation of its inducing agents have gained attention in recent years, since both inhibition and induction of this pathway have been identified as ways to eradicate cells resistant to apoptosis [61].

In addition to cell death pathways, mitochondria actively participate in maintaining the redox environment of cells [48]. To assess the role of AHEAS in the dynamics of ROS and its antioxidant potential, ROS production were analyzed using the DCFH-DA probe. The treatment of A549 and MRC-5 with the highest cytotoxic concentrations of the AHEAS (200 and 400 µg/mL) showed a reduction in ROS levels, while lower concentrations of the extract (25 µg/mL) showed increase in ROS levels. These effects can be justified by the redox balance between the cell death and the AHEAS capability to scavenge free radicals.

This occurs because the oxidative stress generated during cell death processes tend to significantly increase the ROS production [61]. However, studies have demonstrated the saponins present in extracts of A. Sisalana, including AHEAS, can scavenge these free radicals [41,62]. In this sense, we demonstrated that higher concentrations of AHEAS reduced the ROS levels in MRC-5 and A549 cell lines, whereas the MRC-5 cells treated with the lowest AHEAS concentration (25 µg/mL) presented an increased ROS production, indicating that in this concentration is not enough to scavenge the ROS produced as consequence of the cell death induced by the extract.

Beside the cytotoxicity, the selective genotoxicity is another important parameter to be considered in the discovery of novel drugs. In this regarding, many of the routinely used chemotherapeutics, including cyclophosphamide, have mutagenic potential [63-66], which can increase the genomic instability in both cancer and non-cancer cells, increasing the risk for secondary malignancies [67]. For this reason, we also investigated the clastogenic potential of AHEAS in NSCLC-derived cell lines and healthy lung fibroblast cells using the γ-H2AX assay – method recognized as a gold-standard to identify DSBs (the most dramatic type of DNA damage) [68]. For this analysis, we excluded the AHEAS at a concentration of 400 µg/mL, since this concentration was highly cytotoxic for non-cancer cells (MRC-5 cell line). The AHEAS at 25 µg/mL and 75 µg/mL were also excluded to this assay for presenting low or null responses in all assays/cell lines, or for presenting responses very similar to the other intermediate concentrations (50 and 100 µg/mL). Interesting, the results of γ-H2AX assay demonstrated that MRC-5 cell line is susceptible to the DSBs caused by both CP (used as positive control) and AHEAS (50, 100, and 200 µg/mL). This data confirms that the AHEAS has a similar adverse (clastogenic) effect of CP for non-cancer cells. However, we observed that the CP did not have clastogenic potential for A549 cell line. By contrast, the CP significantly increase the number of DSB foci in H460 cell line, although the chemotherapeutic had not present cytotoxic effect for this cell line. Curiously, we observed the AHEAS increased the DSB foci in A549 cells. However, this effect was only observed in cells treated with 50 µg/mL of the AHEAS.

Conclusion

In summary, our data demonstrated that the molecular profiler of NSCLC can lead to different response to the chemotherapeutics. In this sense, we observed that the cyclophosphamide can be considered a useful drug for patients with c.34G>A mutation in KRAS gene, since the CP significantly reduced the number of live A549 cells. Although the AHEAS had shown a cytotoxic effect, especially for A549 cell line, this effect was observed with the use of highest concentrations of these drugs. Moreover, the AHEAS showed a dose-dependent cytotoxic effect, as well as induced DSBs in health lung fibroblasts (MRC-5 cell line), demonstrating that antineoplastic effect of saponin-rich AHEAS is not specific for cancer cells.

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