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Cytotoxic and apoptotic effects of *Annona squamosa* (Atis) crude leaf extract against A549 (Human Lung Adenocarcinoma) cell line

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ABSTRACT

Cancer is a leading issue within the medical community and in the world, specifically lung cancer cells which affect thousands of people each day. Hence, this study aims to investigate the cytotoxic and apoptotic effects of *Annona squamosa* (Atis) crude leaf extract/sample that has not yet been studied against lung cancer cells. *A. squamosa* (Atis) leaf extract was acquired through maceration and then tested using MTT Cytotoxicity Assay to determine % inhibition and the half maximal inhibitory concentration (IC_{50}) after exposure to the extract and Annexin V-Propidium Iodide (PI) Staining Assay to determine the percentage of cells undergoing early and late apoptosis and necrosis. Testing was conducted on the A549 (human lung adenocarcinoma) cell line in UP Diliman Mammalian Cell Culture Laboratory. Among the concentrations, 1.5625 ug/mL resulted in the highest % inhibition and was highly cytotoxic against the A549 cells while the 100 ug/ml concentration resulted in the higher % total cell death and % early and late apoptosis compared to the 1.5625 ug/mL concentrations. Therefore, *A. squamosa* (Atis) leaf extract may be developed into a potentially sustainable and affordable novel drug for treating A549 (human lung adenocarcinoma) cells in the future.

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INTRODUCTION

Cancer is the third leading cause of death in the Philippines, with lung cancer as the second most common case in 2020 (International Agency for Research on Cancer [IARC], 2021). Dr. Rosario Pitargue, the president of the Philippine Society of Medical Oncology, stated that 184 cancer cases occur in every 100,000 patients in hospitals, and 96 cancer deaths nationwide are reported daily (Montemayor, 2023). The disease begins with many symptoms but eventually leads to a malignant or benign tumor, a lump of cancerous tissue dividing uncontrollably. These tumors can metastasize (spread) and damage nearby tissue (National Cancer Institute [NCI], 2021). Cancer is treated by detecting and eliminating these tumors early to prevent them from metastasizing, which is usually performed with the use of chemotherapeutic drugs that induce cell apoptosis (Habtemariam and Lentini, 2018).

Cell apoptosis is the controlled or programmed death of a cell (Gupta, 2022), which serves as the foundation of the treatment of cancer by many chemotherapeutic drugs. A variety of plants can induce cell apoptosis on various cancer cell lines through bioactive compounds in their extracts, such as macerated leaf extracts. A study found that the macerated extract of *Teucrium ramosissimum* induces apoptotic effects and is effective against Chronic myelogenous leukemia (Sghaier *et al.*, 2012), and another study proved the effectiveness of *Tulbaghia violacea* acetonic macerated leaf extract against human oral cancer cells (Takaidza *et al.*, 2018). Moreover, a study investigated the effectiveness of *Sclerocarya birrea* acetonic macerated extract against Breast Cancer Cell Lines or MCF-7 cancer cells (Tanih and Ndip, 2013). Furthermore, studies by Al-Nemari *et al.* (2020) and Al-Nemari *et al.* (2022) have found that the acetonic maceration of *Annona squamosa* (Atis) leaves was effective against colon cancer cells (Lovo and HCT-116) and breast cancer cells (MCF-7 and MDA-MB-231), respectively, through clear evidence of the induction of cytotoxic activity, antiproliferation, anti-migration, and cell-apoptosis.

It has been proven that *A. squamosa* or Atis and other plants possess cell apoptosis-inducing properties through their macerated leaf extracts. The inherent feasibility and evident anti-cancer capabilities of the *A. squamosa* acetonic macerated leaf extract, in most papers such as those previously mentioned, has been shown through its ability to induce apoptotic effects on different cancer cell lines. However, its effects against A549 lung carcinoma epithelial cells have not been investigated. Hence, this study is vital to determine the apoptotic effects of *A. squamosa* (Atis) acetonic macerated leaf extract using cell culture against A549 (human lung adenocarcinoma) cells.

MATERIALS AND METHODS

Research samples

A total of 350 grams of healthy *A. squamosa* (Atis) mixed-leaves was procured by hand from Upper Dulian, Bungiao, Zamboanga city, Zamboanga del sur. Before transportation, leaf samples were placed in a clean and dry plastic bag and away from direct sunlight until used for the preparation of leaf powder. Before the sample preparation, the leaves were brought to the Department of Agriculture, Bureau of Plant Industry-Plant Quarantine Service Region 9 for necessary species confirmation and certification. The A549 (human lung adenocarcinoma) cell line was procured from the MCCL (Mammalian Cell Culture Laboraory) at the Institute of Biology of UP Diliman for use in the experiment.

Research design

This study utilized the Completely Randomized Design (CRD) for the MTT Cytotoxicity Assay, where one (1) sample was subjected to eight (8) treatments (T_n) of different concentrations of *A. squamosa* (Atis) crude leaf extract (T_1 – 100 µg/mL; T_2- 50 µg/mL; T_3- 25 µg/mL; T_4- 12.5 µg/mL; T_5- 6.25 µg/mL; T_6 – 3.125 µg/mL; T_7- 1.5625 µg/mL; T_8- 0.78125 µg/mL) arranged into three (3) replicates. Randomization for this assay was done using the lottery method. While for the Annexin V-PI Staining Assay the study also utilized the Completely Randomized Design (CRD), where one (1) sample was subjected to four (4) treatments (optimal concentrations of the extract

based on MTT Cytotoxicity Assay [T_1- 100 µg/mL; T_2- 1.5625 µg/mL]; T_3- 1 mL Doxorubicin [positive control group]; T_4- 1 mL dimethylsulfoxide [DMSO] [negative control group]) arranged into three (3) replicates. Randomization for this assay was also done using the lottery method.

Research procedures

The healthy mixed-leaves of *A. squamosa* (Atis) were hand-washed gently with running water and then shade-dried for 7 days. The dried leaves were crushed into pieces by hand, powdered finely using an electric blender, and strained until 190 grams of the powder was acquired; this was to maximize the surface area for extraction. The leaf powder was stored in a clean and dry closed container in the dark at room temperature until extraction was performed. The powder was protected from light, air, moisture, and any other form of contamination, and the container was labeled accordingly. The modification done to this process was the storage temperature of the leaf powder. The cell culture procedures described are the methodologies employed by the MCCL at the Institute of Biology of UP Diliman, which was also adapted from Mosmann (1983). The A549 (human lung adenocarcinoma) cell line prepared was seeded at 8,000 cells per well in sterile 96-well microtiter plates. The cell culture medium used was Kaighn's F-12 Medium containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic mix. The plates were incubated overnight in a humidified incubator at 37°C with an atmosphere of 5% CO_2 to allow for cell attachment.

Maceration and concentration of extract

The finely powdered leaves were left to soak in acetone which was acquired through simple distillation from nail polish remover for 48 hours with a 1:10 w/v then sealed. Periodic shaking was done. After 48 hours, the extract was filtered and then the filtrate allowed to sit under a fume hood until completely evaporated. The extract was transferred to a screw-top plastic vial that was labeled accordingly and secured. Samples were dissolved in sterile dimethylsulfoxide (DMSO) at 10mg/mL. Eight two-fold dilutions of the *A. squamosa* (Atis) crude leaf extract were measured in preparation for the application of treatments on the cell culture, which were 0.78125 µg/mL, 1.5625 µg/mL, 3.125 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL *A. squamosa* (Atis) crude leaf extract. After overnight attachment, the cultured cells were treated with the prepared concentrations of samples and controls and were again incubated for 72 hours at 37°C and 5% CO_2 for the MTT Cytotoxicity Assay (Al-Nemari *et al.*, 2020; Abd-Elghany *et al.*; 2022; Al-Nemari *et al.*, 2022).

Data collection

Note that the procedure performed within this section such as the MTT Cytotoxicity Assay (Jokhadze *et al.*, 2007), Annexin V-Propidium Iodide (PI) Apoptosis/Necrosis Staining Assay (Aubry *et al.*, 1999), Statistical Analysis, and Waste Disposal are the methodology employed by the MCCL at the Institute of Biology of UP Diliman.

MTT cytotoxicity assay

The data was collected through a cell viability assay, specifically, the MTT Cytotoxicity Assay. After 72-hour incubation, 0.5mg/mL of 3-(4,5-dimethylethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye was added. The MTT-treated cells were incubated at 37°C and 5% CO_2 for 3 hours. After which, dimethylsulfoxide (DMSO) was used to dissolve the metabolized formazan crystals formed through the reduction of the MTT dye.

Absorbances were read at 570 nm wavelength (OD570) using a spectrophotometric microplate reader. Percent Inhibition was computed through the following formula:

$$\% \text{ Inhibition} = \left[\frac{\text{OD570 (DMSO)} - \text{OD570 (sample)}}{\text{OD570 (DMSO)}}\right] \times 100,$$

where "% Inhibition" represents the percentage of the number of nonviable cells in a sample treated with the extract after 72-hour incubation, "OD570 (DMSO)" represents the absorbance value of the formazan solution of the dimethylsulfoxide, and "OD570 (sample)" represents the absorbance value of the formazan solution of the respective treatment group for the sample.

Based on the inhibition value, the half-maximal inhibitory concentration (IC_{50}) was calculated to assess the strength of the cytotoxic activity of the sample. Biological activity was assessed based on the criteria by Jokhadze *et al.* (2007). Table 1 shows the values of half-maximal inhibitory concentration that correspond with their respective strength levels of cytotoxic activity.

Table 1. IC_{50} value ranges and their interpretation

IC_{50} (µg/ml)	Cytotoxic activity
< 10	Highly active
10 – 30	Active
> 30 – 100	Inactive

Annexin v-propidium iodide apoptosis/necrosis staining assay

The Annexin V-Propidium Iodide (PI) Apoptosis/Necrosis staining assay used the optimal concentration determined by the MTT Cytotoxicity assay. Annexin V with a green fluorescent dye (FITC) and propidium iodide were introduced to the sample to distinguish between the different stages of apoptosis. Annexin V with the green fluorescent dye (FITC) bound to a lipid found in the inner part of the cell membrane called phosphatidylserine (PS) during the early stages of apoptosis, which showed a green fluorescent light under a microscope. Co-staining using propidium iodide was introduced along with the previous chemicals to detect whether the cells had undergone late apoptosis or not; this staining showed a red fluorescent dye under the microscope. Necrosis was detected only through the appearance of the red fluorescent dye due to the cellular membrane disintegrating. In the Annexin V-PI staining assay, live healthy cells with undamaged cell membranes did not show fluorescence at all, cells undergoing early apoptosis showed green fluorescence, cells undergoing necrosis showed red fluorescence, and cells undergoing late apoptosis showed both green and red fluorescence. Toward the end of the assay, nuclear fluorescent stains such as DPI or Hoechst 33342 were used to stain all cells for quantification. After 3-day incubation in three (3) trials, an inverted fluorescence phase contrast microscope was used to visually count cells for quantification. Apoptotic ability was then computed as % Early Apoptotic cells, % Late Apoptotic cells, and % Necrotic cells using the data provided through quantification based on the formulas:

$$\% \text{ Early Apoptotic cells} = \left[\frac{\text{Annexin V/FITC + PI – cell count per field}}{\text{DAPI + cell count per field}}\right] \text{x } 100$$

$$\% \text{ Late Apoptotic cells} = \left[\frac{\text{Annexin V/FITC + PI + cell count per field}}{\text{DAPI + cell count per field}}\right] \text{x } 100$$

$$\% \text{ Necrotic cells} = \left[\frac{\text{Annexin V/FITC – PI + cell count per field}}{\text{DAPI + cell count per field}}\right] \text{x } 100,$$

whereas "% Early Apoptotic cells" represents the percentage of the number of cells in a field that had undergone early apoptosis after treatment with the extract, "% Late Apoptotic cells" represents the percentage of the number of cells in a field that had undergone late apoptosis after treatment with the extract, "% Necrotic cells" represents the percentage of the number of cells in a field that had undergone necrosis after treatment with the extract, "Annexin V/FITC" represents the number of cells in the field that had been stained with Annexin V added with the green fluorescent dye (FITC), "PI" represents the number of cells in the field that had been stained with propidium iodide, "DAPI" represents the number of cells in the field that had been stained with the nuclear fluorescent stain DAPI, and "cell count per field" represents the total number of cells in the field.

Statistical analysis

This method was adapted from Awada *et al.* (2023). Microsoft Excel was used to perform statistical analysis on all data collected in the experiment. The results of each treatment group were calculated into the mean ± standard deviation (SD). The half-maximal inhibitory concentration (IC_{50}) was computed using GraphPad Prism 10 via non-linear regression (curve fit) formula for Absolute IC_{50}, wherein X = log (concentration) and Y = % Inhibition (with top value [Y] = 100% inhibition and baseline = 0%).

RESULTS

MTT cytotoxicity assay, inhibitory activity and IC_{50}

Based on the specific criteria by Jokhadze *et al.* (2007), the results of the absolute IC_{50} ug/ml of the *A. squamosa* (Atis) acetonic leaf showed that it is highly cytotoxic due to its value of 9.7967 being less

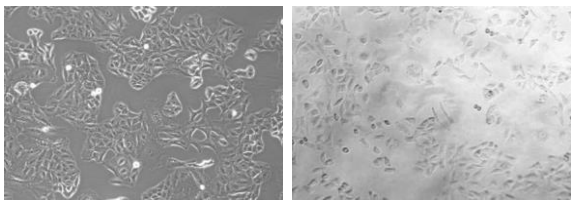
than 10. The results of the assay can be seen in Table 2. The concentrations of 1.5625 ug/mL and 0.78125 ug/mL of the *A. squamosa* (Atis) crude leaf extract yielded the highest % Inhibition of 89.82% and 87.77%, respectively, while concentrations above 3.125 ug/mL did not significantly increase in cytotoxicity; 12.5 ug/mL having the lowest % inhibition and only increasing in cytotoxicity at 3.125 ug/mL below. The data presents a clear U-shaped dose response curve or biphasic response when graphed. As the treatment concentration decreases from 100 ug/ml to 12.5 ug/ml, the % inhibition decreases but rapidly increases in effectiveness after 12.5 ug/ml.

Table 2. Cytotoxicity activity of *A. squamosa* (Atis) acetonic crude leaf extract at different concentrations by MTT Assay

Plant concentration (ug/ml)	Plant extract % inhibition
100	72.62 ± 7.90
50	65.24 ± 11.04
25	52.30 ± 5.69
12.5	48.01 ± 3.38
6.25	50.38 ± 7.89
3.125	68.93 ± 6.42
1.5625	89.82 ± 2.30
0.78125	87.77 ± 2.91
Absolute IC_{50} ug/mL (n=3)	9.7967

Treatment effect against A549 (Human lung adenocarcinoma) cell lines

The following figures provide a visual effect on the cancer cells after application of each treatment.



A B

Fig. 1. A: A549 Cells in T-25 culture flask before seeding, B: A549 Cells in 96-well microplates after seeding and prior to treatment. All images were captured at 100X magnification using a Carl Zeiss Axiovert A.1 inverted phase-contrast microscope

Prior to application of the plant extract, the cells were observed to be numerous, intact, attached to one another, and with a large cytoplasmic volume. Even before and after seeding, the cells share similar morphological characteristics that are indicative of healthy and properly functioning cellular structure. Fig. 1A shows the A549 cells in a T-25 culture flask before seeding, and Fig. 1B shows the A549 cells in 96-well microplates after seeding prior treatment.

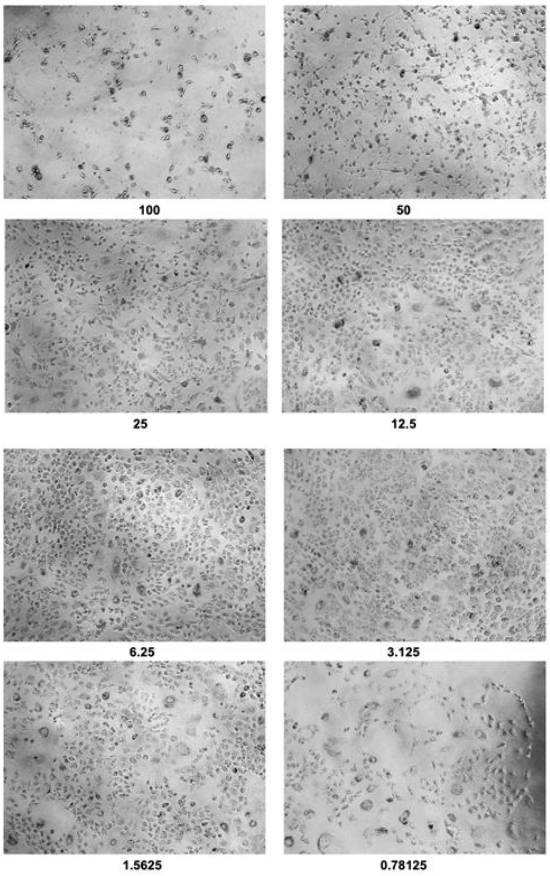


Fig. 2. A549 cells treated with eight concentrations (µg/mL) of *A. squamosa* (Atis) acetonic crude leaf extract

Contradictory to what is expected from the most effective concentration; Treatment 7 (Fig. 2, 1.5625 µg/mL) seems to only have moderate visible effect on the treated cells, many of which are still intact, attached to each other, and with only slight noticeable morphological changes. Cells under Treatment 8 (Fig. 2, 0.78125 µg/mL) display significant morphological changes, many of which are similar to those found in the cells under Treatment 1 (Fig. 2, 100 µg/mL). Nonetheless, there are still some cells that show swelling of the cytoplasmic structure, which have not been present in Treatment 1.

Annexin V-PI apoptosis/necrosis staining assay

Percent early apoptotic, late apoptotic and necrotic cells

The results of the apoptotic and necrotic assessments of two different concentrations of *A. squamosa* acetonic crude leaf extract are presented in Table 3.

Table 3. Apoptotic and necrotic activity of *A. squamosa* (Atis) acetonic crude leaf extract at two concentrations by Annexin V-PI Assay

Category	Sample concentration (ug/ml)	
	1.5625	100
Total cells	100.0%	100.0%
Non-apoptotic	48.8%	36.4%
Early apoptotic	6.5%	9.0%
Late apoptotic	9.8%	25.4%
Necrotic	34.8%	29.2%
Total dead/dying cells	51.2%	63.6%

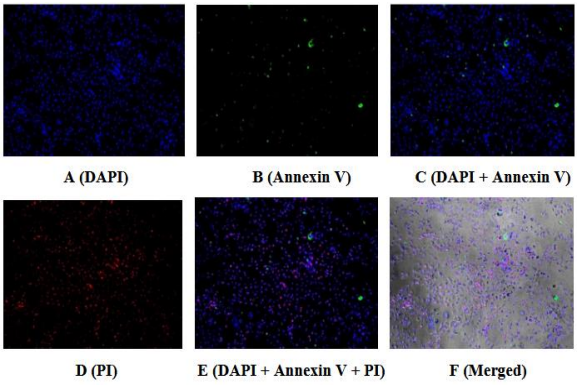


Fig. 3. Annexin V-PI staining images of A549 cells treated with 1.5625 ug/ml *A. squamosa* leaf extract after 72 hours. Images of the fluorescently stained cells post application of treatments

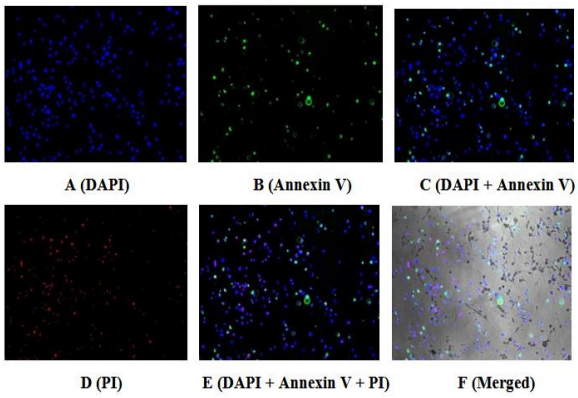


Fig. 4. Annexin V-PI staining images of A549 cells treated with 100 ug/ml *A. squamosa* leaf extract after 72 hours

The 1.5625 ug/ml treatment resulted in a higher percentage of necrotic cells but a lower percentage of apoptotic cells while the opposite is true for the 100 ug/ml treatment. When cells under 1.5625 ug/ml and 100 ug/ml of *A. squamosa* (Atis) acetonic crude leaf extract were applied with a nuclear stain (DAPI), a visualization of the total cell population was shown in the color blue (Fig. 3A [1.5625 ug/ml]; Fig. 4A [100 ug/ml]). When stained with Annexin V, early apoptotic cells appeared green (Figs 3B and 4C [1.5625 ug/ml]; Figs 4B and 5C [100 ug/ml]). When stained with propidium iodide, necrotic cells appeared red (Fig. 3D [1.5625 ug/ml]; Fig. 4D [100 ug/ml]) and late apoptotic cells appeared violet (Fig. 3E [1.5625 ug/ml]; Fig 4E [100 ug/ml]). Figs 3F and 4F show the merged visualization of the stained cells at 1.5625 ug/ml and 100 ug/ml, respectively.

DISCUSSION

It was interpreted from the data that the cytotoxicity trend was bell-shaped, which means that the sample's cytotoxicity levels were highly effective from both ends of the treatment concentration range. Specifically, below 3.125 ug/mL, which were the lowest concentration levels of the sample are where the cytotoxicity levels were observed to be the highest.

However, approaching a certain point in the middle of the range, the cytotoxicity level started to decrease in effectiveness. This trend is distinct from other studies that found the cytotoxicity level of their respective extracts to be dose-dependent, which means that as the concentration level of the extract increases, the cytotoxicity also increases, or in other words, a direct relationship. The studies that inhibited cell proliferation or had a cytotoxic effect in a dose-dependent manner are the following: *Teucrium ramosissimum* (Lamiaceae) against K562 cell-line proliferation (Sghaier *et al.*, 2012), *Sclerocarya birrea* against MCF-7 cells in a dose-dependent manner (Tanih and Ndip, 2013), and *Tulbaghia violacea* against KB cell line (Takaidza *et al.*, 2018). The findings of this study are contradictory to the results of the study conducted by Al-Nemari *et al.* (2020), which also used *A. squamosa* (Atis) crude leaf

extract. The study found that as the extract concentration increased, the cytotoxicity of the treatments also significantly increased, with the highest concentration of 100 ug/mL being the most cytotoxic against Lovo and HCT-116 cells. The results of the said study yielded 58% cytotoxicity against Lovo cells and 81% cytotoxicity against HCT-116 cells. In another paper by Al-Nemari *et al.* (2022), similar results were found; the highest concentration of the extract yielded the most cytotoxicity. The extract was effective against 100% of the MCF-7 cells, inducing cell-death in all the treated cells. Moreover, the extract had 80% effectivity against MDA-MB-231 cells, also inducing cell death.

This study, compared to those similar to it in terms of certain variables such as type of plant extract, extraction method and solvent, and cancer cell line, have opposing findings. The results of this study state that an indirect relationship was found wherein lower concentrations of the extract lead to greater cytotoxicity and increased inhibiting effect while also presenting a non-linear relationship and a biphasic dose response which is graphically represented as a U-shaped dose-response curve wherein the effectiveness of the plant extract is greater at lower or higher concentrations due to certain biological mechanisms (Jodynis-Liebert and Kujawska, 2020). In a study by Lombardi *et al.* (2017), the induction of antiproliferative effects, apoptosis, modulation of the expression of proapoptotic genes like p53 and Bax, and extent of gene expression were only present at 10 µg/mL concentration of the cell culture treatment. Similarly, Campoccia *et al.* (2021) found that one of their samples was able to induce apoptosis in THP-1 cells compared to the positive control at a low concentration. This is most likely due to certain mechanisms such as greater affinity to receptors or activation of certain pathways only at low concentration. However, further investigation is required to determine the specific reasoning as to why lower concentration may cause higher cytotoxic effects from plant extracts.

Although the results of the MTT cytotoxicity assay provide an idea of the cytotoxic effect of the *A. squamosa* (Atis) crude leaf extract, these results only provide the % inhibition and IC_{50} value. There is no confirmation of the specific effects of the cell morphology and the mechanisms behind the cytotoxicity of the extract except for visual observation. Al-Nemari *et al.* (2020) and Al-Nemari *et al.* (2022) found that through nuclear staining, many of the cells had damaged nuclei which suggested that the antiproliferative effect of the extract was through the induction of apoptosis via caspase-3 activation and cell cycle arrest. Moreover, Tanih and Ndip (2013) and Takaidza *et al.* (2018) observed changes such as chromatin condensation and fragmentation, cytoplasm condensation with a noticeable reduction in cell volume, blebbing of the plasma membrane, and nucleus degeneration into membrane-bound apoptotic particles. Further analysis on the specific morphological characteristics and the mechanisms behind the cytotoxicity of the extract are required to determine if these were present after application of the treatment groups. Morphological characteristics were indeed observed from the cells, either apoptotic, necrotic, or both. Prior to treatment, characteritics that are indicative of healthy and fully functioning cells such as a single and inconspicuous nucleolus, large cytoplasmic volume, intact structure, an attachment to other adjacent cells, etc, were present in the untreated cells (Duddukuri *et al.*, 2018; Lansdowne, 2020). On one hand, treatments such as 7 and 8, all of the cells have undergone through numerous changes such as cell shrinkage through nuclear and cytoplasmic condensation, blebbing of the plasma membrane, structural disintegration, and cellular fragmentation, which are signs of apoptosis. While on the other hand, every other treatment from treatment 1 to treatment 6 displays not only signs of apoptosis but also signs of necrosis in the form of swelling in the cell's cytoplasmic structure (Luo *et al.*, 2017; Miller and Zachary, 2017).

Potential reasons for the difference in the results between this study and past studies could be due to extraneous variables or conditions. It is possible that, individually, the variables of this study are not significant enough to explain why the study led to a bell-shaped cytotoxicity trend or why the lower concentrations of the study are the most cytotoxic. It must be a combination of the plant species, the extraction method (maceration), and the cancer cell line used. Another extraneous variable that could have affected the result is the location where the second batch of plant samples were sourced from, as they were larger and thicker than the leaf samples first acquired near urban areas. It is entirely probable that the location of the plant provided conditions such as soil quality, climate, temperature, and lack of pollution which may have led to more potent phytochemicals within the leaf samples.

The current study showed that both treatments induced necrosis and apoptosis based on the results of the Annexin V-PI staining assay, this agrees with the findings of Al-Nemari *et al.* (2020; 2022). The 100 ug/ml *A. squamosa* crude leaf extract was more effective against the A549 cells compared to that of the 1.5625 ug/ml treatment, specifically the former found 63.6% of the treated cells to be dead or dying while the latter was determined to be 51.2%.

This still supports the hormetic response or non-linear relationship of the extract as interpreted from the findings of the MTT cytotoxicity assay, as even though the 1.5625 ug/ml treatment was miniscule in terms of concentration compared to the 100 ug/ml treatment, it was found to only be slightly less effective. Furthermore, it must be noted that the cells affected by the 1.5625 ug/ml treatment were mostly dead or dying due to necrosis, unlike that of the 100 ug/ml treatment that had a similar percentage of cells undergoing either necrosis or apoptosis.

CONCLUSION

In summary, an assessment on the cytotoxic activity of the acetonic crude leaf extract of *A. squamosa* (Atis) was done *in vitro* against A549 (human lung adenocarcinoma) cell lines by MTT Cytotoxicity Assay and Annexin V-PI Apoptosis/Necrosis Staining Assay using different *A. squamosa* acetonic crude leaf extract concentrations. Based on the results, it was shown that the study succeeded in proving that the *A. squamosa* (Atis) crude leaf extract induced high cytotoxicity and apoptosis against A549 (human lung adenocarcinoma) cells.

Treatments 1–8 of the MTT Cytotoxicity Assay (*A. squamosa* [Atis] crude leaf extract) showed significant levels of cytotoxicity and percent inhibition, specifically treatment 7 showed the highest cytotoxicity level among the treatment groups. The half-maximal inhibitory concentration (IC_{50}) of *A. squamosa* (Atis) acetonic crude leaf extract was found to be a value of 9.7967 which is interpreted as highly cytotoxic based on the criteria by Jokhadze *et al.* (2007). Both treatments of the Annexin V-PI Staining Assay (*A. squamosa* [Atis] crude leaf extract) showed significant percentages of apoptotic cells. Treatment 1 of the Annexin V-PI Staining Assay (100 µg/mL *A. squamosa* [Atis] crude leaf extract) induced the highest percentage of apoptotic cells. Notably, over half of the cells treated with the *A. squamosa* Atis crude leaf extract crude leaf extract were found to be dead/dying.

RECOMMENDATIONS

The study would like to recommend the following considerations that are either useful of themselves or not within the scope of the study. Firstly, the utilization of Treatment 1 of the Annexin V-PI Staining Assay (100 µg/mL *A. squamosa* [Atis] crude leaf extract) as a moderately apoptotic-inducing plant compound against cancer cells, specifically the A549 (human lung adenocarcinoma) cell line. Secondly, the utilization of the same treatment tested against other cancer cells such as prostate and skin cancer cell lines. Thirdly, the utilization of Treatments 1–8 (*A. squamosa* [Atis] crude leaf extract) tested against normal human cell lines to determine if the sample is non-cytotoxic against these cells. Fourtly, the addition of parameters such as, Antibacterial Assays, Antioxidant Assays, Cell Migration and Invasion

Assays, Gene Expression Assays, and Angiogenesis Assays to further analyze the effectiveness of *A. squamosa* (Atis) crude leaf extract as a medically usable and anti-cancer plant compound. Fifthly, the testing and analysis of morphological changes that occurred to cell line a result of exposure to the extract. Sixthly, the development of a chemotherapeutic drug using *A. squamosa* (Atis) crude leaf extract tested against the A549 (human lung adenocarcinoma) cell line, which involves a cost analysis of the production of the drug.

Lastly, the utilization of solely young *A. squamosa* (Atis) leaves as the study sample, as well as other parts of the plant such as the fruit, stem, and roots.

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