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*Original Research Article*

# Cytotoxicity Assay of Andrographolide Isolated from Sambiloto Herb [A*ndrographis paniculata* (burm.f.)] against Lung Cancer A-549 and Prostate Cancer DU-145 Cell Lines

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ABSTRACT

Lung and prostate cancer have high prevalence rates worldwide, including in Indonesia. Therefore, exploring alternative drug therapies, particularly those from natural sources in the form of extracts or isolates with relatively low side effects, is necessary. One natural substance exhibiting cytotoxic activity is sambiloto [*Andrographis paniculata* (Burm.F.)]. This plant contains the bioactive compound andrographolide, known for its cytotoxic activity. This study was performed to examine the cytotoxicity of andrographolide isolates against the A-549 lung cancer cell line and the DU-145 prostate cancer cell line. The cytotoxicity test was conducted in vitro using the resazurin reduction method using PrestoBlue™ reagent. Eight concentration series with a 1:2 ratio were employed, ranging from 1,000 ppm to 7.81 ppm, with doxorubicin HCl and cisplatin as the positive control for A-549 and DU-145, respectively. The cytotoxicity parameter $IC_{50}$ was measured using GraphPad Prism version 8 software. The study results showed moderate cytotoxicity properties of andrographolide isolates with $IC_{50}$ values of 47.28 µg/mL for A-549 and 49.35 µg/mL for DU-145 cancer cell lines, respectively. Despite showing weaker values than the ones in positive controls, the moderate cytotoxicity could be further explored in cancer research. Further investigations are warranted to fully ascertain the therapeutic potential of andrographolide, which makes it a compelling candidate for future anticancer studies.

***Keywords*:** Andrographolide, anticancer, resazurin reduction, half-maximal inhibitory concentration ($IC_{50}$), cytotoxicity

## Introduction

Cancer is a leading cause of death worldwide, accounting for nearly 10 million deaths in 2020.[1] The World Health Organization (WHO) in 2020 reported that lung cancer (1.43 million cases) and prostate cancer (1.41 million cases) as the two most common types of cancer in men.[2]

Various clinical cancer management have been employed, including the use of phytopharmaceuticals as natural anticancer agents. Phytopharmaceuticals are known to have efficacy in treating malignancies with fewer or even no adverse side effects.[3,4] Andrographolide, a bioactive compound derived from the sambiloto plant (*Andrographis paniculata*), exhibits diverse potential. Numerous studies have reported its extensive use as an effective anticancer agent.[5] Prior research showed that *A. paniculata* and its primary diterpenoid components have been shown to significantly inhibit cell proliferation, induce cell cycle arrest, and promote apoptosis in various cancer cells.[6-8] Several in vitro and in vivo studies also reported the anticancer properties of *A. paniculata*.[9]

A study conducted by Lee, *et al*. demonstrated that andrographolide exhibits inhibitory activity against A-549 lung cancer cells, as determined by the MTT (Microtetrazolium) colorimetric method.[10] Additionally, Mir, *et al*. also confirmed that andrographolide compounds can inhibit the growth of prostate cancer cells.[11] Regarding those research findings, andrographolide's cytotoxicity needs to be evaluated as an anticancer.

Cell cytotoxicity assays are considered indispensable in the development of anticancer therapeutic drugs.[12] One commonly used cytotoxicity assay method is the resazurin reduction assay.[13,14] This method relies on the principle of resazurin's reduction to resorufin when it penetrates living cells. The resulting resorufin compound can be detected by measuring either the fluorescence intensity or absorbance it generates.[15]

The half-maximal inhibitory concentration ($IC_{50}$) parameter is a crucial measure for assessing toxicity. A higher $IC_{50}$ value indicates lower toxicity, while a lower $IC_{50}$ value suggests higher toxicity. This cytotoxicity test provides information on the percentage of viable cells.[16] Cytotoxicity test results for isolates or pure compounds are categorized into five groups: An $IC_{50}$ value > 501 µg/mL indicates no cytotoxic effect; $IC_{50}$ values between 201-500 µg/mL are considered weak cytotoxicity; $IC_{50}$ values between 21-200 µg/mL indicate moderate cytotoxicity; $IC_{50}$ values less than 20 µg/mL are classified as having high cytotoxic properties.[17]

The research novelty of this retrospective study is highlighted in examining the cytotoxic activity of andrographolide isolates extracted from the sambiloto herb against the lung cancer cell line A-549 and the prostate cancer cell line DU-145. By determining the respective $IC_{50}$ values of andrographolide against these cell lines, this study provides valuable insights into the compound's potential as a natural anticancer agent that adds to the literature on cancer.

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## Material and Methods

*Materials*

Andrographolide with a 91.89% purity level was prepared and isolated at the laboratory Sekolah Tinggi Farmasi Indonesia, Bandung, Indonesia. Human lung carcinoma cell line A-549 and prostate carcinoma cancer cell line DU-145 were obtained from the central laboratory of Universitas Padjajaran, Bandung, Indonesia. Several materials were also used in this research; Roosevelt Park Memorial Institute (RPMI) 1640 medium (Gibco™ Thermo Fisher Scientific); Fetal Bovine Serum (FBS) (Gibco™ Thermo Fisher Scientific); Penicillin Streptomycin (Penstrep) (Gibco™ Thermo Fisher Scientific); Trypan blue solution (Safc® Sigma-Aldrich); Resazurin-based solution for cell viability assay (PrestoBlue™ Thermo Fisher Scientific); Positive control doxorubicin HCl CRS (Council of Europe EDQM) for A-549 cell line; Positive control cisplatin CRS (Council of Europe EDQM) for DU-145 cell line; Dimethyl Sulfoxide (DMSO) (Emsure® Merck); Phosphate Buffered Saline (PBS) (Gibco™ Thermo Fisher Scientific); and Trypsin-EDTA (Gibco™ Thermo Fisher Scientific).

*Cell lines preparation*

The experiment started with the preparation of the Culture Media (CM) by mixing RPMI to constitute 89% of the total MK volume with 10% FBS and 1% Penstrep in a conical tube. Following this, cancer cells were thawed in a 37°C water bath, and 500 µL of the thawed cancer cell line was combined with 500 µL of MK. Subsequently, centrifugation (centrifuge, MicroCL 17, Thermo Fisher Scientific, USA) was performed for 5 minutes at 3,000 RPM to obtain the cancer cell line pellet. A cancer cell suspension was then created by mixing the pellet with 2.5 mL of CM and cultured in a petri dish. These cells were incubated ($CO_2$ incubator, Series 8000 DH, Thermo Fisher Scientific, USA) at 37°C in an atmosphere containing 5% $CO_2$ for 24 hours until they reached approximately 80% confluence, as determined by visual assessment using a microscope.[16,18]

Once confluence reached around 80%, the cancer cells were re-cultured into a T-25 culture flask using 4 mL of CM. The re-culturing procedure was followed by incubation for another 24 hours under the same temperature and air conditions, reaching approximately 80% confluence. The number of cancer cell lines was then counted using a hemocytometer (Z359629, Bright-Line™ Sigma-Aldrich, Germany).[19] Next, the cell suspension was diluted to reach a concentration of 170 cells/µL and prepared in a volume of 4,000 µL. This cell suspension was then placed into a 96-well plate, with each well containing 100 µL of the suspension. Subsequently, the cells were incubated at 37°C with 5% $CO_2$ for 24 hours.[16,18]

*Sample & control media preparation*

Isolate samples were created in eight concentrations from 1,000 ppm to 7.81 ppm. Meanwhile, the negative control media was prepared by mixing CM with 2% DMSO in 1 mL, while positive control media for the A-549 and DU-145 cancer cell lines were formulated by mixing them in CM according to the specified concentration.[16]

*Resazurin reduction assay*

After the incubating, the cell morphology was observed and documented. After that, CM residues were removed before adding 100 µL of the isolate sample and control media based on the order as specified on the 96-well plate. The cells were incubated at a temperature of 37°C with air containing 5% $CO_2$ and for 48 hours. Resazurin testing was then conducted after the completion of incubation. The residue in each well was removed, and then 100 µL of resazurin reagent was added to each well. Next, the cells were incubated again under the same conditions for 4 hours. Immediately after the completion of incubation, absorbance was analyzed using a multimode microplate reader (Infinite 200 Pro Nano Quant, Tecan Trading, Ltd., Switzerland) at 570 nm to 600 nm wavelength. The percentage of living cells was calculated using the following equation:

% Cell viability = (Average absorbances of triplicate treated cells / Average absorbances of control cells) x 100%.

Next, the $IC_{50}$ value was determined by creating a graph in the GraphPad Prism 8 application. The processed data were subsequently analyzed and discussed.[16,18]

## Result and Discussion

In this study, the cell culture confluency was set and maintained approximately 70-80%. Within this range, cells are typically in the logarithmic growth phase, where their growth process is optimized. On the contrary, during the stationary phase, the cell growth substantially decreases and cell morphology changes. Moreover, cells are more difficult to separate during the stationary phase. Conversely, harvesting cells during the lag phase or early log phase, when they are still adapting to environmental conditions, requires careful monitoring of confluence to ensure uniformity and high viability. In this research, the confluence was determined through visual qualitative assessment for efficiency and to minimize exposure to non-sterile conditions or time outside the biological safety cabinet (BSC), thereby reducing contamination risks.[16] At a magnification of 20x using a microscope (EVOS XL Core® Thermo Fisher Scientific, USA), the cells were observed and a precise examination of cell morphology was performed. The morphological characteristics of A-549 and DU-145 cells, as depicted in Figure 1, were compared with the reference morphology provided by ATCC (ATCC, 2022a; ATCC, 2022b) The visual analysis revealed conspicuous size variations between A-549 and DU-145 cancer cell lines, with A-549 cells exhibiting larger dimensions. Additionally, distinctive features such as intercellular tissue structure, filopodia, and cytoneme structures were also observed, showing morphological differences between these cancer cell lines.[20]

*Cell viability after sample treatment*

The microscopic observations, as shown in Figure 2 and Figure 3, display a consistent pattern of cell death in both the A-549 and DU-145 cancer cell lines. Specifically, in A-549, exposure to isolates at a concentration of 31.25 ppm resulted in significant cell death, with live cells becoming almost imperceptible at 62.5 ppm. A similar response was observed in the DU-145 cancer cell line, where cell death commenced at 31.25 ppm and viable cells were nearly undetectable at 62.5 ppm. Consequently, the preliminary estimates for the IC50 values of the isolates against both A-549 and DU-145 align with the concentration range defined by these observations.

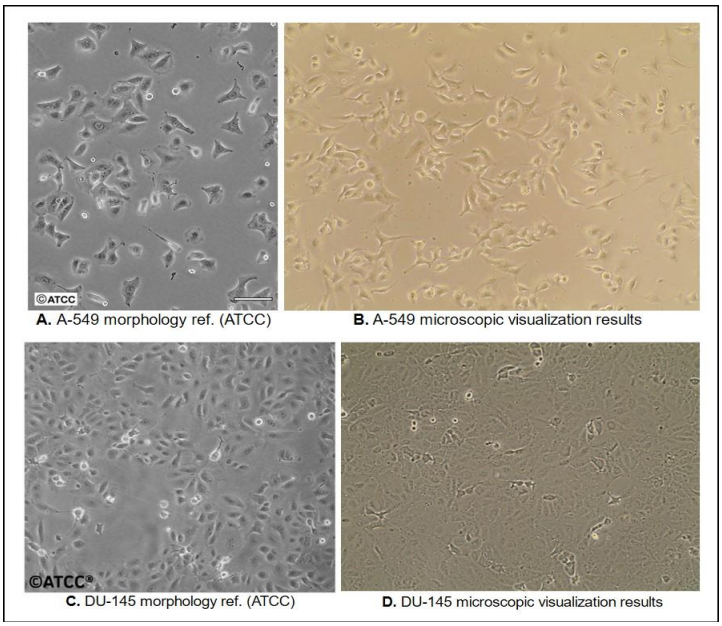


**Figure 1:** Microscopic visualization results for morphology in A-549 and DU-145 cancer cell lines. (**Notes:** 20x magnification using EVOS XL Core® Thermo Fisher Scientific; Results are compared to the reference. **Abbreviations:** Ref, reference; ATCC, The American Type Culture Collection).

During the two cytotoxicity assays conducted, no cell death was observed in the control cells and negative controls. The cytotoxicity tests performed were regarded as accurate and did not show false positives due to exposure to CM and DMSO, the mixture of test isolates. Therefore, both CM and DMSO are non-toxic to the tested cancer cell lines. However, in the positive control, cell death was noticeable at the concentration used.

*Half-maximal inhibitory concentration ($IC_{50}$)*

The percentage of cell viability was determined using a resazurin reagent, the results of which showed a color change in the cells, as shown in Figure 4 for A-549 and Figure 5 for DU-145.

Both cells clearly showed distinct color changes in specific wells. The initial blue color indicates the state before the reaction, signifying a low or complete absence of viable cells post-treatment. As the number of viable cells increases, the color shifts to pink. Both A-549 and DU-145 cell lines demonstrate that in the concentration range of 31.25 – 62.5 ppm, the transition color to pink stopped, which indicates that a significant number of cells died in the range of concentration.

Table 1 shows that higher andrographolide concentration corresponds with a decrease in living cell percentage. It is revealed that the concentration of 1000 ppm resulted in the highest level of cell death. Table 1 also provides insight into the minimum inhibitory concentration required to affect half the population of living cells ($IC_{50}$). The data confirms that the $IC_{50}$ value falls between 31.25 ppm and 62.5 ppm.

The precision level of assay data varies from 0 to 5, depending on the variability in the results. A smaller standard error of the mean (SEM) indicates a higher precision of data. In this result, the SEM value is small, indicating highly precise results (Figure 6).[21]

Recent studies have focused on the anticancer properties of pure compounds, particularly andrographolide, the main active component of *A. paniculata.* This research examined its effects through in vitro analysis. As seen in the logarithmic graph in Figure 6, the IC50 value for andrographolide against the A-549 cancer cell line is 47.28 µg/ml. The value indicates that andrographolide can inhibit 50% of cell growth at this concentration after a 48-hour incubation with a cell density of 170 cells/µL.

Prior researchers have examined the $IC_{50}$ properties of andrographolide against A-549 and DU-145 cancer cell lines. Lee *et al*. found that the $IC_{50}$ value for andrographolide against A-549 cells (at a density of 100 cells/mL) was 74.5 µM (approximately 26.1 ppm) after 24 hours of incubation. Similarly, in a study conducted by Mir *et al*., the $IC_{50}$ of andrographolide against DU-145 cells (at a density of 200 cells/mL) was reported as 20 µM (approximately 7 ppm) following 48 hours of incubation. The results of prior research show consistent results of cytotoxic properties of andrographolide in these cell lines.[10,11]

The result of our cytotoxicity test showed that andrographolide isolate is categorized as having 'moderate' cytotoxicity. In comparison to the positive control, doxorubicin, with an $IC_{50}$ of 2.1 µM or equivalent to 1.2 ppm, the andrographolide isolate exhibits lower cytotoxicity. Similarly, the $IC_{50}$ value of the andrographolide isolate against the DU-145 cancer cell line is determined to be 49.35 µg/ml, indicating that it inhibits the growth of DU-145 by 50% at this concentration. The $IC_{50}$ value shows '*moderate*' cytotoxicity. Compared to the positive control, cisplatin, which has an $IC_{50}$ of 24 µM (equivalent to 7.2 ppm), the andrographolide isolate demonstrates lower cytotoxicity.

The variation in cytotoxic effects between the positive control and the isolate in the two cytotoxicity assays can be attributed to differences in chemical structure, bonding types, and the number of functional groups contributing to cytotoxic activity in cells.[22,23]

A correlation was observed between the absorbance values and the percentage of living cells in both treatments. The data indicates a direct proportionality: higher absorbance values correspond to a greater percentage of living cells. In essence, increased absorbance signifies an increased percentage of viable cells.

The cytotoxic activity of andrographolide isolates against the A-549 and DU-145 cancer cell lines was categorized as moderate. This result shows the potential of andrographolide isolates at high purity to exceed the outcomes of prior research. The isolate produced by our institution has a purity level of 91.89%, which might affect the results of the cytotoxicity test. Further investigations are warranted to quantify the impact of this purity level.

## Conclusion

In summary, BALB/c mice can be used as an animal model for sub-clinical HuNoV infection, although further research is needed to elucidate the mechanism of viral replication and infected organs. Animal models are crucial in the research and development of vaccines or drugs. This research provides valuable new insights into animal models for HuNoV infection, identifying the Mus musculus strain BALB/c as a potential model. This is characterized by an increase in the viral genome. However, the infection observed in BALB/c mice does not replicate all aspects of human Norovirus infection, such as fecal-oral transmission.

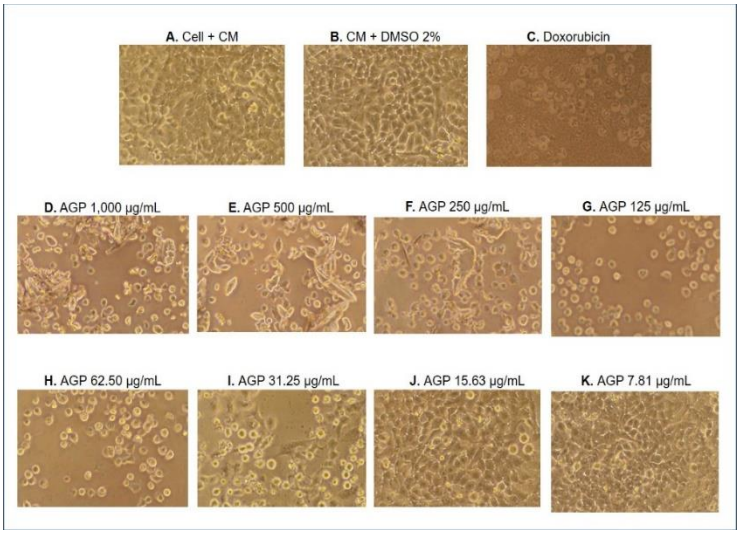


**Figure 2:** Microscopic visualization results after sample treatment of A-549. [**Notes:** 20x magnification using EVOS XL Core® Thermo Fisher Scientific; Cell control (cell + CM); Negative control (CM + 2% DMSO mixture); Positive control (doxorubicin). **Abbreviations:** CM, culture media; DMSO, Dimethyl Sulfoxide; AGP, andrographolide].

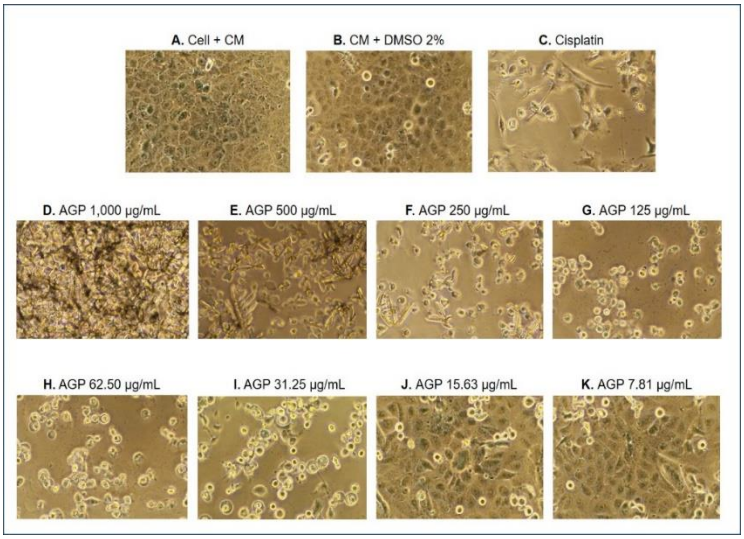


**Figure 3:** Microscopic visualization results after sample treatment of DU-145. [**Notes:** 20x magnification using EVOS XL Core® Thermo Fisher Scientific; Cell control (cell + CM); Negative control (CM + 2% DMSO mixture); Positive control (cisplatin). **Abbreviations:** CM, culture media; DMSO, Dimethyl Sulfoxide; AGP, andrographolide].

**Table 1:** Percentage Viability of Cancer Cell Lines A-549 and DU-145 in Response to Andrographolide Isolate

| **AGPC** | **A-549 Cancer Cell Line** | | **DU-145 Cancer Cell Line** | |
|---|---|---|---|---|
| **(µg/mL)** | $\bar{x}$ % CV | SEM | $\bar{x}$ % CV | SEM |
| 7.81 | 96.00 | 0.57 | 103.30 | 3.02 |
| 15.63 | 92.27 | 1.78 | 98.79 | 2.10 |
| 31.25 | 74.10 | 5.44 | 80.81 | 2.13 |
| 62.5 | 40.83 | 1.19 | 36.88 | 3.15 |
| 125 | 22.40 | 0.61 | 17.89 | 2.18 |
| 250 | 18.84 | 0.96 | 3.24 | 0.46 |
| 500 | 10.65 | 0.34 | 2.21 | 0.20 |
| 1,000 | 8.45 | 1.12 | 2.24 | 0.24 |
| (+) Control | 38.58 | 10.35 | 47.21 | 1.00 |
| Cell Control | 98.88 | 0.35 | 108.06 | 0.16 |
| (-) Control | 100.00 | 0.16 | 100.00 | 0.93 |
| **Log.*f*** | y = -85ln(x) + 95.995 | | y = -101.9ln(x) + 103.75 | |
| | x = *e*^((y – 95.995) / -85)) | | x = *e*^((y – 103.75) / -101.9)) | |

**Abbreviations:** AGPC, andrographolide isolate concentration; CV, cell viability; SEM, standard error of the mean; Log.*f*, logarithmic function.

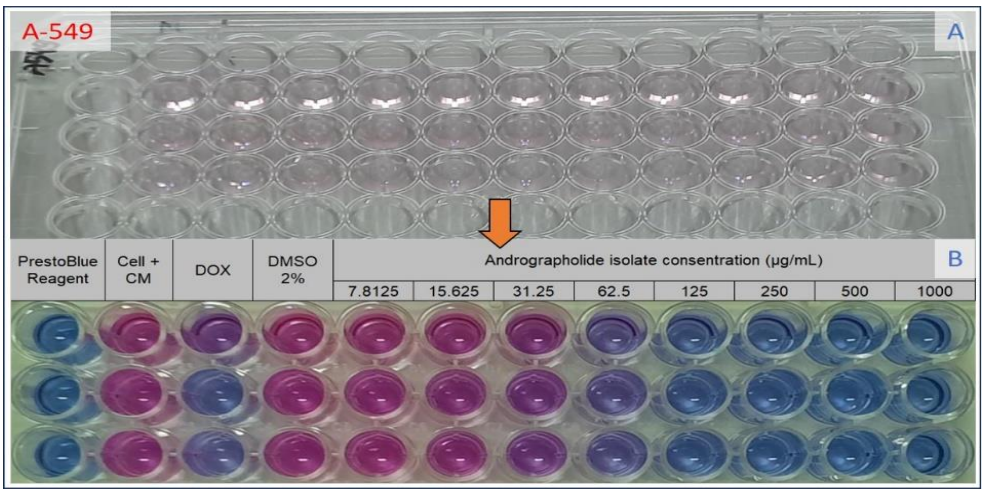


**Figure 4:** Resazurin cell viability results of A-549 cancer cell lin. [**Notes:** (**A**) Post-treatment sample; (**B**) After treatment sample. **Abbreviations:** CM, culture media; DOX, doxorubicin HCl; DMSO, Dimethyl Sulfoxide].

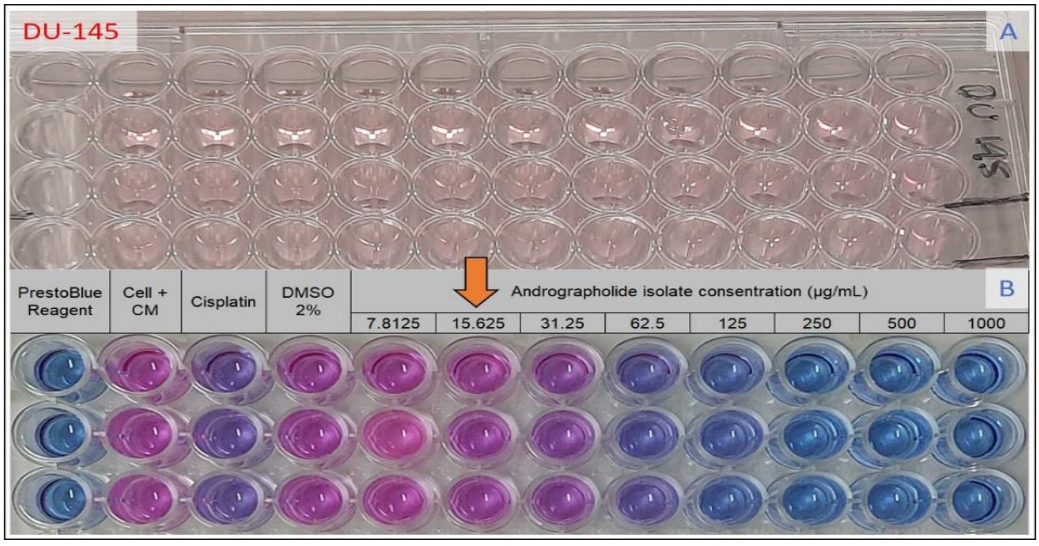


**Figure 5:** Resazurin cell viability results of DU-145 cancer cell line**.** [**Notes:** (**A**) Post-treatment sample; (**B**) After treatment sample. **Abbreviations:** CM, culture media; DMSO, Dimethyl Sulfoxide].

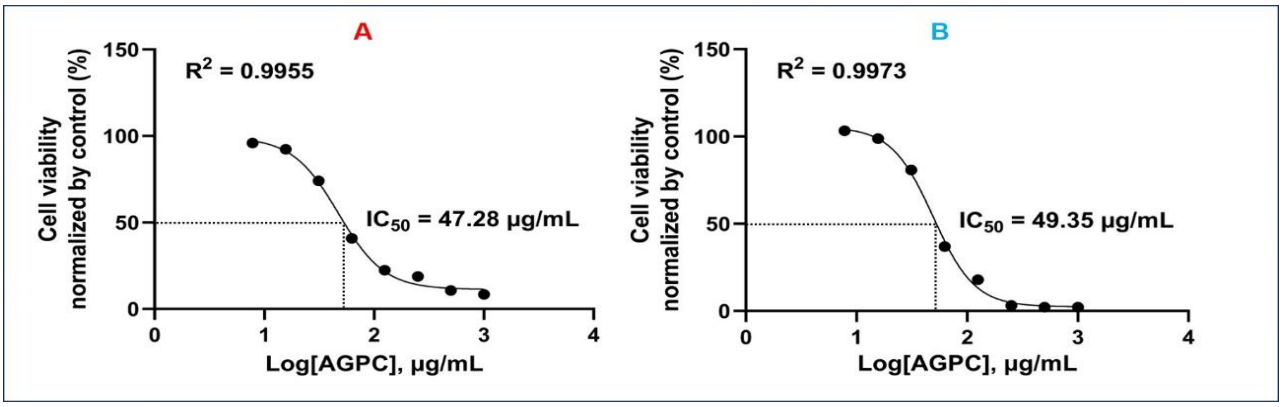


**Figure 6:** $IC_{50}$ of andrographolide isolate against A-549 and DU-145 cancer cell lines. [**Notes:** (**A**) Andrographolide isolate against A-549; (**B**) Andrographolide isolate against DU-145; Line graphs represent 48-hour treatment data. **Abbreviations:** $IC_{50}$, minimum inhibitory concentration for half the population; AGPC, andrographolide isolate concentration].

## Conflict of Interest

The authors declare no conflict of interest.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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