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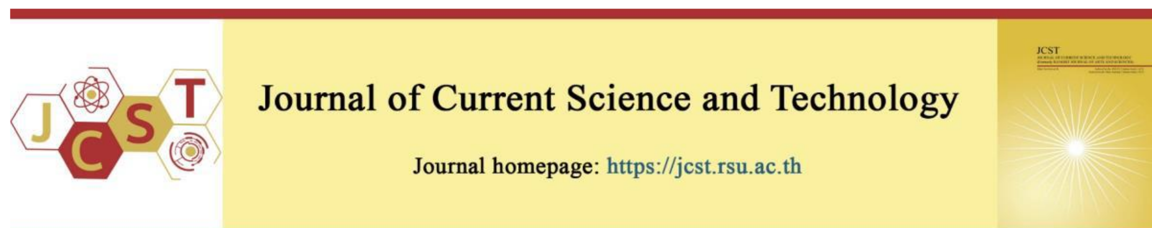

The Effect of 1'-acetoxychavicol Acetate on A549 Human Non-small Cell Lung Cancer

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Abstract

Lung cancer, a global leading cause of cancer mortality, requires safer and more effective treatments. 1'-acetoxychavicol acetate (ACA) is notable for its wide-ranging therapeutic properties, spanning anticancer, anti-inflammatory, and anti-obesity effects. While its efficacy has been explored in various cell lines, its potential against lung cancer remains few reported. This research aimed to investigate the impact of ACA on cytotoxicity, anti-proliferation, and antimigration potential in A549 human non-small cell lung cell lines. The investigation utilized MTT assays to assess cell viability and determined the IC_{50} values of ACA at 24, 48, and 72 hours to be 50.42 µM, 33.22 µM, and 21.66 µM, respectively. These results reveal a concentration- and time-dependent reduction in cell viability following ACA treatment. In addition, anti-proliferation was performed using colony-forming assays, and ACA showed notable efficacy in significantly decreasing colony formation, highlighting its strong impact on cell proliferation and viability. In the final part, ACA had promising inhibitory effects on migration, which varied depending on the dose used in the scratch assay. In conclusion, ACA highlighted cytotoxic and anti-proliferation properties of A549 cell line, supporting its potential role in lung cancer therapy pending further investigation and development.

Keywords: *Lung Cancer; 1'-acetoxychavicol acetate; Anti-proliferative activity; Antimigration; Colony forming*

1. Introduction

In 2021, the most common causes of death globally included heart disease (33%), cancer (18%), and chronic respiratory diseases (7%), but in Thailand, cancer is a leading cause of death (Saelee et al., 2022). Lung Cancer was found to be in the top five causes of death in Thailand and globally (Siegel et al., 2014). Lung cancer is a complex and often life-threatening medical condition characterized by uncontrolled cell growth, leading to the formation of colonies and masses, or tumors. Cancer cells sometimes express the epithelial-mesenchymal transition (EMT) process and avoid apoptosis to survive (Adorno et al., 2009). Additionally, cell migration initiates by moving towards neighboring cells, enabling metastasis throughout the body. Typically categorized, lung cancer is split into two primary types: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). NSCLC accounts for around 85% of instances, while SCLC makes up 15%. In clinical settings, NSCLC divides subtypes into Adenocarcinoma (40%), Squamous cell carcinoma (25%), and large cell carcinoma (10%).

Previous studies have discovered gene mutations that promote cancer cell growth and resistance to disease control. Many studies focus on adenocarcinoma due to the existence of NSCLC, and they found the gene *EGFR* and *KRAS* to play a significant role in proto-oncogene, which is crucial in regulatory cell growth and proliferation. A549 cell lines represent adenocarcinoma and express gene mutations such as *EGFR* (10-15%) and 25% of *KRAS* (Luo et al, 2014; Addeo et al., 2021). Frequently, the disease progresses inconspicuously over ten years, with symptoms becoming noticeable only in the later stages, but early identification can result in more efficient disease control. Regrettably, a significant proportion of diagnoses are made during advanced stages, which in turn leads to elevated mortality rates (Jones, & Baldwin, 2018). Various factors, including genetics, environmental exposures, and lifestyle choices, influence cancer development. The conventional treatment protocol for lung cancer may encompass surgical intervention, chemotherapy, radiation therapy, immunotherapy, or targeted therapy, contingent upon the individual situation (Kurgan et al, 2017). Although there have been improvements in treatment, lung cancer remains a major global health issue, emphasizing the immediate requirement for continuous research and a comprehensive healthcare approach.

1'-acetoxychavicol acetate (ACA) is one of the bioactive compounds found in the isolated rhizomes of *Alpinia galanga* (L.) Willd. ACA has several pharmacological effects, including anticancer, anti- allergy, antimicrobial, gastro-protective, and anti- inflammatory activities (Kojima-Yuasa, & Matsui-Yuasa, 2021). Previous studies discovered synergistic anticancer effects by combining ACA with standard chemotherapy drugs on cervix carcinoma cells (Phuah et al., 2017). Pradubyat et al. (2022) and team found the effects of ACA on breast cancer cell lines (MCF7, MCF7/LCC2, MCF7/LCC9) included inhibiting cell proliferation through the downregulation of HER2 receptor, estrogen receptor coactivator (NCOA3), pERK1/2, pAKT, CCND1, C-myc (Pradubyat et al., 2022). ACA was shown to inhibit cell proliferation of SW480 colorectal adenocarcinoma with an IC50 of 80 µM after 48 hours. Additionally, ACA induced cell apoptosis and interfered with a checkpoint at G0/G1 of the cell cycle, leading to DNA damage (Nelson et al, 2020; Jongrungruangchok et al., 2019). In addition, the A549 human non-small cell lung cancer with ACA showed cytotoxicity effect and induction through the Beclin-1-independent pathway, which is related to the pro-autophagy pathway (Sok et al., 2017). However, multiple pathways can affect the progression of cancer cells. Conducting more studies can help us better understand the activities of ACA. While its efficacy potential against lung cancer remains sparsely reported. This research aimed to investigate the impact of ACA on cytotoxicity, anti-proliferation, and antimigration potential in A549 human non-small cell lung cell lines. The purpose of these insights is to enhance our knowledge of the biology of tumor cells and aid in the development of more effective treatment methods for cancer patients using ACA. Although there have been improvements in therapy, lung cancer remains a significant global health issue, emphasizing the immediate requirement for continuous research and a comprehensive healthcare approach.

2. Objectives

This investigation aimed to determine the effects of ACA on cytotoxicity, anti-proliferation, and antimigration potentials in A549 human non-small cell lung cancer cell lines.

3. Materials and Methods

3.1 Drugs and Chemicals

0.4% Trypan blue dye (Sigma, USA), 1′-acetoxychavicol acetate bioactive compound, ethanol and dimethyl sulfoxide (DMSO). Roswell Park Memorial Institute-1640 (RPMI-1640) medium (GE Healthcare HyClone, USA). Fetal bovine serum (FBS) (Sigma, USA). MTT (methylthiazolyldiphenyl–tetrazolium bromide) and crystal violet.

3.2 1′-acetoxychavicol acetate bioactive compound

1′ - acetoxychavicol acetate (ACA) is obtained from the Department of Pharmacognosy, College of Pharmacy, Rangsit University, Thailand. ACA was isolated from fresh *Alpinia galanga* rhizomes in Bangkok, Thailand (November 2017) and identified by a Specialist in Botany (No. specimen CP-Ag-29). Pradubyat et al. (2022) and the team approved the extraction and purification of ACA in 2022 and resisted the patent (Pub. No.: US 2002/0192262 A1).

3.3 The cytotoxicity activities testing (Viability assay)

The cytotoxic effects of ACA on the A549 cell line were evaluated using an MTT assay, following a modified technique described by Carmichael et al. (1987). Initially, the cell suspension (5×10^5 cells/mL) was seeded in 96-well plates and left undisturbed overnight. Afterward, the cells were directly exposed to different doses of ACA, ranging from 0 to 60 µM. The negative control in the whole medium contained 0.2% v/v ethanol (0.2%EtOH in RPMI medium). The study also investigated the influence of vehicle control on cells under untreated control conditions, using just complete media. Following a 24-hour ACA treatment, 200 µl MTT reagent (0.5 mg/mL) was substituted for the medium, incubated for 4 hours at 37°C, and DMSO solution was added to dissolve the purple formazan crystals. The absorbance values at 570 nm were measured using a microplate reader (BioTek Synergy, USA) to determine the number of live cells. Following initial testing, triplicate MTT assays were conducted for each concentration range (0-60 µM) to determine the IC_{50} value. The techniques mentioned above were replicated in three separate experiments to confirm the reliability of the results. The cell viability was calculated from the ratio of mean absorbance treated cells to mean absorbance control cells (Pradubyat et al., 2022).

3.3 Colony formation assay

The previously described colony-forming assay was a methodology to determine the capability of colony formation (Liew et al., 2017). Seeded cells (5000 cells/well) were placed in 6-well plates and then incubated overnight with a humidity maintained, along with a 5% CO_2 concentration. The cultural media was replaced every three days for seven days. After incubation, the developed colonies were fixed with acetic acid methanol (1:3) for 30 minutes and stained with 0.1% crystal violet for 30 minutes. After rinsing with PBS, pictures of colonies were captured to facilitate the comparison and analysis of the results.

3.4 Migration assay

A scratch test, also called a wound healing assay, uses the approach described by Kaewpiboon and Boonnak (2018). A549 cells were cultivated in 6-well plates until they achieved confluency. Create a wound approximately 1 mm wide with a sterile 200-µl pipette tip. All wells were washed with incomplete medium to eliminate any cells that had become detached. Subsequently, images of the "wounds" formed were taken at the starting point mark (0-hour), substituted with ACA at concentrations ranging from 5 to 10 µM in a culture medium, then incubated for 48 hours to observe cell migration. Following the conclusion of this time frame, a subsequent series of images was captured from the same areas to record the advancement of the migration. Image-J 1.45 software was used to determine the quantitative changes in wound size. The magnitude of cell migration was assessed by comparing the absence of cells in the wound area 48 hours after scratching to the initial size of the gap. The findings were presented as a percentage relative to the initial gap size at 0 hours. The experiment was performed three times, and the analysis included the computation of the overall surface area, the area occupied by migrating cells, and the percentage of wound closure calculated the ratio of migrated cell surface area with Total surface area (Pradubyat et al., 2022).

3.5 Statistical analysis

Mean ± standard error of mean (SEM) was expressed in all data. All findings were performed in triplicates (n=3). A statistical hypothesis was used one-way ANOVA, followed by the Tukey HSD post-hoc test. The SPSS 22.0 software was analysis significance accepted at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

3.6 Ethical consideration

The A549 cell line was obtained from the Institutional Review Board.

4. Results

4.1 Cytotoxicity effect of ACA on A549 cell line

These studies determine the effect of ACA in repressing cell proliferation. The results revealed the anti-proliferative activity of ACA on A549 cell lines, while negative control did not damage cancer cell growth. The percentage cell viability of A549 significantly decreased after exposure to ACA (0–60 µM) in a concentration- and time-dependent manner with distinct IC_{50} values in each incubation at 24-72 hours in Figure 1 and Table 1.

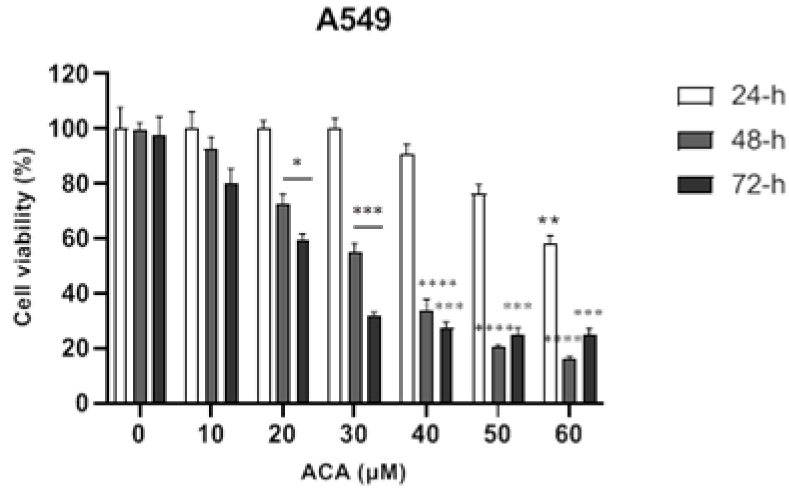

Figure 1 Percentage of A549 cell viability after treatment with ACA for 24 to 72 hours. Significance levels are indicated as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$

Table 1 The cytotoxic activity (IC_{50}) of ACA on A549 cell lines at 24-72 hours.

Cell line	IC_{50} of ACA		
	24 hours	48 hours	72 hours
A549	50.42 µM ± 3.79*,**	33.22 µM ± 2.84*	21.66 µM ± 2.79

*$P < 0.05$ and ** $P < 0.01$ compared at 72 hours incubation

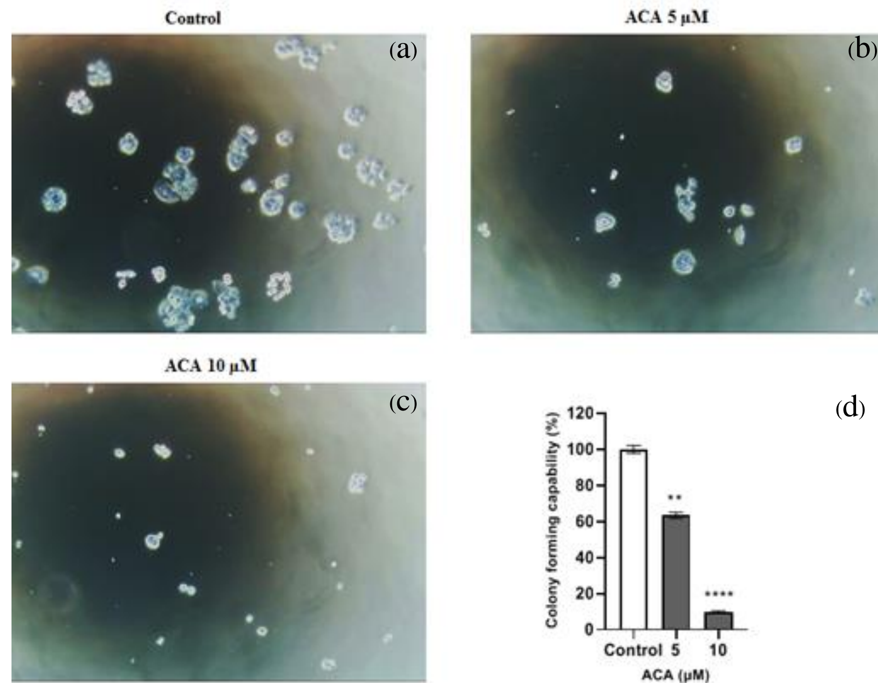

Figure 2 Colony formation in A549 cells (a) untreated control, (b) treated with 5 µM ACA, and (c) treated with 10 µM ACA; (d) quantified colony formation with statistical significance indicated by **$P < 0.01$ and ****$P < 0.0001$

4.2 Anti-proliferation effect of ACA on A549 cell line

The result demonstrated that the number of colonies formed and percentage of colony forming capability after being treated with ACA at 5 and 10 µM significantly decreased in A549 cell lines compared to the control is shown in the Figure 2.

4.3 Antimigration effect on A549 cell lines

The impact of ACA on the A549 cell line was performed using a Scratch assay to determine the migration of cancer cells. Concentrations of ACA were used to provide the safety of the experiment as value IC_{50} at 24 hours. More precisely, doses ranging from 5 to 10 µM, determined through a two-fold dilution technique, maintained over 70% cell viability in four different cell lines. This method follows the guidelines of the INTERNATIONAL ISO STANDARD (Vidal, & Granjeiro, 2017), ensuring that the ACA dosages are non-harmful and maintain over 70% viability in all evaluated cell lines. Figure 3 and Figure 4 demonstrate the impact of ACA on the mobility of the A549 cell line, specifically by depicting the progression of the healing process over 48 hours. ACA doses of 5 and 10 µM showed promising inhibitory effects on migration but not significantly compared to control, which reduced wound closure areas to 38.74% and 36.44%, respectively.

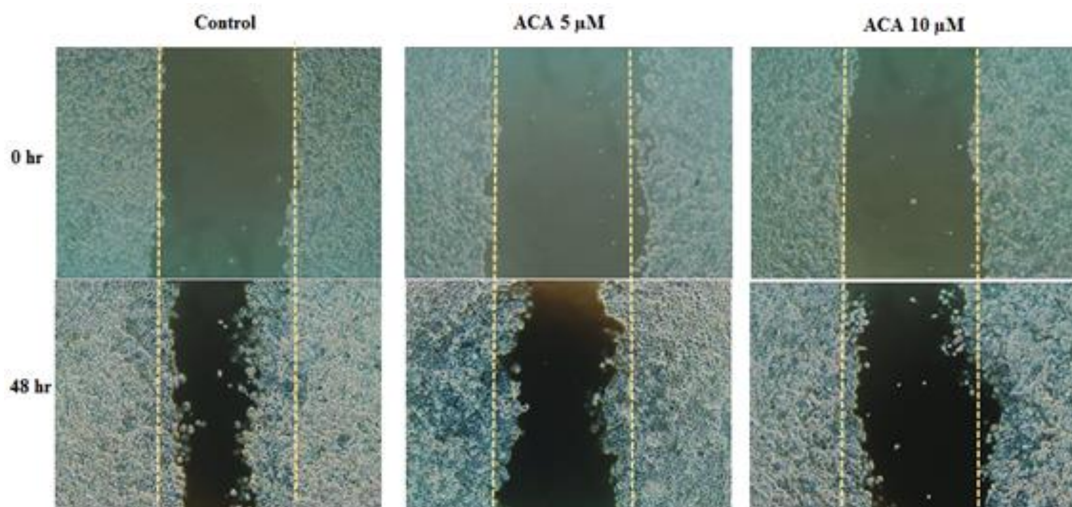

Figure 3 The comparison of the wound closure area of A549 cells before and after being treated with ACA at 5 and 10 µM for 48 hours

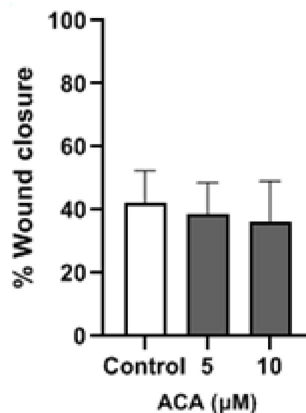

Figure 4 Wound closure (%) of A549 after treatment with ACA at 5, 10 µM for 48 hours

5. Discussion

Herbal remedies have a long-established track record of boosting well-being and treating diseases on a global scale (Prisa, 2022). However, their extensive usage and lack of thorough qualitative and quantitative data verifying their safety and effectiveness have restricted their international acceptance (Chotchoungchatchai et al., 2012; In et al., 2012). Traditional herbal treatments have played a significant role in healthcare systems, both in rural and urban areas, in several nations, including Thailand (Srithi et al., 2012). *Alpinia galanga* has been used for cooking and traditional medicine for a long time, which means it is safe to consume. The phytochemicals, such as alkaloids, tannins, flavonoids, and phenylpropanoids, have high antioxidant, anti-inflammatory, and anticancer properties (Xu et al., 2008; Wang et al., 2014). Also, ACA is a bioactive compound isolated from this plant. Awang et al. (2010) conducted a study to investigate the safety of ACA in laboratory conditions (in vitro) and animal models (in vivo). Their research revealed that ACA concentrations close to 80 μM did not cause any detriment to normal HMEC cells. Lastly, published investi-gations revealed that ACA possesses significant anticancer properties, exerting its effects through many mechanisms in multiple cancer cells (Pradubyat et al., 2022). In contrast, NSCLC is not clear. This research proposes to consider the anticancer effects of ACA on A549 human non-small cell lung cancer cells by relating the stage of cancer development from the initial to metastasis. The results demonstrate the cytotoxicity effect of ACA at 24, 48, and 72 hours to be 50.42 μM, 33.22 μM, and 21.66 μM, respectively, which depends on the dose and time duration of treatment. ACA is expected to have some level of cytotoxicity to achieve the desired effect. We must use a strong enough dose to ensure the cancer cells are affected by controlling cell growth, division, and proliferation (Baradwaj et al., 2017). Due to the difference in the doubling time of each cell line, which mainly produces at least 24-72 hours, we used an action that covers the doubling time of cell growth (Sok et al., 2017). In addition, the anti-proliferative effects of ACA significantly decreased colony formation in the A549 cell line compared to the control group, which had the best effect at high doses, emphasizing its strong influence on cell proliferation and viability. Further, cell migration contributes to or is a key step in initiating metastasis and developing secondary tumors (Kalluri, 2009). ACA had promising inhibitory effects on migration, which varied depending on the dose. Ongoing research investigates the consequences of ACA, which found flavonoid content and its impact on cancer cells (Kopustinskiene et al., 2020). The results of our study suggest that ACA has excellent promise as an anticancer drug because of the combined effects of its phytochemicals. The results indicate that ACA possesses noteworthy anticancer characteristics, which justifies additional molecular-level research on its mechanisms, especially underlying cell growth and proliferation.

6. Conclusion

This study established that 1'-acetoxychavicol acetate (ACA) exhibits cytotoxicity and anti-proliferation effects against cancer. Although the precise mechanisms by which ACA wields its therapeutic and health impacts are not yet fully understood, the evidence reported in this study suggests that ACA has the potential to be a valuable addition to cancer therapy efforts. Additional investigation is required to reveal the intricate mechanisms that underlie its effects.

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