No.	K24-2121	
研究課題名	Cell Technology for Testing Indonesian Natural Products as Anticancer and Antiaging Agents	
研究代表者	Dwiranti Astari (Universitas Indonesia・准教授)	
研究組織	受入教員	中西 真 (東京大学医科学研究所・教授)
	分担者	Anom Bowolaksono (Universitas Indonesia・Professor)
	分担者	Murdani Abdullah (Department of Internal Medicine, Dr. Cipto Mangunkusumo General Hospital, Faculty of Medicine, Universitas Indonesia・Professor, Medical Doctor in Gastroenterology, Hepatology)
	分担者	Erwin Danil Yulian (Division of Surgical Oncology, Department of Surgery, Dr. Cipto Mangunkusumo General Hospital, Faculty of Medicine, Universitas Indonesia・Medical doctor in oncology)
	分担者	Jamal Zaini (Division of Thoracic Oncology, Department of Pulmonary Disease, Persahabatan Central General Hospital, Faculty of Medicine, Universitas Indonesia・Medical doctor in Pulmonology, Respiratory Medicine)
	分担者	Takeharu Nagai (Osaka University, Department of Biomolecular Science and Engineering, The Institute of Scientific and Industrial Research, Osaka University・Professor)
	分担者	Qanita Hana Amira (Universitas Indonesia・Graduate student)
	分担者	Shadira Anindieta Irdianto (Universitas Indonesia・Graduate student)
	分担者	Frieska Amelia Widya Ananda (University of Indonesia/ The Department of Biology at the Faculty of Mathematics and Natural Sciences・Research Assistant)

(Form 2)

Principal Investigator
Astari Dwiranti, Ph.D.

IMSUT International Joint Usage/Research Center Project <International>

Joint Research Report (Annual/Project Completion)

Project Completion Report

Report

Reporting Period:
December 18, 2024 –March 8, 2025

Summary of Activities:

During the research stay at IMSUT, a comprehensive series of in vitro and in vivo experiments were conducted to evaluate the anticancer effects of Berberine, Cardamonin, and their combination on the MDA-MB-231 human breast cancer cell line. The in vitro phase involved cytotoxicity assessment using the INCell Analyzer, employing Propidium Iodide (PI) and Hoechst staining. Apoptosis analysis was performed using flow cytometry with DAPI and Annexin APC staining, while cell cycle analysis was conducted via flow cytometry using PI staining and RNase treatment. Additionally, protein expression studies were carried out using western blot to examine the molecular effects of Cardamonin treatment on key signaling pathways in MDA-MB-231 cells. Experimental workflows included compound administration, viability assays, apoptosis profiling, cell cycle distribution, and protein-level pathway analysis. Following the in vitro experiments, an in vivo study was conducted using two groups of mice—one treated with Cardamonin and the other as a placebo control. A total of six doses were administered every two days, and tissue samples were subsequently collected and preserved in paraffin blocks (total of 24). Histological analysis included Sirius Red staining and Hematoxylin & Eosin (H&E) staining to evaluate tissue morphology and fibrosis. This study aimed to provide a holistic understanding of the potential anticancer and antifibrotic effects of the tested compounds and to explore possible synergistic mechanisms of action.

Progress Toward Goals:

1. **Successfully established and maintained MDA-MB-231 cell cultures,** ensuring consistent cell growth and viability throughout the experimental period for both control and treatment groups.

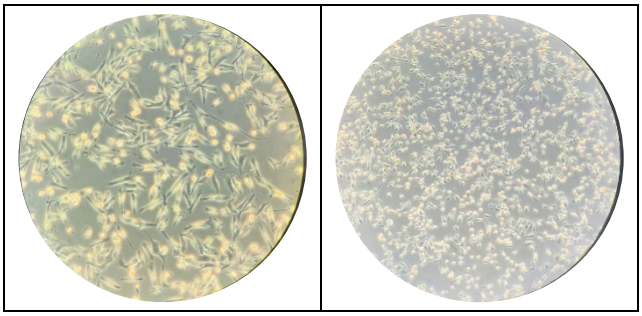

Fig 1. MDA-MB-231 Cells Observed Under Inverted Light Microscope

2. **Conducted comprehensive single and combination compound treatments using** Berberine, Cardamonin, and their synergistic pairing across multiple time points and concentrations to investigate their effects on cell viability and behavior.

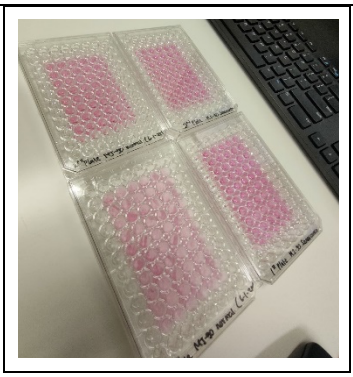

Fig 2. Treatment of MDA-MB-231 Cells with Berberine, Cardamonin, and Their Combination

3. **Performed cytotoxicity assays** using the **INCell Analyzer,** utilizing **Propidium Iodide (PI)** to stain non-viable cells and **Hoechst** to label all nuclei. High-resolution imaging and quantitative analysis were completed for all treatment groups to assess dose-dependent effects on cell viability and morphology.

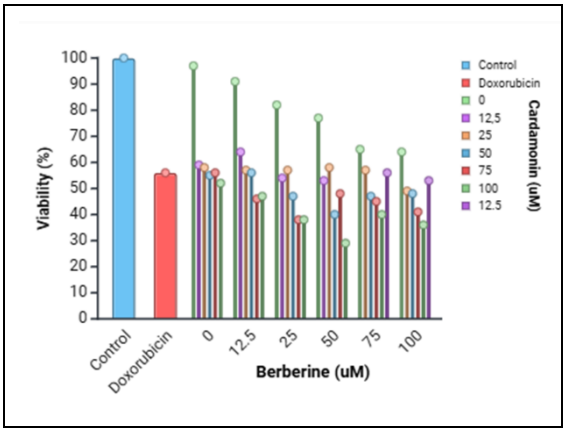

Fig 3. Effects of Berberine, Cardamonin, and Their Combination on Cell Viability

4. **Completed apoptosis analysis** via **flow cytometry,** employing **DAPI and Annexin APC staining** to quantitatively evaluate apoptotic, necrotic, and viable cell populations across the different treatment conditions. Distinct population shifts were observed, indicating compound-specific induction of programmed cell death.

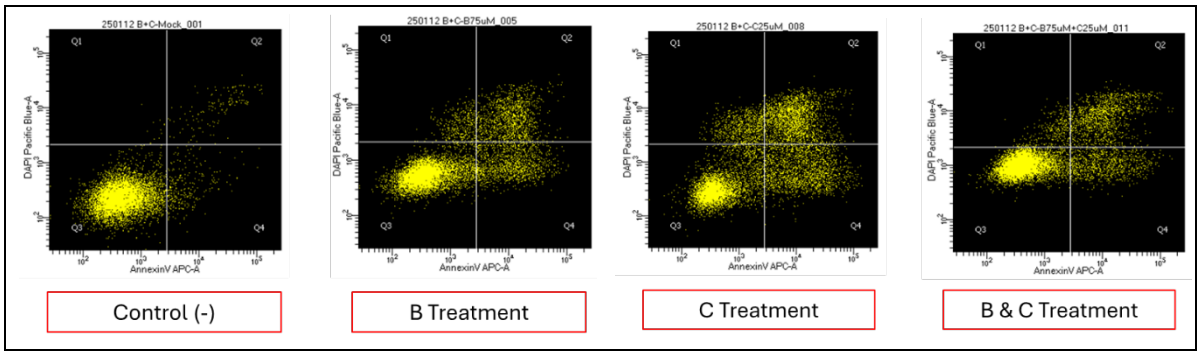

Fig 4. Apoptosis Analysis Following Treatment with Berberine, Cardamonin, and Their Combination

5. **Performed cell cycle analysis** using **flow cytometry** with **PI staining and RNase treatment**. Data was collected and analyzed to determine the distribution of cells in the **G0/G1, S, and G2/M phases**, revealing cell cycle arrest patterns associated with each treatment.

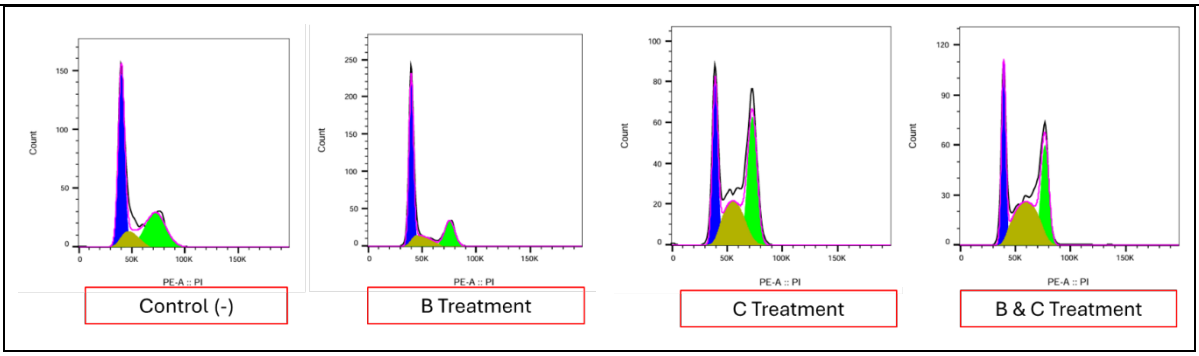

Fig 5. Cell Cycle Analysis After Treatment with Berberine, Cardamonin, and Their Combination

6. **Conducted protein expression profiling** using **Western blotting**, targeting key regulatory and tumor-related proteins, including:
   - **β-actin** as the internal loading control
   - **Rb (Retinoblastoma protein)** and **p21** to assess cell cycle regulation
   - **p53**, a tumor suppressor protein linked to apoptosis
   - **p55** (possible TNFR-related marker) for cell death signaling
   - **PD-L1** for immune checkpoint expression analysis

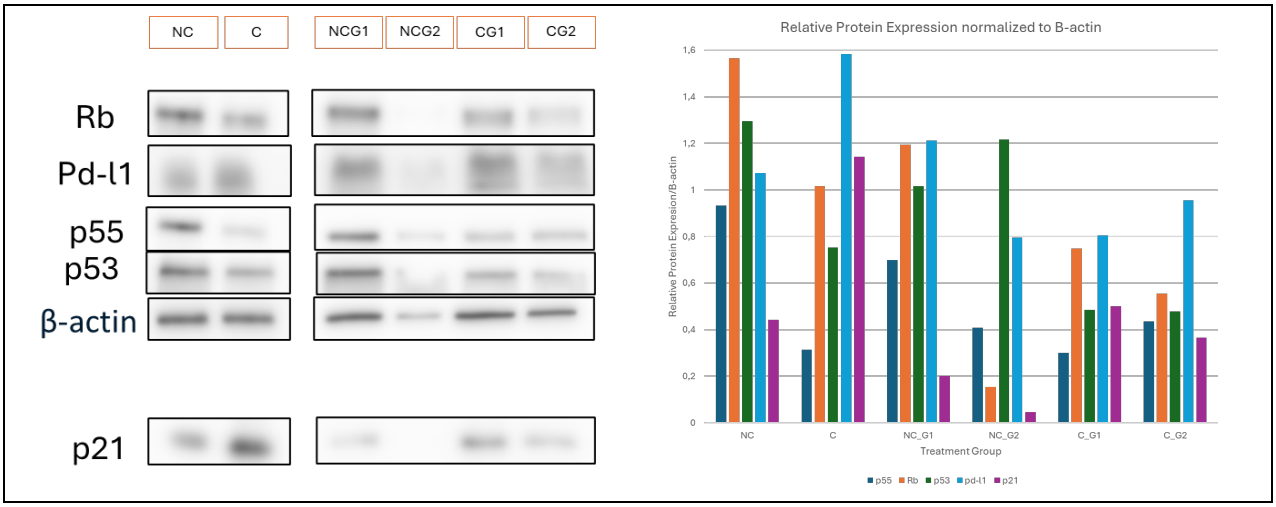

Fig 6. Western Blot Analysis of MDA-MB-231 Cells Treated with Cardamonin

7. **Conducted an in vivo study using two mouse groups (Cardamonin-treated and placebo controls), each receiving six intraperitoneal injections over the treatment period.** Following the treatment, tissue samples were collected and processed into 24 paraffin blocks for histological evaluation. Two staining techniques were applied: **Hematoxylin and Eosin (H&E)** to assess tissue structure and cellular morphology, and **Sirius Red staining** to evaluate collagen deposition and fibrosis. Initial observations revealed structural and fibrotic differences between treated and control groups, suggesting potential tissue-level effects of Cardamonin. Further analysis is ongoing to confirm these findings quantitatively.

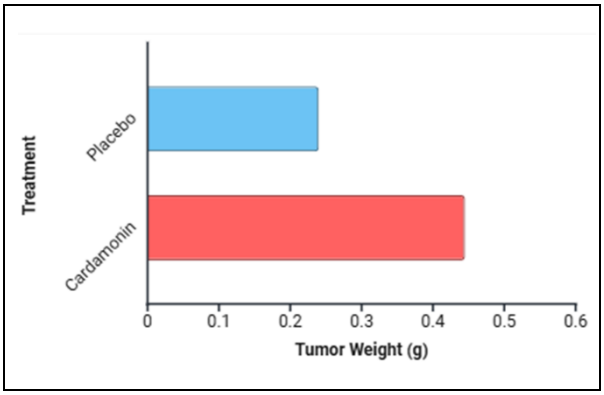

Fig 7. Tumor Weight Results Following Six Doses of In Vivo Cardamonin Treatment

Challenges and Solution:

During the research at IMSUT, several challenges emerged that required methodical troubleshooting and adaptive solutions:

1. **Viability Assay Interference**

   The initial plan involved using the CCK-8 assay to measure cell viability. However, it was discovered that the intrinsic color of the compounds—particularly Berberine and Cardamonin—interfered with the colorimetric readout, leading to biased and unreliable results.

   Solution: To address this, we shifted to imaging-based cytotoxicity analysis using the INCell Analyzer, coupled with Propidium Iodide (PI) to stain dead cells and Hoechst to stain all nuclei. This provided more accurate and visual confirmation of compound effects on cell viability.

2. **Inconsistent Cytotoxicity Results**

   Inconsistencies in viability data were observed, suspected to be due to the compounds' tendency to precipitate easily, potentially causing pipetting errors and uneven distribution in the culture medium.

   Solution: Additional replicate experiments were performed to improve data reliability and ensure reproducibility, with heightened attention to pipetting technique and compound preparation.

3. **Cell Cycle Analysis Complications**

   The initial attempt at cell cycle analysis using Hoechst and PI staining without RNase treatment resulted in suboptimal and noisy data. This could have been due to RNA contamination, which overlaps with DNA staining and interferes with accurate cell cycle phase distinction.

   Solution: The protocol was revised to include PI staining in combination with RNase treatment, which successfully resolved the issue and yielded clearer, more interpretable results.

4. **Western Blot Detection Failure**

   Repeated attempts to detect specific protein targets by Western blotting failed to yield visible bands using certain antibodies. One notable issue was with the antibody for cleaved caspase-3, which appeared to be non-functional—possibly due to degradation or poor specificity.

   Solution: Multiple repetitions of the Western blot were carried out, and antibody stocks were re-evaluated. Eventually, the problematic antibody was excluded from the final analysis due to irreproducible results, and alternative protein markers were considered.

5. **Time Constraints and Intensive Scheduling**

   The overall timeline for the research project was limited to a relatively short period. This posed significant logistical challenges, as experimental procedures—especially those involving multiple replicates and in vivo work—required tight scheduling and rapid execution.

   Solution: Careful planning and prioritization of critical experiments allowed for completion within the time frame, though some additional experiments had to be excluded from the final dataset.

6. **In Vivo vs In Vitro Discrepancy**

   A major challenge was the discrepancy between the in vitro and in vivo results. While the in vitro studies showed promising anticancer activity, the in vivo results did not align, suggesting the presence of unexpected biological or pharmacokinetic factors influencing the treatment outcomes.

   Solution: Although this discrepancy could not be fully resolved within the project duration, it was acknowledged as an important observation that warrants further investigation, possibly involving deeper analysis of metabolism, bioavailability, or immune system interaction.

Research Outputs

1. **All raw experimental data**—including imaging results, flow cytometry files, and Western blot documentation—have been **systematically archived** and **securely stored** for ongoing analysis and reproducibility purposes. These datasets are currently **under internal review and validation** to ensure data quality and consistency.
2. **Initial findings and key observations** from both in vitro and in vivo experiments have been **extensively discussed with the supervising professor**, laying the groundwork for deeper investigation and the development of **follow-up research directions**. These discussions have sparked interest in exploring additional molecular targets and refining treatment strategies in future studies.
3. A **preliminary manuscript outline** has been initiated, with the aim of preparing a **collaborative research publication targeted for release in 2025**. The manuscript will focus on the mechanistic insights into the anticancer effects of Berberine and Cardamonin, integrating both the cellular and molecular data generated during this research stay.

Plans for the Next Projects (2025)

Building upon the findings from the 2024 research project at IMSUT, the upcoming study in 2025 will aim to further explore the **biological mechanisms and therapeutic potential of natural compounds**, with a specific shift in focus toward **anti-aging research**. The planned project will either continue utilizing **Berberine, Cardamonin**, or potentially introduce **new candidate compounds**—currently still under discussion with the supervising professor and research collaborators.

The future study will focus on examining **cellular senescence, oxidative stress responses, and aging-associated molecular pathways** using advanced in vitro techniques, and possibly in vivo models. The integration of insights gained from the anticancer study is expected to provide a valuable foundation for understanding **overlapping molecular mechanisms between cancer progression and cellular aging**, allowing for a more targeted and innovative approach to compound screening and therapeutic evaluation. This project is currently in the **planning and design phase**, with discussions ongoing to determine the most relevant models, endpoints, and compound candidates for effective anti-aging intervention research.

Other Remarks

The research stay at IMSUT has provided valuable international research experience and collaborative learning. Gratitude is extended to Professor Nakanishi, Dr. David, Sugimoto-san and all the laboratory team for their support and guidance throughout the project.