No.	K24-2121	
Project Title	Cell Technology for Testing Indonesian Natural Products as Anticancer and Antiaging Agents	
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(Form 2)

Principal Investigator
Astari Dwiranti

IMSUT International Joint Usage/Research Center Project <International>

Joint Research Report (Annual/Project Completion)

Project Completion Report

Report

Research activities at the Institute of Medical Sciences, University of Tokyo (IMSUT), were generally organized into three phases: reconfirmation, experimentation, and analysis, with the aim of evaluating the effects of cardamonin and its anticancer activity against triple-negative breast cancer. The reconfirmation phase involved assessing PD-L1 expression in MDA-MB-231 cells treated with 25 µM cardamonin. This step was essential to verify the effects of cardamonin in both in vitro and in vivo settings. At the cellular and molecular levels, analyses using FACS and Western blotting demonstrated an increase in PD-L1 expression in cardamonin-treated cells. Subsequently, experimental studies were conducted to analyze cellular responses following cardamonin treatment, including cell cycle progression, apoptosis, protein expression, and gene expression. Further investigations were extended to in vivo models. PY230 cells were transplanted into C57BL/6 mice to establish tumor-bearing allograft models. The mice received seven doses of cardamonin administered every two days before being sacrificed. The results showed a reduction in both tumor size and weight following cardamonin treatment.

Goals:

1. **Successfully reconfirm effect of cardamonin on Triple Negative Breast Cancer (TNBC) cell line, MDA-MB-231.** PD-L1 expression were confirmed to be increased after 25 µM cardamonin treatment.

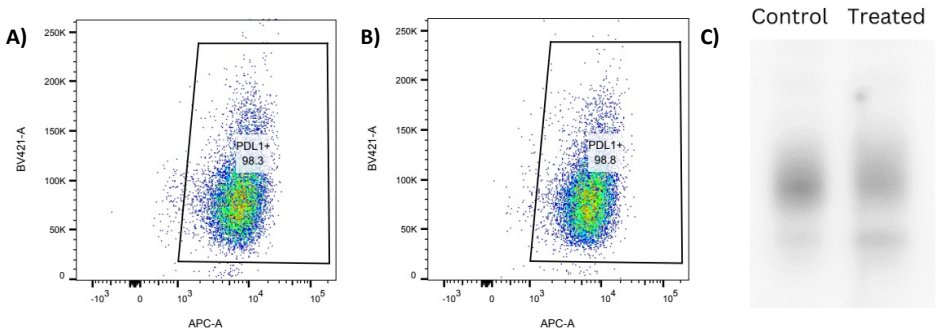

Fig 1. A) PD-L1 Expression in control group; B) PD-L1 Expression in cardamonin treated group; C) Protein expression of MDA-MB-231 in control and cardamonin treated

2. **Profiled protein Expression of MDA-MB-231 through western blot analysis.** Cell cycle and cell death regulatory protein were checked upon cardamonin reducing MDA-MB-231 viability.

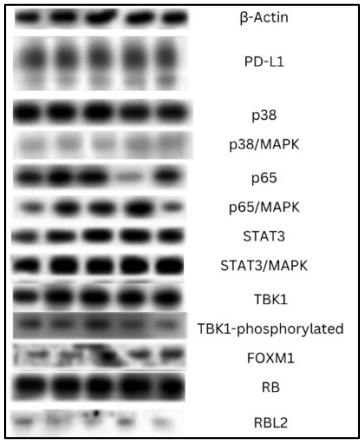

Fig 2. Protein expression of MDA-MB-231 after 0, 1, 5, 10, and 25 µM cardamonin treatment (left to right)

3. **Extracted RNA and analyzed using R.** RNA-Seq were employed to uncover gene profiling on MDA-MB-231 after 0 µM, 10 µM, and 25 µM cardamonin treatment on MDA-MB-231.

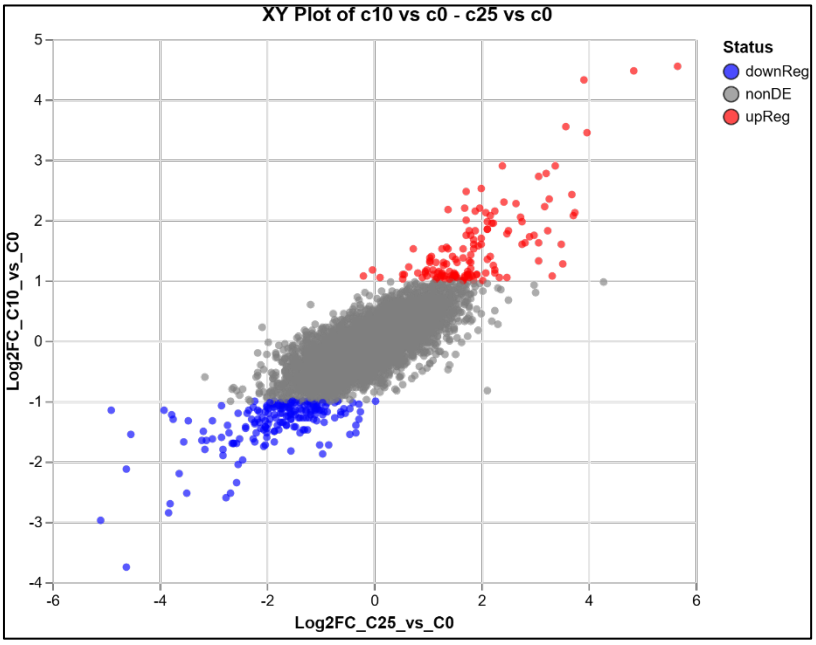

Fig 3. Expressed gene after 0, 10, and 25 µM cardamonin treatment were assayed using RNA-Seq. Upregulated and downregulated expression were shown above

4. **Affected pathway in MDA-MB-231 analyzed by Differentially Expressed Gene (DEGs) and Gene Ontology (GO) Analysis.** RNA-seq and GO analysis shows cardamonin affect cell response to chemical and oxidative stress due to cardamonin treatment.

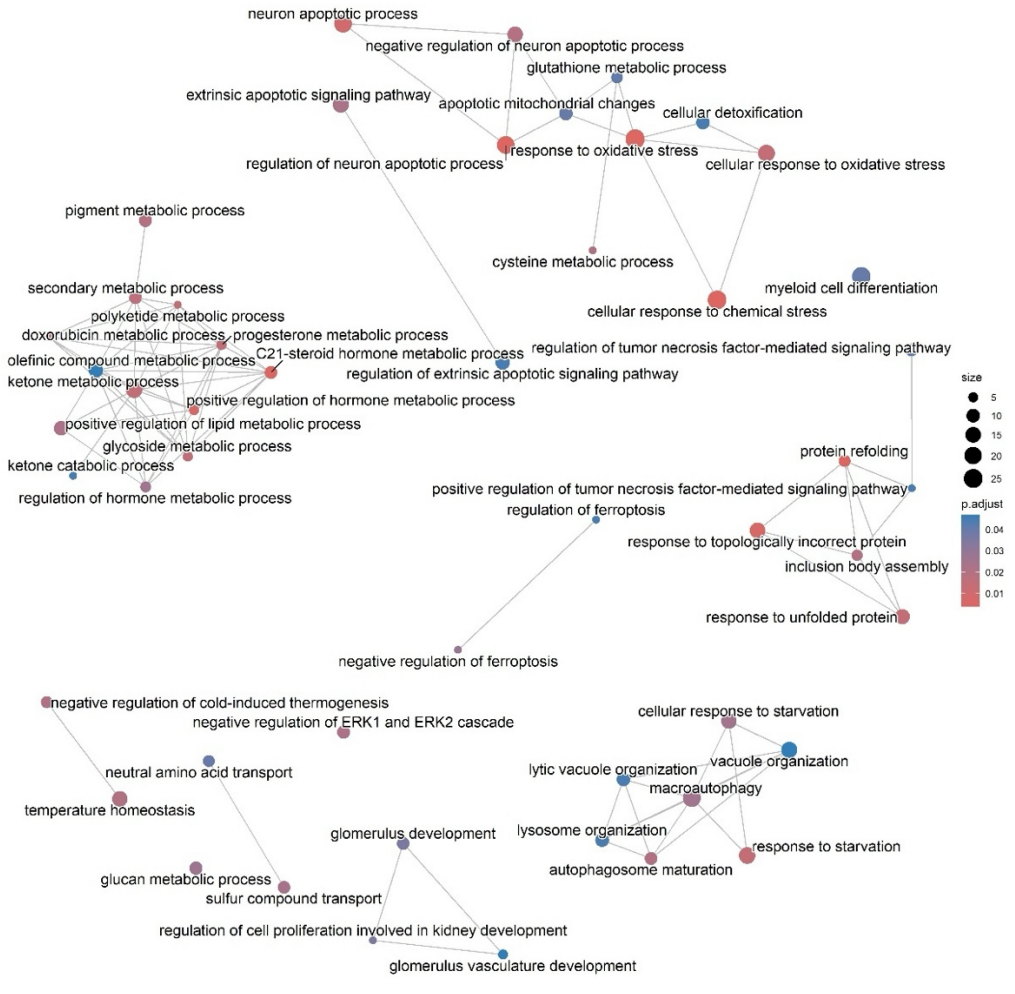

Fig. 4 Emapplot of GO related expression after cardamonin treatment on MDA-MB-231

5. **Effect of cardamonin further analyzed using western blot by testing effect of AP-1 inhibitor T5524.** Protein that involved in ferroptosis, oxidative stress, and cell progression were assayed.

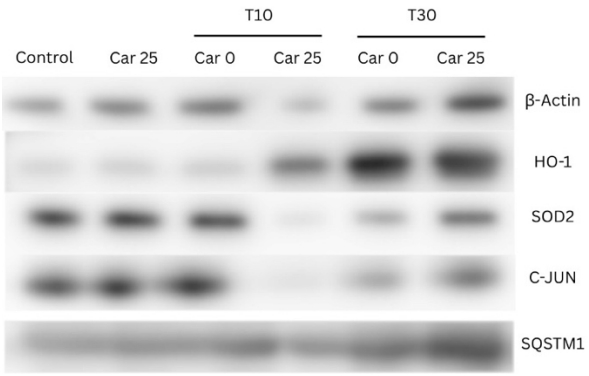

Fig. 5 Effect of AP-1 inhibitor (T10 and T30) and cardamonin (Car 25) on MDA-MB-231 ferroptosis, oxidative stress, and cell progression protein expressions

6. **Validated gene expressions of ferroptosis and oxidative stress through qPCR**. Genes were screened by RNA-seq analysis and amplified through qPCR reaction.

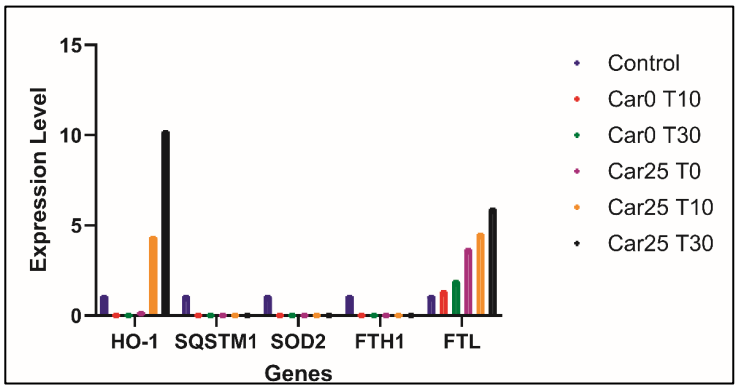

Fig. 6 Gene expression change after combined treatment of cardamonin and AP-1 inhibitor

7. **Iron sensitivity of MDA-MB-231 were checked by employing FACS for apoptosis analysis.** Annexin-V used for staining and checking each quadrant for apoptotic profiling shifts.

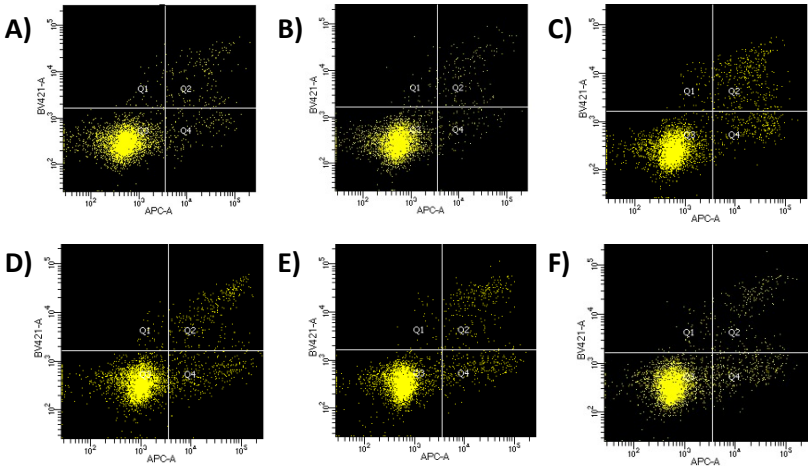

Fig. 7 Apoptotic profiling shift of MDA-MB-231 after A) control treatment; B) ferritin 1 µM treatment; C) AP-1 inhibitor 30 µM treatment; D) cardamonin 25 µM treatment; E) cardamonin 25 µM and ferritin 1 µM treatment; and D) cardamonin 25 µM and AP-1 inhibitor 30 µM treatment.

8. **Proved caradamonin reduce tumor size and weight in tumor bearing mice allograft.** Ten mice allograft were sacrificed after cardamonin dose injection every 2 days and 7 shots in total. The dissected tumors were under analysis and for immunostaining in Pathological Core Lab Institute of Science, University of Tokyo.

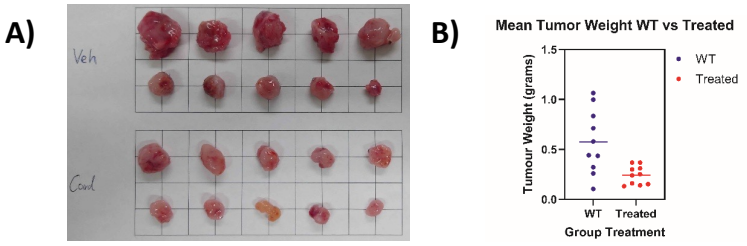

Fig. 8 Dissected tumors from mice allograft A) arranged from biggest to smallest size; and B) tumor weight, untreated (WT) and cardamonin treated (Treated) mice groups

Challenges and Solutions:

1. **Direct Interaction of Ferritin and Cardamonin**

   In the total population of stained events in FACS. There are high occurrences of small particles that suggest it was conjugation of ferritin and cardamonin.

   Solution:

   On this occasion, a proper gating and subset the small particle that shown as events to be more accurate in size of cells.

2. **Western Blot Protein Detection Background**

   Some of western blot imaging were having a high background noise, it might look like stain on PVDF paper to be darker.

   Solution:

   Repeat the process and optimize washing process of antibody. After more than 3-4 repetition, the results validated by reference protein β-actin and determined if the protein were expressed or suppressed due to the treatment.

Research Outputs:

1. **All raw experimental data—including imaging results, flow cytometry files, qPCR, RNA sequencing, 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 manuscript for publications** that in the process of writing and further analysis while on waiting of additional data. These findings on this research period could be valuable for future application and deeper research understanding in cell technology.

Acknowledgement:

We sincerely appreciate and are grateful for this research collaboration between Universitas Indonesia and IMSUT.

We would like to thank Professor Makoto Nakanishi, Dr. Wang Teh-Wei, Sugimoto-san, Sato-san, and all laboratory members for their support, guidance, and warm hospitality during the research project. Our gratitude also goes to the IMSUT for the financial support through the international joint research grant.