Artocarpanone isolated from Artocarpus heterophyllus heartwoods enhances cytotoxic effect of cisplatin against H460 and MCF-7 cell lines

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Abstract. Artocarpus heterophyllus has been used as a folk medicine in Asian countries. Flavonoid possessed a wide-range of therapeutic compounds for the treatment of several diseases including cancer prevention. Artocarpanone is a flavonoid isolated from the heartwoods of A. heterophyllus. This study was undertaken to investigate the interaction of natural compound and commercial anticancer, cisplatin against two cancer cell lines. MTT assay was performed to determine the cytotoxic effect of the combination. Isobologram analysis was used to predict the interaction of combination against cancer cell lines. The effect of this combination was then confirmed using AO/PI staining method. It showed that artocarpanone demonstrated cytotoxic effect against H460 and MCF-7 cell lines with IC50 values of 16.88 and 23.34 µg/mL, respectively. While cisplatin exhibited the cytotoxic effect against H460 and MCF-7 cell lines with IC50 values of 12.95 and 10.29 µg/mL. The combination of these compounds produced the synergistic effect on MCF-7 cell lines with a combination index (CI) of 0.09 and 0.13, respectively. Fluorescence microscope using AO/PI staining showed that the combination caused the morphological change of cancer cell lines and lead to necrosis and apoptosis. This result indicated that artocarpanone may enhance the cytotoxic effect of cisplatin against H460 and MCF-7 cell lines.

1. Introduction
Cancer is still a major cause of death all around the world. Lung cancer is one of the most common cancer found particularly in men. There are 2 types of lung cancer cells which are non-small cell lung cancer and small cell lung cancer cells. About 85% of non-small cell lung cancer cells have occurred in women regardless of smokers or non-smokers [1]. It has been reported 1 million people death worldwide due to this cancer. In the case of breast cancer, it has been known that this cancer is the most common type of cancer found in women, and caused high rate of the mortality and morbidity [2]. Conventional cancer therapy methods including surgery, radiotherapy, and chemotherapy can offer major curative effects against the localized tumors [3,4]. Hence, the use of those therapies may cause the incidence of tumor recurrence and lead to the development of any malignancy as well as drug resistance. Therefore,
it needs to find alternative approach to overcome the problem. One of appealing strategy is the use of combination drug with natural compound that possessed anticancer activity. The interaction of those combination may lead the synergistic effect of commercial drugs toward cancer cell development.

Figure 1. Chemical structure of artocarpanone

Natural product is a source of alternative therapeutic agents that can be used in treating of several diseases. *Artocarpus heterophyllus* has long been used as traditional medicine in many Asian countries. This plant is the main source of many bioactive compounds including flavonoid [5]. Artocarpanone (Figure 1) is flavonoid compound found in the heartwood of this plant. This compound possessed several pharmacological properties including for antibacterial, immunomodulator, and cancer prevention [6-8]. Although anticancer activity of this compound has been reported, however less information available about the effect of artocarpanone in combination with anticancer against cancer cell lines. This study was undertaken to investigate the interaction of artocarpanone in combination with cisplatin against two cancer cell lines including H460 and MCF-7 cell lines.

2. Materials and methods

2.1. Materials and Cell Culture

RPMI-1640, Fetal Bovine Serum, Penicillin-Streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and dimethylsulfoxide (DMSO), and cisplatin were from Sigma Aldrich, St. Louis, MO. Acridine orange (AO) and propopidium iodode (PI) were obtained from Merck. Artocarpanone was isolated from *A. heterophyllus* as previously described [5].

2.2. Cell Culture

In brief, MCF-7 and H460 cell lines (ATC.HTB-177) were purchased from the American Type Culture Collection (ATCC) and cultured in complete medium containing RPMI-1640 media supplemented with 10% v/v fetal bovine serum, and 1% v/v penicillin-streptomycin in 25 cm2 T-Flask (GIBCO, USA). The cells were humidified atmosphere with 5% CO2 at 37°C.

2.3. Cytotoxic assay

The assay was conducted as previously described a slightly modification [9]. Briefly, 5 × 10³ cells/well of H460 and MCF7 were plated in a microplate, incubated for 24 h. Then, compounds (31.25, 15.63, 7.81, 3.91, 1.95 µg/mL) with five different concentrations were added into each well, and incubated for 72 h in 37°C at 5% CO2. Commercial anticancer (cisplatin) as positive control and untreated cell was used as a negative control. After incubation, 20 µL of thiazolyl blue tetrazolium bromide (MTT) reagent (5 mg/mL) was plated into the 96-wells plate, incubated for 4 h. Next, 150 µL of DMSO was added after aspiration of media. The cytotoxicity activity was counted using microplate reader at 570 nm. The study was performed in triplicate and the percentage of inhibition was counted using the following formula:
Percentage inhibition (%) = ((Abs control-Abs treated))/((Abs control) ) × 100%

2.4. Synergistic study
Isobologram analysis was performed to assess the interaction of artocarpanone with cisplatin for its cytotoxic effect against H460 and MCF7 cell lines. The assay was conducted according to previous method with slight modification [9]. In brief, the dose-dependent effects of artocarpanone and fixed concentrations of cisplatin were evaluated using MTT assay. The tested cells were exposed with a mixture of cisplatin and artocarpanone. The combination index (CI) was counted to evaluate their interaction; synergy, antagonism, and additive by using the following formula:

\[ CI = (D_1)/(Da_1) + (D_2)/(Da_2) \]

Da1 = the concentration of artocarpanone alone required to cause certain percentage, D1 = the concentration of artocarpanone in equal percentage when combined with cisplatin. Da2 = the concentration of cisplatin alone required to cause certain percentage, D2 = the concentration of cisplatin in equal percentage when combined with artocarpanone. The CI values were defined as synergy if CI < 1, additive if CI = 1, and antagonist if CI > 1.

2.5. Fluorescence microscope
Morphological changes and apoptosis features of cells were evaluated using fluorescence microscope according to Goh et al., with slight modifications [10]. In brief, H460 and MCF-7 cell lines after treated with compounds were seeded and incubated for 72 h of incubation. Next, the medium was discarded. The treated cells were stained using combination dye containing 10 µL of 1 mg/mL acridine orange (AO) and 10 µL of 1 mg/mL of propidium iodode (PI). The stained cells were observed under inverted fluorescence microscope.

2.6. Statistical analysis
The results were in triplicate and express as the means ± standard error of the mean (SEM). Any significance different was considered where a value of p < 0.05.

3. Results and Discussion
The cytotoxic effect of artocarpanone and cisplatin on H460 and MCF7 cell lines was evaluated using MTT assay. Artocarpanone exhibited strong activity with IC\(_{50}\) values of 16.88 and 23.34 µg/mL, respectively (Table 1). Meanwhile, cisplatin showed the cytotoxic effect against H460 and MCF7 cell lines with IC\(_{50}\) values of 12.96 and 10.29 µg/mL, respectively. These results were confirmed using fluorescence microscope, morphological changes of those tested cell line were observed after treatment with artocarpanone and cisplatin for 72 h. This result indicated that those compounds cause cell damage which in turn inducing cell death. It has been reported that natural products possess anticancer activity. Several reports have confirmed the potency of natural product, particularly flavonoid as anticancer as well as cancer prevention. Artocarpin, a flavonoid compound isolated from Artocarpus sp., demonstrated strong cytotoxic effect against a wide-range of cancer cell lines including T47D, MCF7 and H460 [9]. In addition, artocarpin also exhibited strong anticancer activity in non-small lung cancer cell line by inducing apoptosis through ROS mediated MAPKs and Akt activation [11].

Primary cancer treatments have incorporated both chemotherapeutic agents and ionizing radiation to eliminate the bulk of tumor mass. There is the evidence, however, that the combination of these therapies brings the incidence of tumor relapse that the results in development of drugs resistance in tumor cells. Therefore, alternative approach is needed to overcome this problem [12,13]. One of the strategies is combination two drugs all together to eliminate cancer cells. The use of drug combination may increase the biological activities of those compound. It may be due to different compounds have different target
of action as well as the different compounds may have the same target of action that lead to agonistic activity [14]. In the present study, we combined artocarpanone and cisplatin against two tested cancer cell lines. The interaction of the mixture was then analysed using isobologram analysis. Combination index (CI) represents the interaction of two compounds in combination, CI less than 1 indicates the synergistic effect of compounds in the mixture, on the other hand, CI more than 1 indicates the antagonistic.

Table 1. IC₅₀ values of artocarpanone and cisplatin on H460 and MCF7 cancer cell lines

| No. | Compounds   | IC₅₀ (µg/mL) H460 | IC₅₀ (µg/mL) MCF7 |
|-----|-------------|-------------------|-------------------|
| 1.  | Artocarpanone | 16.88 (55.89 µM) | 23.34 (77.28 µM) |
| 2.  | Cisplatin   | 12.96 (43.18 µM) | 10.29 (34.29 µM) |

Figure 2. Isobologram analysis of H460 (a) and MCF-7 (b) when treated with combination of artocarpanone and cisplatin.

As shown in Figure 2 (a), IC₅₀ value of the mixture artocarpanone and cisplatin against H460 cell lines was below than straight line. The combination index (CI) using isoblogram analysis was then used
to confirm the interaction. It showed that CI of the mixture of artocarpanone and cisplatin was 0.09, and it represented the synergistic effect. This result indicated that artocarpanone may enhance the cytotoxic effect of cisplatin against H460 non-small lung cancer cell line. In the case of cytotoxic effect against MCF7 (Figure 2b), the combination of artocarpanone and cisplatin produced an IC₅₀ which was bellow than straight line. Isobologram analysis was then calculated to confirm the interaction. It found that CI of the mixture was 0.13. This result indicated that a synergistic effect of those compounds in the combination by which artocarpanone may enhance the activity of cisplatin against breast cancer cell line.

![Artocarpanone, Cisplatin, Artocarpanone + Cisplatin](image)

**Figure 3.** Morphological changes of H460 (a) and MCF7 (b) induced by cisplatin, artocarpanone, and cisplatin-artocarpanone in combination. Arrows show (CI) Combination Index, (FL) Fluorescence, (AB) Apoptotic Body, (MB) Membrane Blebbing, (EA) Early Apoptosis, (LA) Late Apoptosis, (CC) Chromatin Condensation, (VI) Viable Cells, (DF) DNA Fragmentation.

In order to confirm any synergistic effect of the combination against H460 and MCF7 cell lines, fluorescence microscope analysis was then performed. Morphological change was observed using dual staining AO/PI method. This AO/PI staining method was performed to observe any morphological changes after treating with compounds such as viable, necrotic, or apoptosis cells [15]. It has been known that AO will bind to live cells via intercalation with DNA and RNA, while PI is able to bind into
DNA and RNA of dead cells. Dual staining AO/PI was relatively stable for image-based viability analysis [16]. The results exhibited that the treatment with combination of artocarpanone and cisplatin (Figure 3 a), the number of H460 cells was lower than treatment with single compound. The morphological observation after treatment with the mixture for 72 h demonstrated that the combination lead to significant changes in apoptotic cell and some membrane blebbing compared to treatment with single compound. On the other hand, treatment with the combination of artocarpanone and cisplatin against MCF7 cell line (Figure 3 b) also indicated significant changes in cell morphology compared to compound when used alone. It can be observed that the mixture of artocarpanone and cisplatin cause some morphological changes in MCF7 cell line including membrane blebbing (MB), early apoptosis (EA), and chromatin condensation (CC).

The result of this study proved the potency of artocarpanone in enhancing cytotoxic effect cisplatin against cancer cell lines. In this combination, low concentration of cisplatin as well as artocarpanone to achieve their therapeutic concentration on cancer cell lines. It is important in order to reduce and avoid drug resistance due to the use of drugs in high concentration. It has been known that cisplatin is platinum anticancer drug in which this compound works via DNA damage signal transduction in cancer cells, also induce apoptosis and necrosis. Meanwhile, the mechanism of action of artocarpanone on cancer cells is not fully understood. The result of this study was consistent with another previous work, it has been recorded that flavonoid deguelin enable to enhance cytotoxic activity of cisplatin on cancer cell lines [17]. In addition, a diterpene compound named triptolide also demonstrated a synergistic effect when combined with cisplatin in inducing cancer cells apoptosis [18]. The mechanism of synergistic interaction, however, needs to be explore through molecular study.

4. Conclusion
It can be concluded that artocarpanone may enhance cytotoxic effect of cisplatin in a synergy interaction. In the combination only required low concentration to afford their therapeutic doses against tested cancer cell lines including H460 non-small lung cancer cell lines and MCF7 cell lines. The results of this study propose the potency of artocarpanone as adjuvant of cisplatin *in vitro*. Nevertheless, further experiments which involved molecular study are needed to justify the mechanism of action in this combination.

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