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RESEARCH ARTICLE

GALLIC ACID IN COMBINATION WITH CISPLATIN TRIGGERED APOPTOSIS IN MCF-7 BREAST CANCER CELLS

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Abstract. Breast cancer emerges as the most prevalent malignancy among women worldwide. While single-agent chemotherapy is commonly used in cancer treatment, combination therapy is generally considered more effective. Cisplatin is effective against a broad spectrum of cancers, though several drawbacks limit its clinical use. Combination treatment has proven to overcome these limitations, which involves pairing conventional drugs with natural compounds. Gallic acid exhibits potent antioxidant and anticancer activities, making it a potential candidate for such combinations. Therefore, this study aims to determine the effect of gallic acid combined with cisplatin on the proliferation of breast cancer cells (MCF-7) through apoptosis induction, characterised by the apoptotic morphology of the treated cells. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine the minimal inhibitory concentration at 50% of cell populations (IC_{50}). The IC_{50} value of gallic acid was combined with cisplatin and applied to the cells to obtain the combination index (CI) value using CompuSyn software. Apoptotic morphology was investigated using AO/PI staining and visualised under a fluorescence microscope. Gallic acid and cisplatin showed potent IC_{50} (< 20 µg/mL) which were 9.765 µg/mL and 0.7869 µg/mL, respectively. A fixed concentration of gallic acid (9.765 µg/mL) combined with cisplatin at concentrations of 0.09836 µg/mL and 0.04918 µg/mL yielded CI values of 0.97423 and 0.65164 indicating a synergistic effect. Apoptotic features were observed morphologically in both single and combined treatments, with a higher number of apoptotic cells noted in the combined treatment, as indicated by bright green and orange fluorescence. This proves the potency of gallic acid in enhancing the antiproliferative effect of cisplatin against MCF-7 cells. In conclusion, a combination of gallic and cisplatin suppressed the MCF-7 cell proliferation through the induction of apoptosis.

Keywords: cisplatin, gallic acid, synergistic effect, apoptosis

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1. INTRODUCTION

Breast cancer has become the most common cancer among women, overtaking lung cancer in terms of incidence, and now stands as the leading cancer burden globally [1]. In 2020, according to International Agency for Research on Cancer, breast cancer is the first most common cancer worldwide with 2.3 million cases (11.7%) from 19 million newly diagnosed cases, followed by lung cancer (11.4%), colorectum cancer (10%) and prostate cancer (7.3%). Meanwhile, in Malaysia, breast cancer incidence accounted for more than a quarter of new cancer cases, with 17.3%, making it the leading cancer, involving 8418 women. The five-year prevalence of breast cancer has accumulated 29453 patients diagnosed with the cancer, and it was estimated that 3503 people died from breast cancer. These data indicate that breast cancer was the most prevalent cancer in Malaysia [2].

Chemotherapy, despite having several limitations, remains a common treatment for breast cancer. One of the drugs commonly used to treat breast cancer is Cisplatin. It is a platinum-based chemotherapeutic agent widely used in the treatment of various cancers, including testicular, kidney, leukemia, head and neck, breast, and ovarian cancers [3]. Chemically known as cis-diamminedichloroplatinum(II), it is a small and structurally simple molecule consisting of a central platinum atom bonded to two amine groups and two chloride ligands [4]. Numerous investigations have indicated that cisplatin induces autophagy, which serves as a protective mechanism against cisplatin-induced apoptosis [5]. Therefore, more effective therapeutic options are required. Thus, the combination treatment is one of the alternative strategies to overcome the problem.

The use of cisplatin-based chemotherapy is facing several challenges such as cell resistance and toxicities. The combination treatment of cisplatin with other types of anticancer agents has been proven to increase the efficacy of the chemotherapy treatment and reduce organ toxicity [6]. Nowadays, natural products have attracted much interest in anticancer research for the development and discovery of new anticancer agents from metabolites of plant sources [7]. The diverse chemical structures and pharmacological effects of natural products serve as an effective component against chemotherapeutic drug resistance.

Gallic acid (3,4,5-trihydroxybenzoic acid) is a phenolic acid that is abundantly present in many plant resources, including apple peels, lemons, green tea, tea leaves, pineapples, strawberries, sumac, bananas, witch hazel, gallnuts, and oak bark. Gallic acid exhibited anticancer activity towards various cancer cells including colon adenocarcinoma, stomach cancer, prostate cancer, breast cancer, and lung cancer [8]. Moreover, the antioxidant and anti-inflammatory effects of gallic acid make it a suitable agent to protect tissues from oxidative stress, which is advantageous for combination with chemotherapeutic drugs [9]. In this study, the cytotoxicity of gallic acid, cisplatin, and their combinations were assessed towards breast cancer cells (MCF-7). The synergistic impact was then determined, and the induction of apoptosis was analysed by confirming the morphological changes of the treated cells using a fluorescence microscope.

2. MATERIALS AND METHODS

2.1 Cell Culture

Breast cancer cell, MCF-7, and Vero, a normal kidney cells, were obtained from stock culture at the School of Health Sciences, Universiti Sultan Zainal Abidin. The cell lines were cultured in a humidified incubator at 37 °C with 5% (v/v) CO_2. Cells were cultured in a complete medium containing the mixture of DMEM, 1% penicillin-streptomycin and 10% FBS. All culture work involving revival, maintenance, passage and cryopreservation of the cells was performed in a sterile biosafety cabinet [10,11].

2.2 Drug Preparation

Gallic acid and cisplatin were purchased from Sigma-Aldrich, USA. 10 mg of gallic acid and cisplatin were dissolved in 1 mL of DMSO to produce stock solutions. It was then aliquoted into 3 tubes and stored at -20 °C for further use. Using serial dilution, working solutions of cisplatin and gallic acid were prepared with concentrations ranging from 1.562 µg/mL to 100 µg/mL [12].

2.3 Cell Viability and IC_{50} Determination

MTT assay was used to perform the cell viability and IC50 determination [13]. In a 96-well plate, 100 µL (5×10^3) of MCF-7 and Vero cells were plated, and incubated in a CO_2 incubator as previously described for 24 hours. Next, cells were observed for 80-90% confluency in each well before treatment. The media used was removed. Following that, the cells were treated with different concentrations of gallic acid and cisplatin ranging from 1.562 µg/mL to 100 µg/mL and incubated in a humidified incubator at 37 °C with 5% CO_2 for 72 hours. A negative control group was set up by adding only 1% DMSO as a vehicle in a complete medium into the respective wells.

The MTT assay solution was prepared by dissolving 5 mg MTT powder in 1 mL PBS producing 5 mg/mL solution. The mixture then was vortexed and filtered using sterile 0.22 µm syringe filter. Without discarding the used media, 20 µL of MTT solution was added into each respective well. The plate was wrapped with aluminium foil to protect from light and incubated in a humidified incubator at 37 °C, 5% CO_2 for 4 hours. Then, the used media was discarded followed by the addition of 100 µL of absolute DMSO into the wells to dissolve the blue formazan crystals formed by viable cells. The optical density (OD) of each well was measured using microplate reader at 570 nm. The percentage of cell viability (%) was calculated using the following formula:

$$Percentage\ of\ cell\ viablity\ (\%) = \frac{Absorbance\ in\ a\ well}{Absorbance\ of\ negative\ control} \times 100 \quad (1)$$

IC_{50} of each compound was determined using a graph of the percentage of viable cells against $\log_{10}$ concentration (µg/mL) of gallic acid and cisplatin. The obtained IC_{50} values for gallic acid and cisplatin then were used for the subsequent combination treatment.

2.4 Combination Treatment

Methods used for combination treatment were adapted from [12]. A volume of 5×10^3 MCF-7 cells were seeded in 96-well plates and incubated for 24 hours. 5 serial concentrations of cisplatin starting range from its IC_{50} value were prepared using two-fold dilution. 100 µL of working solution of these 5 serial concentrations of cisplatin, combined with a fixed concentration of IC_{50} of gallic acid, were added into respective wells (Table 1). These combinations were designed to evaluate the combination of cisplatin that could give high synergistic effects even at its low concentration. In this study, the drug combination aims to enhance the cisplatin cytotoxicity effect and possibly reduce its adverse effects by reducing the concentration. The treated cells were incubated in humidified incubator at 37 °C, 5% CO_2 for 72 hours. Cell viability was determined by MTT assay. 1% DMSO was also added into the respective wells as negative control.

Table 1: Concentration of cisplatin and gallic acid used in combination treatments.

Combination Set	Concentration of cisplatin (µg/mL)	Concentration of gallic acid (µg/mL)
1	0.7869	9.765
2	0.3934	9.765
3	0.1967	9.765
4	0.0983	9.765
5	0.0491	9.765

The combination effect then was calculated using CompuSyn software. The results of the combinations were reported as combination index (CI), a mathematical analysis to quantify the pharmacological interaction of drug combinations. The CI values can be expressed as an antagonism (CI >1), additive (CI =1) and synergism (CI <1). This study aimed to evaluate the combinations that showed synergistic effects as the target is to increase the efficacy of the treatment. In this study, 2 combinations with CI values less than 1 were considered to have synergistic effects. However, only the combination with the lowest concentration of cisplatin and the highest synergistic effect was chosen for further assay.

2.5 AO/PI Staining

AO/PI staining methods were modified from [14]. MCF-7 cells were plated in 8-well cell culture plate with coverslips at the volume of 3×10^4 cells/well, incubated in a humidified incubator CO_2, 5% at 37 °C for 24 hours. The 80-90% confluency of the seeded cells was checked in each well prior to treatment. As for treatments, Gallic acid and cisplatin were added into each well at concentrations of IC_{50} (9.765 µg/mL and 0.7869 µg/mL respectively). The concentration of gallic acid was maintained constant, whereas the concentration of cisplatin was serially diluted to the 5 concentrations (Table 1). The lowest CI index obtained from 0.0491 µg/mL of cisplatin was chosen for combination with a fixed concentration of gallic acid, 9.765 µg/ml then was incubated as previously described.

After 72 hours, the media was discarded, the cells were washed with sterile PBS, trypsinized, and then stop the trypsin reaction by adding 200 µL of complete media, followed by centrifugation for 10 minutes at 1500 rpm. Cells pallets were collected and resuspended in 20 µL of AO solution, followed by 20 µL of PI solution. The mixed solutions were quickly kept in cold condition by placing it in ice box. 10 µL of cell solution was dropped onto a clean slide and mounted with a coverslip and quickly viewed under a fluorescence microscope. The cells were observed for the presence of condensed, fragmented nuclei and apoptotic bodies, that differentiated with green and orange fluorescence. The whole procedure was done without light exposure.

2.6 Statistical Analysis

Statistical analysis was performed by using GraphPad Prism (version 9). Data were presented as mean ± standard deviation (SD) from triplicate experiments (n=3). The combination effects of the compounds were determined using CompuSyn software [12].

3. RESULTS AND DISCUSSION

3.1 Cell Viability Assay

Figure 1 shows the dose-response curve indicating the cytotoxicity activity of gallic acid and cisplatin on MCF-7 cells after 72 hours of incubation. Gallic acid and cisplatin were shown to inhibit the cell growth dose-dependently. Gallic acid exhibited a potent inhibitory effect on MCF-7 cells with IC_{50} value of 9.765 µg/mL. As a positive control and clinical anticancer drug, cisplatin inhibited cell growth at a very low concentration with IC_{50} value of 0.7869 µg/mL as shown in Table 2.

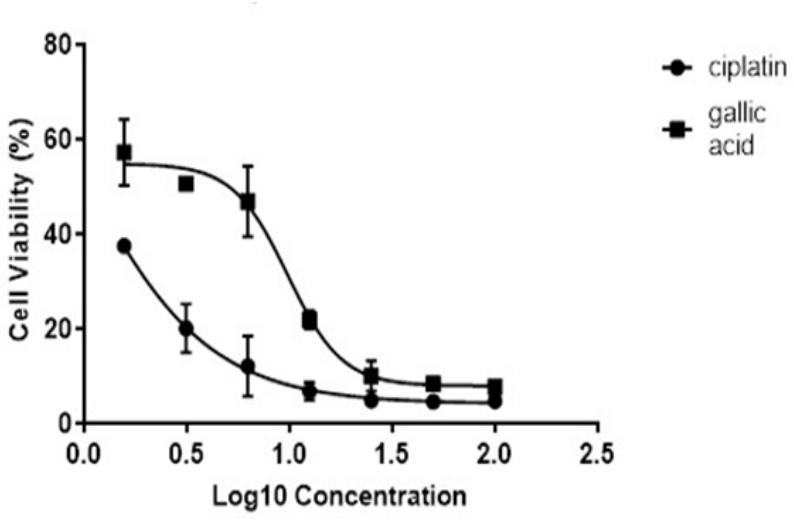

Figure 1: Cell viability of MCF-7 cells treated with cisplatin and gallic acid for 72 hours. All data were presented as the mean ± standard deviation (SD) from triplicate experiments (n=3)

Table 2: IC_{50} values of cisplatin and gallic acid on MCF-7 cells

Compounds	IC_{50} value
Cisplatin	0.7869 µg/mL
Gallic acid	9.765 µg/mL

Additionally, both cisplatin and gallic acid showed dose-dependent growth inhibition on normal kidney cells, Vero (Figure 2). However, the IC_{50} value of gallic acid on Vero cells (31.39 µg/mL), was higher than that for MCF-7 cells, suggesting cytoselective effect on MCF-7 cells. In contrast, cisplatin showed high cytotoxic effect on Vero cells with a very low IC50 value of 0.1640 µg/mL (Table 3).

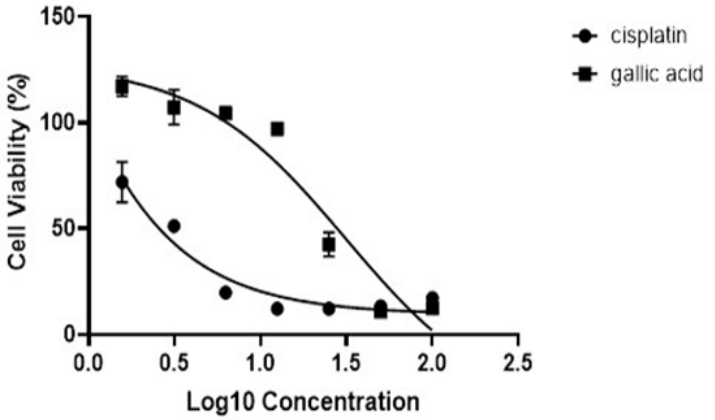

Figure 2: Cell viability of Vero cells treated with cisplatin and gallic acid for 72 hours. All data were presented as the mean ± standard deviation (SD) from triplicate experiments (n=3)

Table 3: IC_{50} values of cisplatin and gallic acid on Vero cells

Compounds	IC_{50} value
Cisplatin	0.1640 µg/mL
Gallic acid	31.39 µg/mL

Gallic acid was reported to act as a selective cytotoxic agent against a variety of tumor cells with low toxicity towards normal cells in numerous studies. Natural gallic acid bearing anticancer effects which are targeted towards molecular mechanisms including apoptosis induction cell cycle arrest, metastasis, invasion and angiogenesis [15]. Cisplatin was used in this study as a combination agent with gallic due to its effective chemotherapeutic drug and is used widely to treat a variety of cancers.

In this current study, a single treatment of gallic acid and cisplatin inhibited the growth of MCF7 cells as the cell viability decreased in a concentration-dependent manner. Both gallic acid and cisplatin showed low IC_{50} values and exhibited potent IC_{50} values (less than 20 µg/ml) [16]. In contrast to cisplatin, gallic acid showed a higher IC_{50} value on Vero cells compared to its IC_{50} on MCF-7 cells indicating its less cytotoxicity on normal cells [17]. The distinct antiproliferative effects of gallic acid seen in cancer and normal cells based on the IC_{50} values of both cells, suggest that gallic acid is more selective towards MCF-7 cells and has lower cytotoxicity effects on normal cells, thus it could be considered as not harmful and safer to normal cells. The pro-oxidant and antioxidant properties of gallic acid might contribute to different cytotoxicity effects on cancer and normal cells [18]. This finding could give new insight into the potential of gallic acid in killing cancer cells and protecting normal cells, thus reducing organ toxicities.

Similarly, gallic acid exhibited a potent cytotoxicity effect on MCF-7 cells with IC_{50} value of 18 µg/mL [19]. Previously, gallic acid and cisplatin had reduced the viability of a non-small lung cancer cell with IC_{50} values of 74.19 µM and 12.13 µM, respectively through the induction of apoptosis by mitochondria-dependent pathway [20]. In another study, gallic acid had shown inhibition of prostate cancer and human oral cancer cells proliferation through apoptosis induction by suppression of DNA repair gene expression and induced DNA damage [21]. Gallic acid also exhibits effective anticancer properties in ovarian cancer and colon cancer [22,23].

3.2 Combination Effect of Cisplatin and Gallic Acid

A non-constant combination treatment was used to evaluate the cytotoxicity of gallic acid and cisplatin on MCF-7 cells. The combination index (CI) (Table 4) obtained was reported as an additive, synergistic and antagonistic effect. The CI values of cisplatin and gallic acid treatments for the first two combination treatments starting with IC_{50} of cisplatin (0.7869 µg/mL and 0.39345 µg/mL) was more than 1, indicating an antagonistic effect. Only for combination of cisplatin and gallic acid at a concentration of 0.19673 µg/mL showed an additive effect and demonstrated CI value as equal to 1. Moreover, the combination of gallic acid and cisplatin at concentrations of 0.09836 µg/mL and 0.04918 µg/mL, respectively, has proven a CI value of below 1, indicating a synergistic effect. The concentration of cisplatin at 0.04918 µg/mL was chosen as the best combination and was used for the treatment in further assay.

Table 4: Combination index of cisplatin combined with gallic towards MCF-7 cells

Dose cisplatin (µg/mL)	Dose gallic acid (µg/mL)	Effect	Combination Index	Combination effect
0.7869	9.765	0.68520	1.94065	Antagonistic
0.39345	9.765	0.73796	1.12954	Antagonistic
0.19673	9.765	0.73875	1.01456	Additive
0.09836	9.765	0.73659	0.97423	Synergistic
0.04918	9.765	0.78872	0.65164	Synergistic

In cancer treatment, combining anticancer drugs with phytochemicals may provide synergistic, antagonistic, or additive effect outcomes. The advantages of synergism are increasing therapy efficacy while also lowering the dosage of the compound administered, thus effectively reducing the toxicity of the drugs [24]. The current study examines the combination effect of cisplatin and gallic acid on MCF-

7 cells, with variations based on different concentrations of cisplatin. The combination treatment manages to synergize at the lowest CI value obviously.

In a study on non-small cell lung carcinoma, gallic acid enhanced cisplatin's effects by decreasing proliferation, increasing the rate of apoptosis and inducing morphological changes. The elevation of Bax protein expression and the decrease of Bcl-2 and JAK/STAT3 protein expression verified apoptosis [25]. Apart from that, the combination exhibited synergistic effects by modulating apoptosis-related molecule activation and inducing pro-apoptotic mediators. On top of that, gallic acid enhanced cisplatin antitumor activity via ROS-dependent mitochondrial apoptotic pathway on small lung cancer cells. Gallic acid and cisplatin combination also exhibited synergistic reactions towards human cervical cancer cells [12].

Lauryl gallate, a derivative of gallic acid, has been shown in another investigation on breast cancer to be more effective than tamoxifen at suppressing breast cancer cell proliferation through BCL-2 gene downregulation [26]. Moreover, gallic acid from green tea, combined with an anticancer agent, arsenic trioxide, has a significant synergistic effect on breast cancer chemotherapy treatment through the induction of apoptosis involving BCL-2 family members [27].

In another study, gallic acid combined with paclitaxel and carboplatin shows the potential of increased apoptotic effect towards MCF-7. The effect of cell apoptosis was triggered through upregulation of Bax, p53 and CASP-3 protein expression, whereas Bcl-2 protein was downregulated where the combination treatment induced the Pre-G1 apoptosis and arrested and progressed to G2/M phase [28].

3.3 Morphological Observation of Apoptotic Cells (AO/PI staining)

AO/PI staining provides visualization of viable cells and non-viable cells by green and orange, fluorescent dyes. Viable cells are demonstrated as green and uniform nuclei, while cells with apoptosis are demonstrated as irregular bright green nuclei or irregular orange fluorescence. After 72 hours of treatment, MCF-7 cells exhibited many apoptosis characteristics compared to the control. The typical features of apoptotic cells such as irregular or fragmented nucleus, nuclear condensation and cell blebbing presented in green and orange dye observed in the treated cell groups (Figure 3(A) to (C)). Meanwhile in untreated control cell (D), the cells maintained an oval shape and the nucleus was intact and stained uniformly with green fluorescent.

Morphological changes are one of the hallmarks of apoptosis. Distinctive morphological changes, including DNA fragmentation, chromatin condensation, appearance of apoptotic bodies, membrane blebbing and cell shrinkage, will lead to cellular destruction. On top of that, apoptosis involves intrinsic and extrinsic pathways which depend on the molecular target of any type of bioactive compounds. Those pathways will trigger immune surveillance mechanisms, cellular stress, and DNA damage in the cell. The success of clinical cancer therapy highly depends on the competency of anticancer compounds in the elimination of cancerous cells through programmed cell death or apoptosis [28].

In this study, AO/PI staining revealed various morphological characteristics of apoptosis in the treated MCF-7 cells with single and combination treatment. A significant presence of apoptotic cells post-treatment proved the effectiveness of treatments through apoptosis induction. Cells exhibited morphological characteristics such as irregular and fragmented nuclei, loss of cellular membrane integrity, nuclear condensation and damaged cell membrane shown by green and orange color from AO/PI staining. The interpretation of apoptotic cells stained with AO/PI staining is based on the fluorescence light emitted and nucleus morphology. The viable untreated cells have a uniform shape, less green-stained nucleus, with green cytoplasm and intact plasma membrane. AO/PI staining is useful in categorizing the cells into early or late apoptosis. Early apoptosis is demonstrated by the presence of chromatin condensation in the nucleus with bright green mixed with orange areas, while late apoptosis

is shown by the presence of condensed chromatin in the nucleus with dense orange mixed with red areas whereas necrosis is determined as red areas with burst nucleus [29].

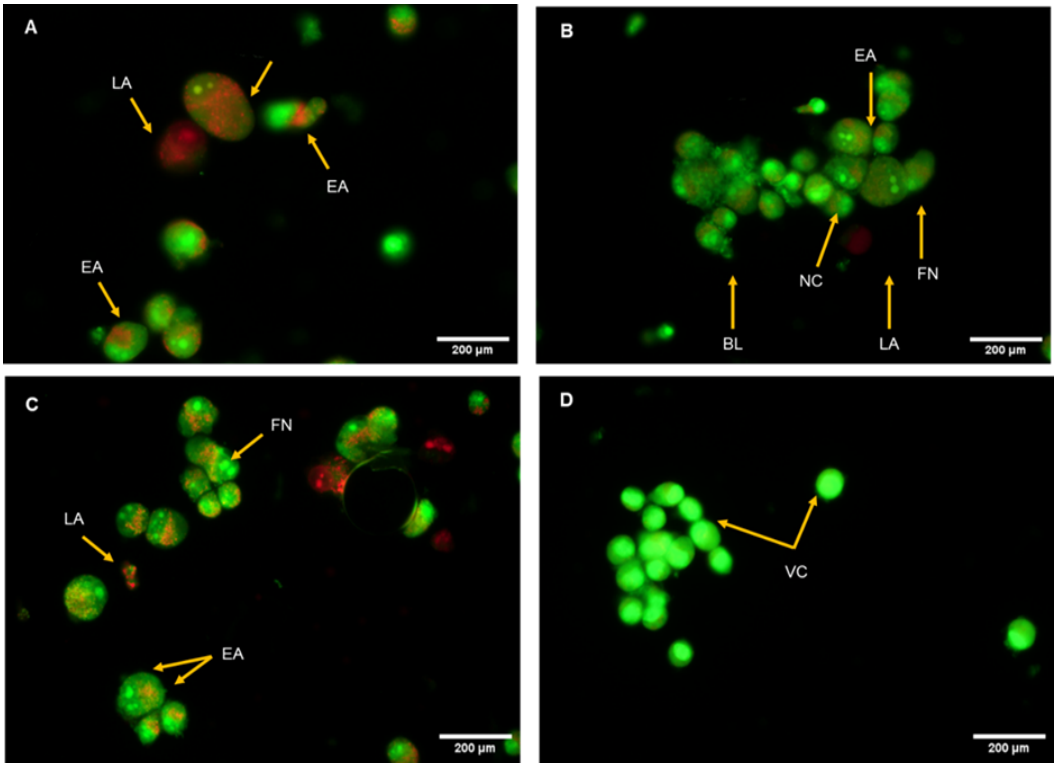

Figure 3: The morphology of apoptotic cells with criteria of early apoptotic (EA) and late apoptotic (LA) with fragmented nucleus (FN), nuclear condensation (NC) and cell blebbing (BL) were presented in (A) cisplatin (0.7869 µg/mL), (B) gallic acid (9.765 µg/mL), and (C) combination of cisplatin and gallic acid groups, stained by AO/PI staining with bright green and orange fluorescence. The oval shape and intact nucleus were maintained in (D) untreated control cell which represented the viable cell (VC). The cells were observed and captured under 400x magnification

This result indicated the treatment of MCF-7 cells with cisplatin, gallic acid and the cisplatin combined with gallic acid triggers cell apoptosis. More cells were stained with bright green and orange fluorescence in the combination treatment group indicating there were more apoptotic cells suggesting their enhanced cytotoxicity effects on MCF-7 cells. Gallic acid exerts its cytotoxic effect mainly by modulating the antioxidant/pro-oxidant balance. Reactive oxygen species (ROS)-induced carcinogenesis can be controlled by gallic acid by increasing the activity of superoxide dismutase, glutathione reductase, catalase and glutathione peroxidase. On the other hand, gallic acid triggers excessive ROS production that leads to cell apoptosis [30]. It has been revealed that small-cell lung cancer (SCLC) cells treated with cisplatin combined with gallic acid showed the highest level of intracellular ROS [31]. Moreover, various studies reported that gallic acid induces apoptosis through the expression of tumor suppressor gene (p53), pro-apoptotic protein (Bax) and anti-apoptotic protein (Bcl-2) [28]. Additionally, gallic acid has shown high activation of caspase activity through the upregulation of the ratio of cleaved caspase-3/pro-caspase-3 and cleaved caspase-9/pro-caspase-9, which indicates the ability of gallic acid to inhibit cell proliferation and promote the cell apoptosis [32].

4. CONCLUSIONS

In conclusion, gallic acid alone and in combination with cisplatin demonstrated inhibitory growth effect towards MCF-7 at lower IC_{50} value (9.765 µg/mL) compared to normal cells, Vero with IC50 value of 31.39 µg/mL. At this stage, it proved cytoselective effect towards breast cancer cells.

Additionally, the combination of gallic acid and cisplatin at 0.09836 µg/mL and 0.04918 µg/mL synergistically suppressed the proliferation of MCF-7 cells. Cell death exhibited apoptotic features such as irregular and fragmentation of nucleus, loss of cellular membrane integrity, nuclear condensation and membrane damage. This finding may support that gallic acid is a great potential natural product candidate to be combined with cisplatin in elucidating its mechanism of action on breast cancer cells through other several in-vitro and in-vivo studies.

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Author Contributions

Hasmah Abdullah: Contributed to the experimental design, wrote, revised, and edited the paper, guided and supervised the entire experimental process, and acquired funding for research. Norlida Mamat: Performed experiments, analysed the data, reviewed and edited the manuscript. Nur Najihah Mahyun: Performed the experiments and conducted data analysis, as well as a literature review and collation. Norhazilah Muhamad: Gave technical advice, reviewed, and validated the methods used. Hermizi Hapidin: Reviewed and confirmed the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Disclosure of Conflict of Interest

The authors have no disclosures to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards

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