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Combination effect of cisplatin and gallic acid on apoptosis and antioxidant enzymes level in cervical cancer (HeLa) cells

Norlida Mamat[1,2], Hasmah Abdullah[2*], Hermizi Hapidin[2], Noor Fatmawati Mokhtar[3]

[1]Faculty of Health Sciences, Gong Badak Campus, Universiti Sultan Zainal Abidin, Terengganu, Malaysia.
[2]School of Health Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
[3]Institute for Research in Molecular Medicine (INFORMM), Health Campus, Universiti Sains Malaysia, Kubang Kerian, Malaysia.

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ABSTRACT

Gallic acid (GA) has demonstrated an antiproliferative effect on different tumor cells. This study was carried out to determine the apoptosis event, oxidative stress, and the related-antioxidant enzyme activity in HeLa cells treated with cisplatin, GA, and their combination. The morphology and percentage of apoptotic cells were evaluated using Hoechst staining and annexin V/PI assay. The intracellular reactive oxygen species (ROS) and activity of antioxidant enzymes were measured using 2′,7′-dichlorofluorescin diacetate assay and spectrophotometric method. Our findings showed that the percentages of early apoptotic cells in cells treated with cisplatin (CIS) (8.8 ± 1.75%), GA (9.6 ± 0.148%), and their combination (12.83 ± 1.44%) were significantly higher than the control group (4.67 ± 0.14%). Moreover, intracellular ROS in HeLa cells treated with CIS (122.7 ± 9.45%), GA (124.7 ± 4.94%), and the combination (137.1 ± 8.99%) were significantly increased compared to the control group. The combination treatment also decreased the activity of superoxide dismutase and catalase. Our findings suggest that in HeLa cells, GA is combined with cisplatin-induced apoptosis by increasing ROS and reducing antioxidant enzyme levels. This result will support further research related to the combination of CIS and GA as a potential cervical cancer chemotherapeutic agent.

INTRODUCTION

Cervical cancer is ranked fourth globally as the most frequently diagnosed cancer in women. Approximately 569,847 new cases were diagnosed in 2018. Other than that, cervical cancer represents 7.5% of cancer deaths accounting for 311,365 deaths worldwide (Bray *et al*., 2018). The incidence and mortality rates of cervical cancer vary among different regions. The majority of incidence cases are attributed to the African region. The highest cervical cancer rates are reported in Eastern Africa and lowest in Western Asia. In the Southeast Asian region, cervical cancer is the second most prevalent cancer in women. It is also the leading cause of death related to cancer among women in low- and middle-income countries (LMICs) (Shrestha *et al*., 2018). In 2015, an estimated 90% of the 270,000 cervical cancer deaths occurred in LMICs where the mortality rate is 18 times greater when compared to developed countries (Cohen *et al*., 2019). A high-risk type of human papillomavirus (HPV) infection is suggested as the most etiological factor for cervical cancer. Approximately half of the cervical cancer cases are associated with HPV type 16 and 10%–20% of the cases involve HPV type 18 (Narisawa-saito and Kiyono, 2007).

There are several treatments for cervical cancers including surgery, radiotherapy, and chemotherapy. Chemotherapy is categorized as a systemic therapy that interrupts cancer cell division, leading to cell death (Smith and Prewett, 2017). Cisplatin (CIS) or cisplatinum, also called *cis*-diamminedichloroplatinum (II), is an effective chemotherapeutic medication used to treat a number of cancers, such as bladder cancer, cervical cancer, ovarian cancer, head and neck (Petrovic and Todorovic, 2016), testicular cancer, and non-small-cell lung cancer (Purena *et al*., 2018). Despite its effectiveness, CIS treatment causes organ toxicity including nephrotoxicity, hepatotoxicity, and ototoxicity. In addition, the patients may develop chemoresistance that

*Corresponding Author
Hasmah Abdullah, School of Health Sciences, Health Campus, Universiti Sains Malaysia, Kubang Kerian, Malaysia.
E-mail: hasmahab @ usm.my

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becomes its limitation in cancer treatment (Sun *et al*., 2019). Because of these problems, CIS is used in combination with other chemotherapeutic drugs as novel therapeutic strategies in cancer therapy (Dasari and Tchounwou, 2014).

Recently, phytochemical compounds derived from natural plants have attracted greater interest among researchers due to their beneficial effects on human health, safety, and cost-effectiveness (Bacanli *et al*., 2017). They are widely distributed in plant-derived fruits, beverages, and herbal remedies. To date, more than 10,000 phytochemicals have been identified; many yet remain unknown and need to be explored (Russo *et al*., 2010). They contain the unique properties that enabled them to be effective anti-inflammatory and anticancer agents. Interestingly, they also synergistically enhance the effect of anticancer drugs and lessen the harmful effects (Tuorkey, 2015).

Gallic acid (3,4,5-trihydroxybenzoic acid, GA) is the ubiquitous phenolic compound distributed in various plants (Figure 1). It is the commonest phenolic acid from the hydroxybenzoic acid group (Fantini *et al*., 2015). GA is found in abundance in red wine, green tea, strawberries, pineapples, bananas, lemons, gallnuts, sumac (Asci *et al*., 2017), mango, honey, vegetables, and beverages (Badhani *et al*., 2015). This compound is considered as one of the most biologically active phenolic compounds. Its antioxidant activity has been revealed previously in numerous studies (Karamac *et al*., 2005). GA also exhibited other biological activities including antimutagenic, antiallergic, anti-inflammatory, antiviral, antibacterial, and antiarteriosclerosis (Choubey *et al*., 2015). However, the attention of GA is given mostly to its antitumor activity. Several studies have reported that GA demonstrated an antiproliferative effect toward various cancer cells, as well as leukemia cells (Locatelli *et al*., 2008), melanoma cells (Lo *et al*., 2010), glioma cells (Lu *et al*., 2010), prostate cancer cells (Heidarian *et al*., 2017), and HeLa cells (Zhao and Hu, 2013). Interestingly, normal cells such as fibroblasts and endothelial cells are least sensitive to GA, indicating that GA's antiproliferative effect is only selective toward cancer cells without causing harm to normal cells (Nam *et al*., 2016).

Recently, we found that CIS synergistically inhibited the proliferation of HeLa cells in combination with GA. Thus, this present study aimed to evaluate morphology and apoptosis event induced by CIS and GA alone and their combination CIS–GA, as well as the intracellular reactive oxygen species (ROS) and related-antioxidant enzyme activity.

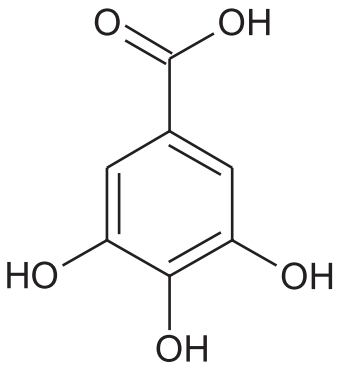

Figure 1. Chemical structure of GA.

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, streptomycin and penicillin (Gibco Thermo scientific), GA and CIS (Sigma Chemical Co.), 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Abcam), catalase and superoxide dismutase assay kit (Cayman Chemical), Hoechst 33342 (Sigma), and Annexin V-Fluorescein isothiocyanate (V-FITC) apoptosis kit (BD Biosciences) were used.

Cell culture

Cervical cancer cells (HeLa) obtained from the American Type Culture Collection were grown in a humidified atmosphere under the condition of 37°C with 5% (*v/v*) CO_2. DMEM complemented with 10% FBS and 1% penicillin–streptomycin was used to grow the cells.

Cell treatment

HeLa cells were treated with CIS and GA at a concentration of IC_{50} and the combination of CIS and GA (CIS–GA) (66.73% of cell inhibition) was chosen based on a previous study (Mamat *et al*., 2020). The concentrations are listed in Table 1.

Hoechst staining

Morphological changes of apoptotic nuclei in HeLa-treated cells were evaluated using Hoechst staining (Hoechst 33342). 2×10^5 of HeLa cells per well were seeded on a coverslip in six-well plates. After 24 hours, cells underwent the single and combination treatment. After 72 hours of incubation, the cells were washed with sterile phosphate buffer saline (PBS). The cold methanol was added to fix the cells for 15 minutes at room temperature, followed by the addition of 0.2% Triton-X-100 solution for 2 minutes. The coverslip then was washed thrice with PBS and incubated with Hoechst staining solution for 30 minutes. The coverslip was covered with aluminum foil to protect from light. The coverslip was washed thrice again with PBS. The presence of apoptotic, condensed, and fragmented nuclei was determined by using a fluorescence microscope (Olympus, Japan).

Annexin V/PI apoptosis assay

The apoptosis of HeLa cells was quantitatively calculated by flow cytometry using the manufacturer's procedure (BD Biosciences, San Jose, CA). HeLa cells were harvested and washed with PBS. Approximately 1×10^6 cells/ml were resuspended in 1× binding buffer and then 100 µl was transferred in a 5 ml flow cytometry tube. 5 µl of FITC annexin and 5 µl of propidium iodide (PI) were added to the cell suspension and

Table 1. The concentrations of single compounds and the combination used to treat HeLa cells.

Compound	Concentration
CIS (IC_{50})	8.04`µg/ml
GA (IC_{50})	13.44 µg/ml
Combination (CIS–GA)	
CIS	0.51 µg/ml
GA	13.44 µg/ml

were vortexed thoroughly. After 15 minutes of incubation at room temperature in the dark, 400 µl of binding buffer was added to the stained cell and was then analyzed immediately with Becton Fluorescence-activated cell sorting (FACSC) flow cytometer (Becton Dickinson Corporation) within 1 hour. Unstained and stained untreated control cells were used for gating fluorescence parameters and 10,000 events were accumulated per sample.

Measurement of intracellular ROS

In brief, HeLa cells (3×10^3 cells per well) were seeded in 96-well plates. After 24 hours, cells were treated with 100 µl of GA, CIS, and a combination of GA–CIS. The treated cells were incubated for 72 hours in 37°C CO_2 incubator. 30–45 minutes prior to completion of the treatment, 100 µl of DCFH-DA was added to each well at a final concentration 20 µM and incubated in the dark. The fluorescence intensity was immediately read using a microplate reader at an excitation wavelength of 485 nm and at an emission wavelength of 535 nm according to the protocol described by the manufacturer's protocol (Abcam, Cambridge, UK). The percentage of intracellular ROS is based on the following formula:

$$\frac{\text{Fluorescence intensity of treated samples}}{\text{Fluorescence intensity of control untreated samples}} \times 100$$

Measurement of superoxide dismutase (SOD) activity

SOD activity was measured with Cayman's superoxide dismutase assay kit (Cayman Chemical, Ann Arbor, MI) using a tetrazolium salt to detect xanthine oxidase and hypoxanthine oxidase-generated superoxide radicals. The amount of enzyme required to produce 50% dismutation of the superoxide radical is described as one unit of SOD. The measurement of SOD activity was carried out using a plate reader at 440–460 nm. Determinations were performed in triplicates.

Measurement of catalase activity

Catalase activity was determined with Cayman's catalase assay kit (Cayman Chemical, Ann Arbor, MI). Briefly, this procedure involves the reaction of the enzyme with methanol at an optimum concentration of H_2O_2. The measurement of formaldehyde production was carried out colorimetrically using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole as the chromogenic reagent. The absorbance of the sample was read at 540 nm using a plate reader. The samples were assayed in triplicate.

Statistical analysis

Statistical analysis was carried out with GraphPad Prism (version 7) statistical software. Experiments were conducted in triplicate and all data were presented as mean ± SD. One-way analysis of variance with Tukey's *post-hoc* test was used to determine the statistical significance of the data. A $p < 0.05$ was considered to be significant.

RESULTS

Apoptosis induction in HeLa cells

After 72 hours of treatment with single and combination compounds, the observation made showed significant morphological alterations in the treated HeLa cell's nuclei as compared to untreated control. As shown in Figure 2A–D, the control untreated cells appeared in an intact oval shape and the nuclei showed less bluish-white fluorescent (Ma *et al*., 2014) denoting regular, intact cells. Cells treated with CIS and GA alone (Figure 2B–2C) exhibited typical features of apoptosis, such as cell shrinkage, condensed and fragmented chromatin, segregated bodies, the formation of apoptotic bodies, and cell decrement with brighter fluorescence compared to control. Interestingly, the combination of CIS–GA (Figure 2D) exhibited clear apoptotic nuclei with highly condensed or fragmented chromatin that was consistently an intense fluorescent.

To further quantify the proportion of apoptotic cells induced by CIS, GA, and CIS–GA, an annexin V-FITC/PI apoptosis assay was performed and quantified using a flow cytometer. Cells were evaluated for the early and late stages of apoptosis. As shown in Figure 3, quadrant 4 (Q4) represents the early apoptotic cells (positive annexin V-FITC) and Q2 represents the late apoptotic and necrotic cells (positive annexin V-FITC and PI) while viable cells were sorted in the lower left quadrant (Q3). The fully apoptotic cells are the total cells in quadrant 2 and quadrant 4. In this study, the number of viable cells treated with CIS (83.15 ± 0.98%), GA (81.66 ± 0.43%), and CIS–GA (76.77 ± 2.65%) was significantly reduced when compared to the control group (87.98 ± 1.73%). The percentages of early apoptotic cells in cells treated with CIS (8.8 ± 1.75%), GA (9.6 ± 0.148%), and CIS–GA (12.83 ± 1.44%) were also significantly higher than the control group (4.67 ± 0.14%). However, there was no significant difference in late apoptotic and necrotic cells between these groups (Figure 4). These data are aligned with the morphological analysis that showed more apoptosis characteristics in cells treated with the combination compared to the single treatment and untreated group.

Intracellular ROS levels in HeLa cells

Intracellular ROS was measured using fluorometric analysis using DCFH-DA as a probe. As shown in Figure 5, intracellular ROS in HeLa cells treated with CIS, GA, and the combination were significantly increased compared to the control group. Moreover, treatment with CIS–GA showed a higher ROS level compared to CIS and GA alone.

Effect of GA and GA/CIS on SOD and catalase levels in HeLa cells

In order to evaluate the effect of treatment on antioxidant enzymes in HeLa cells, SOD and catalase levels were measured. The activity of SOD (Figure 6) and catalase (Figure 7) was significantly reduced in cells treated with CIS and GA as compared to the control. The level of SOD and catalase in CIS–GA group was slightly lower than control even it was not significant.

DISCUSSION

As cancer has now become the leading cause of death worldwide, there is an urgent need to develop a new safer and efficient treatment. Thus, chemotherapy drug combined with a natural product is one of the alternatives to enhance the effectiveness of the chemotherapeutic drug with a lesser side effect. In this study, the apoptosis and antioxidant enzyme levels in HeLa cells treated with GA alone and combined with CIS were investigated. To the best of our knowledge, there is no study reporting the effect of combination of GA and CIS in inducing apoptosis in HeLa cells.

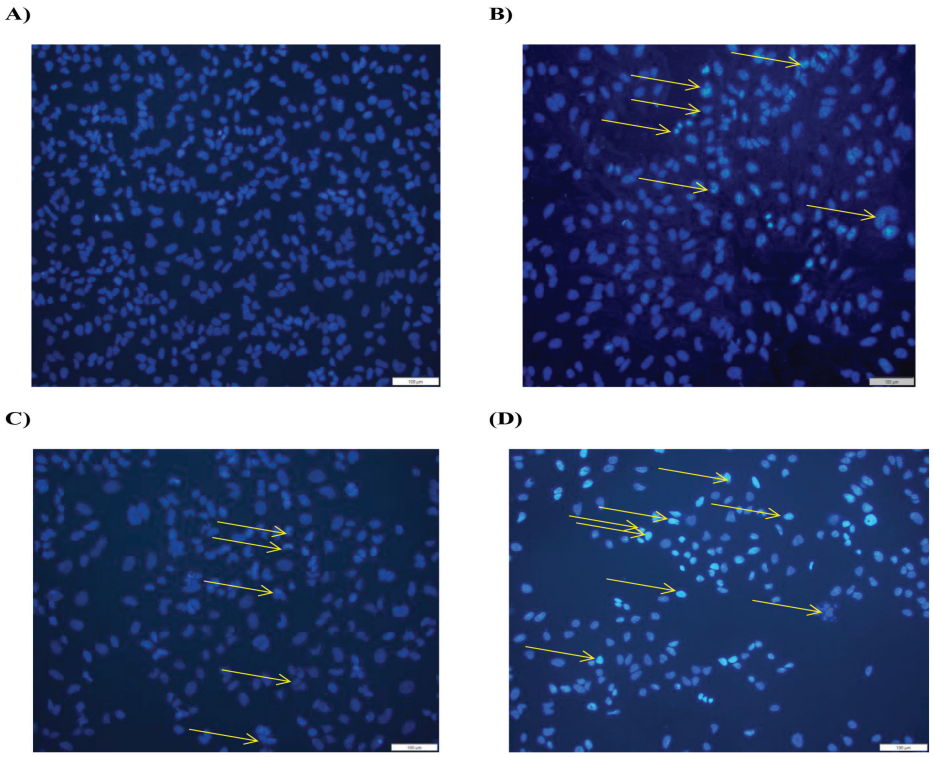

Figure 2. Morphological changes observed using Hoescht (33342) stain. HeLa cells were treated with (A) control cells, (B) cisplatin, (C) GA, and (D) CIS–GA for 72 hours. Photographs were taken under a fluorescence microscope (20× original magnification). The arrows indicate the cells with apoptosis characteristics.

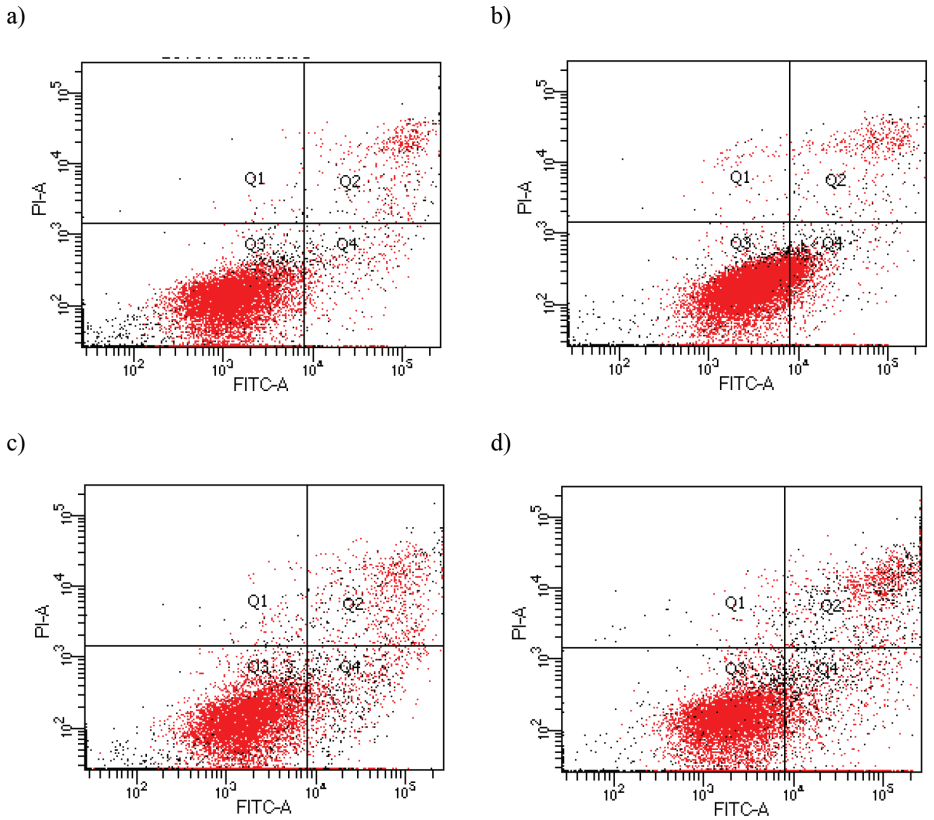

Figure 3. Apoptosis analysis of control (a) and treated HeLa cells with (b) CIS, (c) GA, and (d) CIS–GA using annexin V-FITC and PI stain. Cells were sorted into late apoptotic and necrotic (Q2), viable (Q3), and early apoptotic stages (Q4).

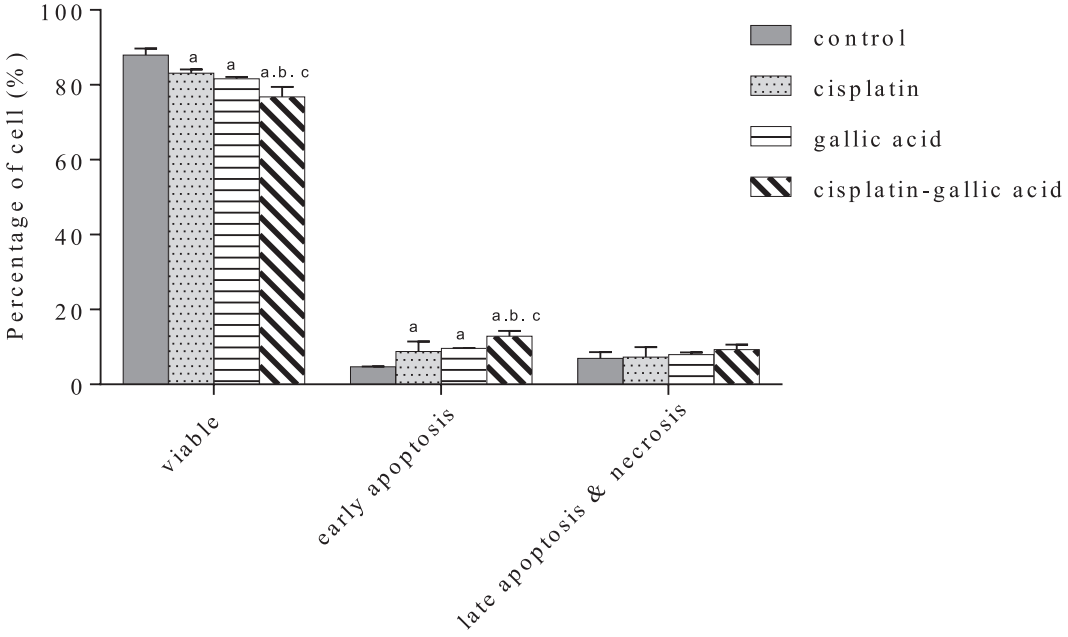

Figure 4. Flow cytometry analysis of untreated and treated HeLa cells using annexin V-FITC and PI stain. Results are presented as mean ± SD from three separate experiments. (a) $p < 0.05$ as compared to the control group. (b) $p < 0.05$ as compared to cisplatin; (c) $p < 0.05$ as compared to GA.

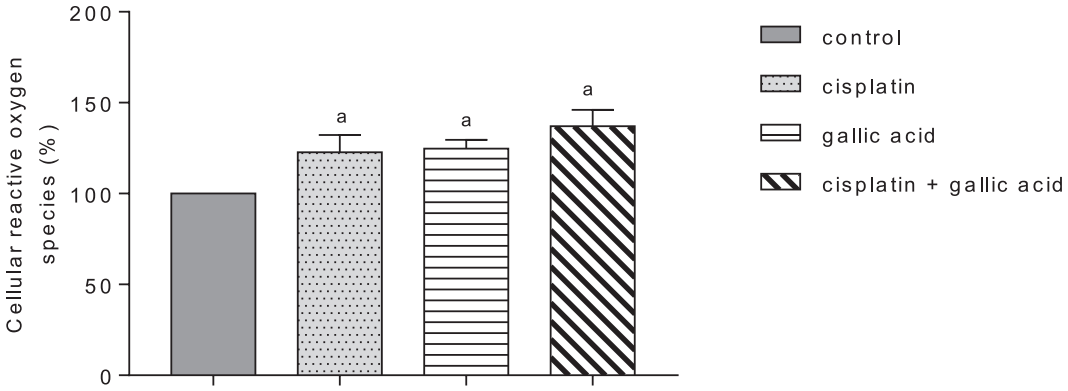

Figure 5. Effect of CIS, GA, and CIS–GA on intracellular ROS levels in HeLa cells. (a) $p < 0.05$ as compared to the control.

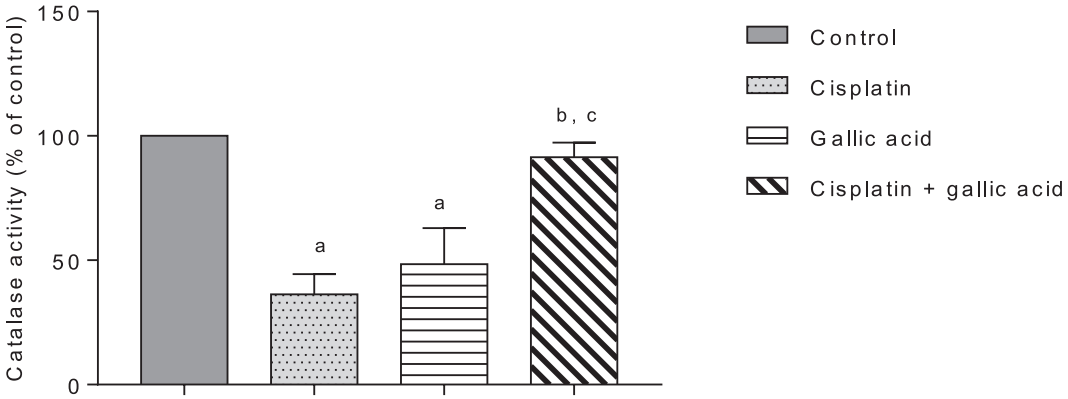

Figure 6. Effect of CIS, GA, and the CIS–GA on activity of catalase in HeLa cells. (a) $p < 0.05$ as compared to the control; (b) $p < 0.05$ as compared to cisplatin; (c) $p < 0.05$ as compared to GA.

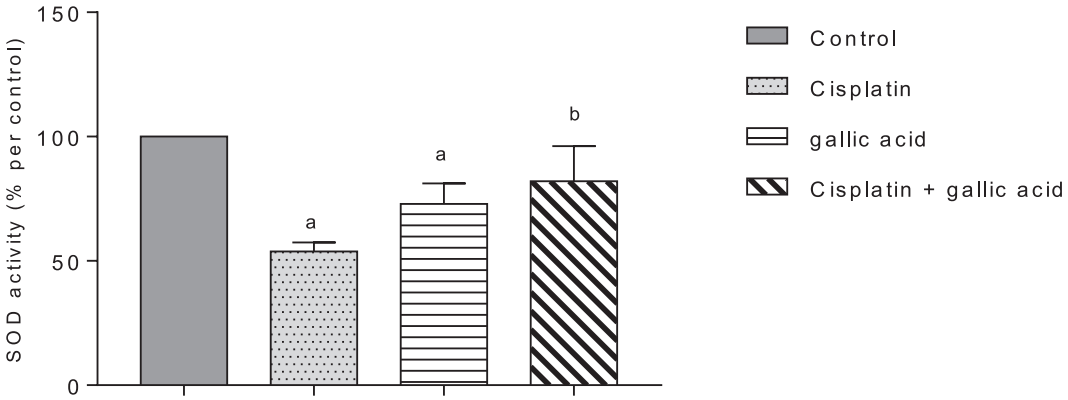

Figure 7. Effect of CIS, GA, and CIS–GA on activity of SOD in HeLa cells. a: $p < 0.05$ as compared to control; b: $p < 0.05$ as compared to cisplatin.

However, the antiproliferative effect and apoptosis induced by GA alone in HeLa cells have been extensively studied (You *et al*., 2010; Zhao and Hu, 2013).

Apoptosis is known as the programmed cell death in regulating cell growth homeostasis. There are two main pathways involved in apoptosis events, namely the extrinsic (death receptor) pathway and the intrinsic (mitochondria) pathway. The imbalance between cell death and cell proliferation may lead to pathological disease or cancer. Thus, the induction of apoptosis is one of the strategies currently used in cancer therapy. Cell morphological changes may occur during apoptosis such as cell shrinkage, chromatin condensation, membrane blebbing, and apoptotic bodies (Elmore, 2007). Apoptosis could be evaluated by observing the morphology of apoptotic cells stained with Hoechst, while the number and stages of apoptosis could be determined by flow cytometry (Yang *et al*., 2014). In the apoptotic cell, phosphatidylserine which is normally located on the cytosolic surface of the plasma membrane was translocated to the outer surface (Lee *et al*., 2011). Annexin V that has a high affinity to phospholipid phosphatidylserine (PS) will bind to the cells that have an exposed PS. Since the externalization of PS occurs at the early stages of apoptosis, FITC annexin V could be used to identify the early stages of apoptosis. In this study, we have found that GA alone induces apoptosis in HeLa cells which is in line with previous studies. Also, more HeLa cells in the combined treatment group exhibited the morphological characteristics of apoptosis as compared to the single treatment group. The result of flow cytometry analysis also showed that the combination treatment demonstrated the highest percentage of apoptotic cells. These results suggested that CIS-GA may induce cell death via apoptosis mechanism.

ROS such as superoxide anion, hydrogen peroxide, and singlet oxygen are required in various biological processes of living organisms. At a low level, ROS acts as messengers in cell signaling and play a role in maintaining homeostasis. However, excessive ROS can cause oxidative stress which results in many pathological processes including cancers (Wang *et al*., 2016). In cancer cells, ROS are considered oncogenic, as they play role in promoting cancer initiation, progression, and spreading through the activation of different signaling pathways. However, the excessive ROS are toxic to cancer cells which potentially induce cell death signaling, senescence, and cell cycle arrest (Glasauer and Chandel, 2014).

Generally, the human body is complemented with antioxidant enzymes which are important to combat disease and prevent the cellular damage caused by excessive ROS. The generation of intracellular H_2O_2 has been recognized as the mediator of apoptosis. Various antioxidant enzymes including SOD, catalase, and peroxidase are involved in H_2O_2 modulation (Valdameri *et al*., 2011). SOD converts superoxide to H_2O_2 (and O_2) while catalase, the glutathione peroxidases, and peroxiredoxins reduce H_2O_2 to H_2O (Helfinger and Schroder, 2018).

CIS, a platinum-based drug, is the most efficient chemotherapeutic medication for advanced cervical cancer treatment. Its antiproliferative effect is achieved in cancer cells by inducing oxidative stress and apoptosis (Ordikhani *et al*., 2016). GA, besides being recognized as a strong antioxidant, has been identified to have a prooxidant effect. It is suggested that GA-induced apoptosis is related to oxidative stresses derived from ROS (Park and Kim, 2012). This study has shown that GA alone generated the production of ROS in HeLa cells. This finding was similar to other studies as revealed by You et al. (2010). It is worrying that GA when combined with CIS may exert as antioxidant and shield the anticancer effect of cisplatin. Nevertheless, this study has shown that level of ROS in cells treated with the combination of CIS and GA was increased compared to untreated cells, suggesting that the elevated ROS may be related to the induction of apoptosis. Furthermore, GA when combined with CIS induces more ROS generation compared to a single treatment. Our results also demonstrated that the single and combination treatment decreased the activity of SOD and catalase. The reduction of the antioxidant enzyme activity may be explained by increased ROS promoted by the prooxidant action of CIS and GA. However, in this study, CIS–GA has reduced the inhibition of catalase and SOD activity as compared to the single treatment group. This might be due to the interaction between GA and CIS when combined together. It has been established that GA can act as an antioxidant and pro-oxidant in a certain condition. Thus, further studies need to be conducted to get more explanation. Besides, the concentration of CIS used in the combination of CIS–GA is not the same as the concentration used for a single treatment.

CONCLUSION

The finding of this present study reveals that the combination of GA and CIS induces apoptosis in HeLa cells by increasing the ROS level. The outcome of this study also provides the basis for further molecular studies to investigate the underlying mechanism involved in this CIS–GA-induced apoptosis.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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