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Gold nanoparticle-*Colocasia gigantea* mixture for enhancing cytotoxic effect against a375 melanoma cell line

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ABSTRACT

Introduction: *Antidesma bunius* is commonly found in Thailand and contains high amounts of antioxidants. The pharmaceutical properties such as antibacterial, anti-inflammatory and anticancer. The aim of this study is to evaluating antibacterial activity against oral pathogens (i.e., *Streptococcus mutans, Straphylococcu saureus* and *Streptococcus pyogenes*) with respect to four different types of A. bunius extraction by water including green fruits, red fruits, black fruits, and leaves. **Method:** The extracts of *A. bunius* were examined percent yield, total phenolic compounds, thin layer chromatography and antimicrobial susceptibility testing. **Result and Conclusion:** The percentage of extract yield was found to be the highest in the black fruits (5.20%), followed by red fruits (3.53%) and the leaves (0.71%). The lowest percentage was observed in green fruit (0.47%). The thin-layer chromatography analysis showed a dark spot of gallic acid, the green and red fruit extract was found to have the same Rf value at 0.92.Total phenolic content was highest in the leaves (59 mg GAE g-1) followed by the green fruits (32 mg GAE g-1) and red fruits (26 mg GAE g-1), respectively, whereas the lowest was found in the black fruits (24 mg GAE g-1). Antibacterial activity of the green fruit extract against *S. aureus* (1.73 cm) was higher than tetracycline (1.05 cm). In contrast, the anti-bacterial activity against *S. pyogenes* was not different (2.46 cm for the green fruits was similar to tetracycline), whereas against *S. mutans*, it was lower (1.96 for the green fruits, 3.86 for tetracycline). Anti-bacterial activity was not observed in the black fruit and leaf extracts. The MIC's of the green fruit extract were 0.0125 mg ml-1 for S. pyogenes and 0.025 mg ml-1 for both *S. mutans* and *S. aureus*. Furthermore, the same results were identical for MBC. Results of the current study indicated that the green and red fruit extracts of *A. bunius* had had an inhibitory effect on oral pathogenic bacteria.

INTRODUCTION

Nature has always been a great source of medicines. Traditional oriental herbs and their extracts have been used to treat many kinds of diseases. The herbal extract, which is one of the main divisions of traditional medicine, can contribute to the health of the individual [1]. The activity of a plant extract depends on all of the herbal components, as every composition has their unique action that provides its therapeutic effectiveness [2]. However, most of herbal medicines have been shown to be of an insoluble character, due to their poor bioavailability and stability [3,4]. Therefore to take the greatest advantage from these natural sources, the herbal formulation may still require some modification.

Found abundantly in tropical countries in Southeast Asia, *Colocasia gigantea* is an edible plant, with a short, stout stem and large heart-shaped leaves characteristic to the genus *Colocasia*. Crude extracts from the plant have been

valued for their varied medicinal uses, such as reducing fever, ameliorating stomach problems, fading melasma, and treating wounds. In addition, previous studies have shown that the bioactive components of *C. gigantea* can have cancer-fighting properties [5].

Nanotechnology has been a rapidly expanding area of research and novel therapeutics in recent years. Among many types of nanomaterials, gold nanoparticles (AuNPs) are remarkable nano-sized elements. AuNPs are fundamentally inert and nontoxic, are simply synthesized with modifiable size, and provide the large surface area for drug targeting, with wide-ranged ligand conjugation and protection. Moreover, this nanoparticle system is currently used for controllable release of drugs and delivery agents with effective cellular uptake and specific cellular targeting [6-8]. These properties give AuNPs applications in optical sensors, improved efficacy and stability of drugs, enhanced radiotherapy, and targeted delivery of drugs and genes [8-10]. A recent study reported that adding plant extract during AuNPs synthesis exhibited the potential cytotoxic effect against many types of cancer cell line [11]. AuNPs, which is synthesized using phytochemical compound from herb extract as a reducing agent, have shown anticancer activity in gastric adenocarcinoma cell line [12]. In addition, the implementation of AuNPs for these widespread applications is a source of great interest in both the medical and pharmaceutical sectors [13].

Having learnt about the feasible applications of both *C. gigantea* and AuNPs, this has led to novel thinking about whether AuNPs can be used as a delivery agent for a *C. gigantea* extract (CGE) to target the malignant cells, and whether a combination of the herb extract and AuNPs would synergistically enhance their cytotoxic activities against cancer cells. Therefore, this study aims to investigate the combined effect of CGE and AuNPs toward the cytotoxicity of melanoma skin cancer cells, as well as to examine the possible underlying mechanism.

MATERIALS AND METHODS

C. gigantea Sample Preparation and Extraction

Samples *of C. gigantea* were collected from Ban paew district, Samut Sakhon province, Thailand. Only underground tubers of the plants were used. At first, tubers were sliced into thin sheets and baked in an oven at 40°C for 1 day to completely dry the samples. The dried samples were then chopped into fine pieces using a grinder. As for the extraction, 100 g of the sample was soaked in 1 L of 95% v/v ethanol solvent, and left for a week at room temperature (during which time the bottle containing the samples would be periodically shaken). After that, the mixture was filtered and the ethanol solvent was evaporated under reduced pressure. The crude extract was then weighed and dissolved in an appropriate volume of ×0.1 phosphate buffered saline (PBS) to form 2000 μg/ml stock CGE solution, for later use in the experiments.

Synthesis of AuNPs

All glassware was first thoroughly washed with an aqua regia solution (HCl: HNO_3 = 3:1), rinsed with deionized (DI) water and then left to dry. 1 ml of 1% hydrogen tetrachloroaurate (III) trihydrate was added to a flask containing 24 ml of Milli-Q water under constant stirring. Without heating, 10 mM sodium borohydride ($NaBH_4$) (Merck, Hohenbrunn, and Germany) solution was added dropwise until the yellow solution turned dark red and then bright red. The resulting AuNPs colloid was kept in a container at 4°C away from light. The final concentration of AuNPs is 200 μg/ml.

Evaluation of AuNPs Stability

Absorption spectra of AuNPs synthesized by $NaBH_4$ reduction methods were measured by ultraviolet (UV)-visible spectrophotometer. These were taken as a reference to compare to the absorption spectra of AuNPs and a mixture of AuNPs and CGE, measured following the treatments in melanoma-seeded Roswell Park Memorial Institute Medium (RPMI) for 24 h.

Culture of Cell Lines

Cell lines used in this study are A375 melanoma cell line. The A375 cells were grown in RPMI with the addition of $NaHCO_3$ (2.7 g), 10% fetal bovine serum, and 1% streptomycin (antibiotic). The cells were kept in culture flasks and incubated at 37°C and 5% CO_2. Passaging (subculturing) of the cells was done once every 3 days.

Treatment of Cells for Cytotoxic Study

The tests for cytotoxic study at different conditions were done on 24-well plates. At first, 10^5 cells in 500 μL RPMI medium were seeded into each well and incubated overnight at 37°C and 5% CO_2. Following this, AuNPs (1, 10, 50, and 100 μg/ml), CGE (100, 250, 500, and 1000 μg/ml), and a mixture between both components at various concentrations were added into different wells. A positive control was set by adding 100% dimethyl sulfoxide (DMSO) which is known to be toxic to the cells, while a negative control was set by simply adding more RPMI medium. Eight replicates were done for each sample treatment and following treatments. All samples were incubated for 24 h before being analyzed for cell viability by MTT assay.

Evaluation of Cell Viability Following Treatments (MTT Assay)

The [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay is a colorimetric assay used to determine the extent of cell viability. After the treated cells in 24-well plates were incubated for 24 h, the liquid mixture in each well was carefully drained away by a micropipette. 350 μL of yellow MTT solution was then added to each well and incubated in an incubator for 1 h 30 min. MTT is a light sensitive compound so this treatment was done under dark conditions. After that, MTT solution in each well was drained away and 1 ml of DMSO/glycine buffer was added to each well to dissolve any formazan in the well, if present, to produce purple coloration. The buffer with dissolved formazan was then measured for optical absorbance at 570 nm with a spectrophotometer. The extent of cell viability is directly proportional to the value of the optical absorbance at 570 nm. Bar charts of mean percentage cell viabilities under different treatments were plotted with

a standard deviation bar. A one-way ANOVA was analyzed for each treatment group to judge if there is any statistically significant difference of mean values in the treatment group. Tukey's method (*post hoc* comparison analysis) was then computed to determine specifically which pairs of the treatment have means which significantly differ in values.

Observation Under Light Microscope

The changes in morphology and population density of the melanoma A375 cells following treatments with AuNPs, CGE and a mixture between both components were observed. This was done under a light microscope, by comparison with untreated healthy melanoma A375 cells (negative control cells). Before the observations, the RPMI medium was first removed from each well and cells were washed with PBS.

Apoptosis Assay by Annexin V and Propidium Iodide (PI) Staining

High doses of AuNPs (100 μg/ml), CGE (1000 μg/ml), and moderate doses of the combined form mixture (50 μg/ml AuNPs + 250 μg/ml CGE) were used to treat A375 melanoma cells for the study of apoptosis by annexin V and PI staining (BD Pharmingen, San Diego, USA). Annexin V is a molecule which binds to phosphatidylserine while PI is a molecule which binds to DNA. Double staining is usually done to evaluate the extent of apoptosis. Apoptotic cells are dyed with FITC-conjugated annexin V only, while necrotic cells are stained with both FITC-conjugated annexin V and PI.

Apoptosis Analysis by Flow Cytometry

The RPMI medium in each well was first removed. The cells were then trypsinized by adding trypsin and washed by adding cold PBS, then centrifuged for 2 cycles. The FITC-conjugated annexin V and PI working solutions were added to the vial containing the collected cells, and then kept in a dark place for 15 min. After that, a Ca^{2+}-containing annexin V buffer was added and the vial was loaded into the flow cytometer for analysis.

Apoptosis Analysis Under Confocal Microscope

The melanoma A375 cells were cultured in a lysine-coated chamber slide, different treatments occurring in different wells. Following the treatment, the medium was removed. FITC-conjugated annexin V solution, PI working solution and Ca^{2+}-containing annexin V buffer were mixed together and added to each chamber, before keeping the chamber slide in a dark place for 15 min. The staining mixture was then removed and the cells were washed with cold PBS, before the glass partition was taken out. A cover glass was mounted onto the slide with a mounting

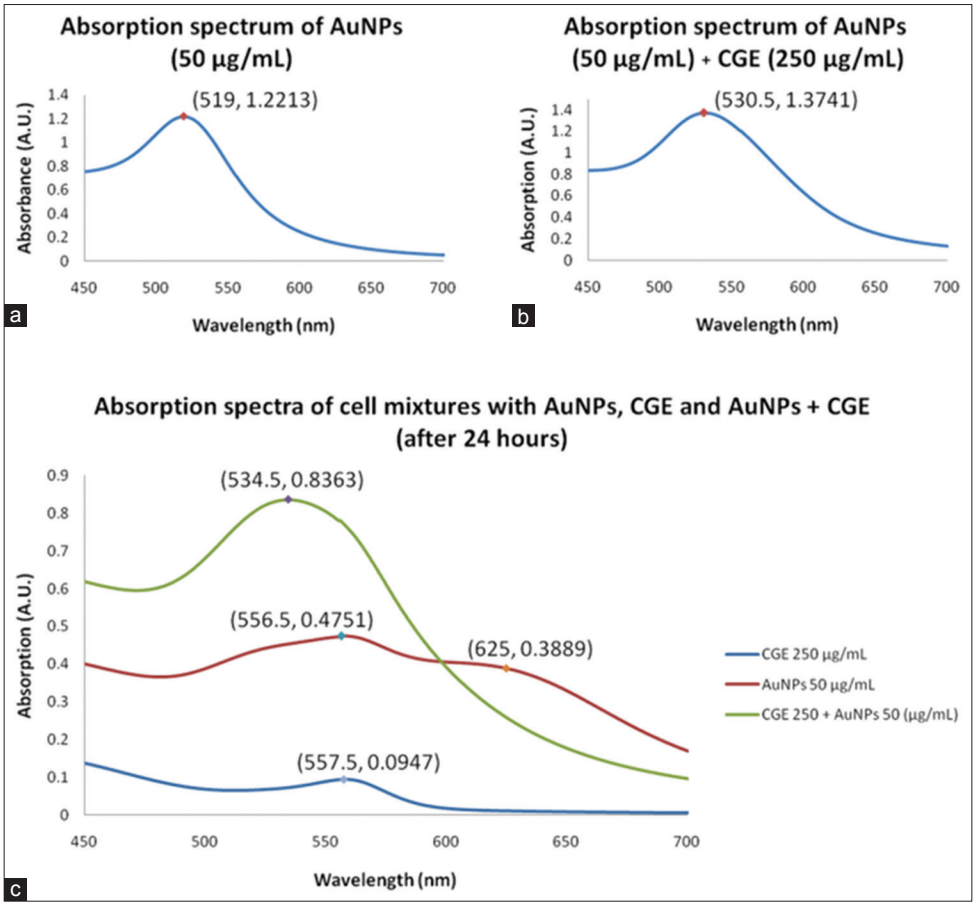

Figure 1: Absorption spectra of (a) gold nanoparticles (AuNPs) ($NaBH_4$ reduced), (b) a mixture AuNPs ($NaBH_4$) and *Colocasia gigantea* extract (CGE) before treatments and (c) absorption spectra of AuNPs ($NaBH_4$), CGE and a mixture of AuNPs ($NaBH_4$ reduced), and CGE after treatments in melanoma-seeded Roswell Park Memorial Institute Medium for 24 h

medium (xylene). The slide was then observed under the confocal microscope. The excitation wavelengths for FITC and PI are 495 nm and 536 nm, respectively, while their emitted wavelengths are 519 nm (green) and 617 nm (orange red), respectively.

RESULTS

AuNPs Stability

The absorption spectra of AuNPs (50 μg/ml)-treated cell mixture and AuNPs (50 μg/ml) and CGE (250 μg/ml)-treated cell mixture were measured under UV/visible spectrophotometer. They were then compared to those of AuNPs solution and AuNPs and CGE mixture as seen in Figure 1. There is a significant shift in the maximum peak, from 519 nm of AuNPs to 625 nm of AuNPs-treated cell mixture, indicating the agglomeration of such AuNPs. On the other hand, CGE-mixed AuNPs have a very slight shift in maximum peak from 530.5 nm to 534.5 nm implying that a greater stability is upheld in the CGE-mixed AuNPs. Note that a peak at 556.5 nm of the AuNPs-treated cell mixture is probably interference from RPMI medium since a similar peak at 557.5 nm is also observed in the absorption spectrum of the CGE-treated cell mixture. Hence, it may be concluded from our spectrophotometric data that CGE-mixed AuNPs are more stable than AuNPs in the RPMI medium environment. The size of CGE-mixed AuNPs was larger than that of AuNPs as inferred from their higher maximum absorption peak.

Cytotoxic Effect of AuNPs and CGE on A375 Melanoma Cells

AuNPs were found to be cytotoxic toward A375 melanoma cells ($P < 0.01$). From Figure 2a, at AuNPs concentrations of 1, 10, and 50 μg/ml, their effect appears to be dose-dependent. The percentage cell viabilities of A375 melanoma cells were 40%, 34%, and 29%, respectively, relative to the negative control. CGE also was found to have a toxic effect against A375 melanoma cells. When statistically compared to the negative control, the 100 μg/ml CGE has the most modest cytotoxic effect toward melanoma cells ($P < 0.05$) while the CGE for the other three concentrations have a much more significant effect ($P < 0.01$), as shown in Figure 2b. All in all, a dose-dependent cell killing activity was observed over the range of the concentrations examined. The mixture of AuNPs and CGE displayed a significantly greater cytotoxic effect toward

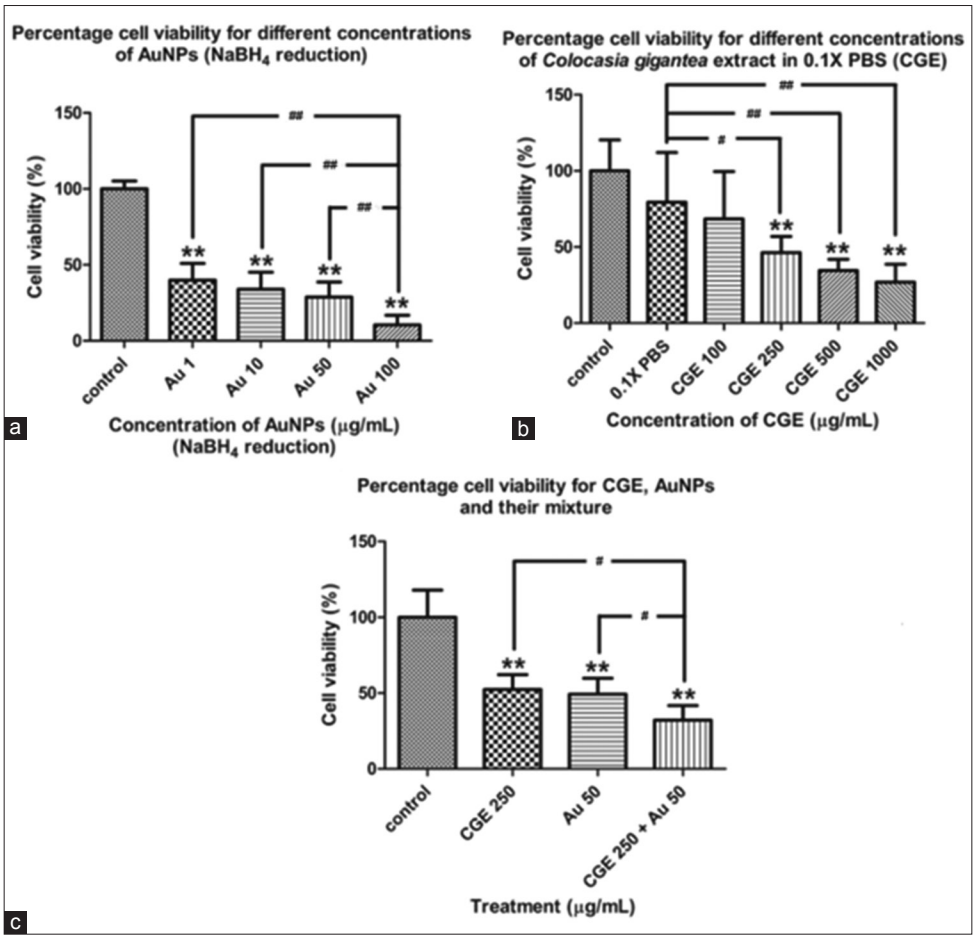

Figure 2: Cell viability studies following treatments with (a) gold nanoparticles (AuNPs), (b) *Colocasia gigantea* extract (CGE), and (c) a mixture of AuNPs and CGE. Data are the average of 8 replicate samples (*$P = 0.05$ and **$P = 0.01$ compared with the control. $^{\#}P = 0.05$ and $^{\#\#}P = 0.01$ compared with the other samples)

A375 melanoma cells than the individual constituents did ($P < 0.05$). This is clearly seen in Figure 2c, which shows that the percentage cell viabilities of A375 melanoma cells treated with 250 μg/ml CGE solution, 50 μg/ml AuNPs solution, and the optimal mixture of the two components are 52%, 49%, and 32%, respectively.

Microscopic Images of A375 Melanoma Cells after AuNPs and CGE Exposure

To confirm the cytotoxic effect of AuNPs and CGE on A375 melanoma cells, we evaluated the morphological changes of A375 melanoma cells that were exposed to AuNPs, CGE, and the combination of AuNPs and CGE. From Figure 3a and b, it is clearly seen that the number of cells remaining on the well decreases, as cells are subjected to higher concentrations of AuNPs and CGE. Compared with the AuNPs and CGE treated alone, the mixture of AuNPs and CGE showed a very large decrease in the number of cells attach to the well plate (Figure 3c). This is in proportion to the decrease in the percentage of viable cells treated with higher doses of AuNPs, as analyzed from the MTT data.

Cell Death Pattern Analysis

To determine whether AuNPs and CGE induce A375 melanoma cell apoptosis, both FITC-conjugated annexin V and PI were employed to investigate the pattern of cell death, under a flow cytometer and confocal microscope. As shown in Figure 4a, the negative control has the highest percentage of normal healthy cells (unstained, 76.92%). The cells treated with high doses of AuNPs, CGE and the mixture of AuNPs and CGE show a predominantly apoptotic behavior, with a minor necrotic behavior as seen in Figure 4b-d. For the confocal images shown in Figure 5, it is clear that the shape of the cells is more rounded as opposed to healthy melanoma cells, which would be spindle-like in shape and spread out more readily. An observation here is the evident breakdown of nuclei into smaller granules, as observed only in the cells treated with the mixture of AuNPs and CGE (Figure 5c). This stands in contrast to the nuclei's shape of cells treated with AuNPs or CGE alone, which is still rounded and seemingly intact (Figure 5a and b). From both confocal microscopy and flow cytometry analyses, it can be concluded that the major mechanism of cell death in cells treated with AuNPs, CGE and their combined form is apoptosis.

DISCUSSION

Herbal medicine is widely used at present for the treatment of various diseases. Crude extracts from plants have been valued for their varied medical uses. *C. gigantea* is one of the most common edible plants used in traditional folklore. In addition, there have been scientific reports of the

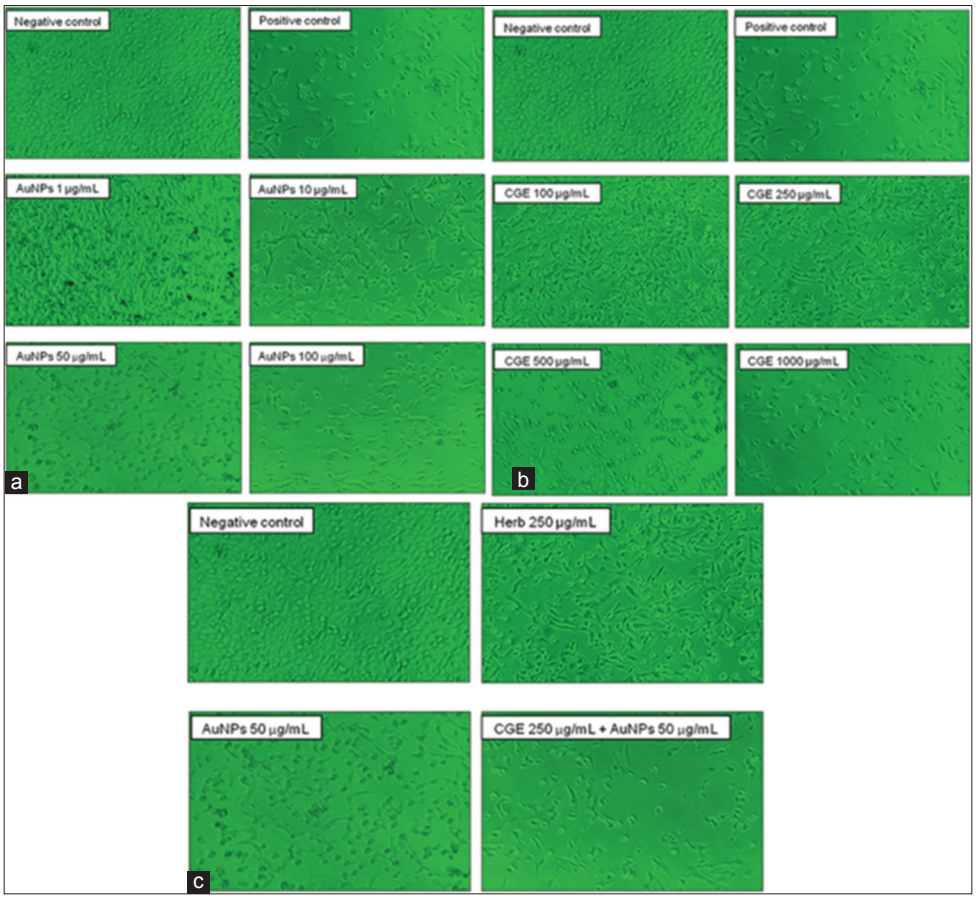

Figure 3: Microscopic images of melanoma cells treated with (a) different concentrations of gold nanoparticles (AuNPs), (b) different concentrations of *Colocasia gigantea* extract (CGE), and (c) a mixture of AuNPs and CGE

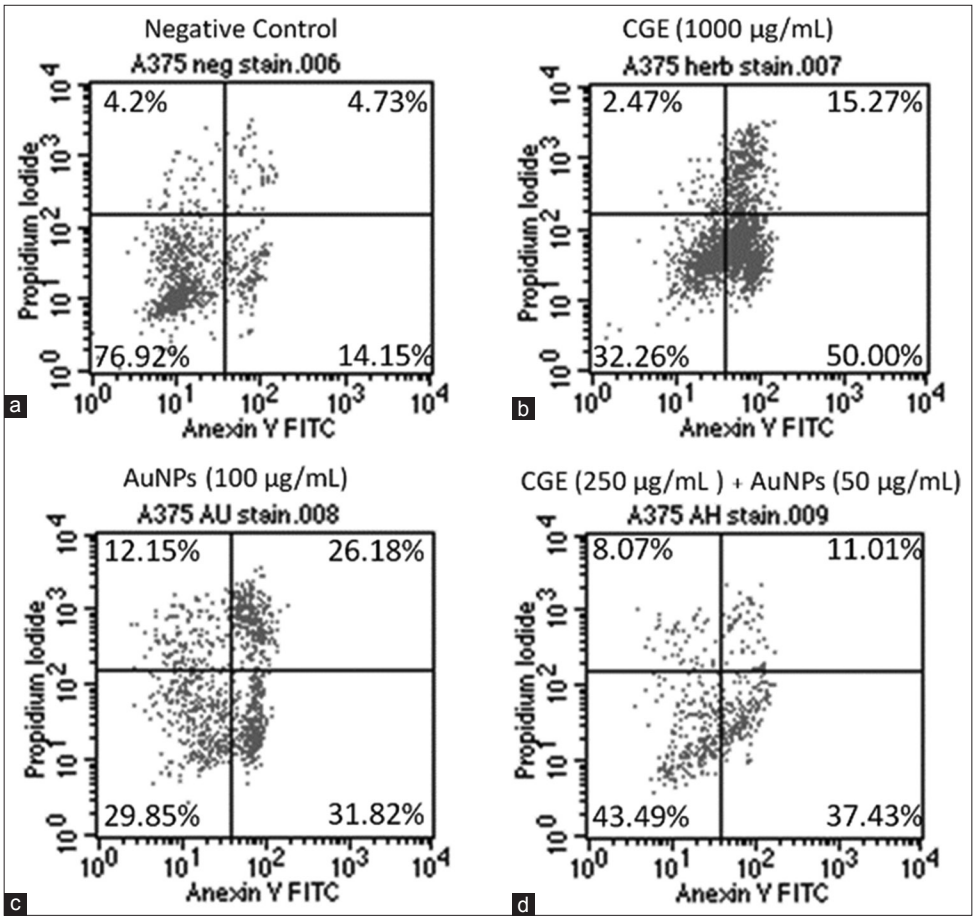

Figure 4: Flow cytometric analysis showing dot plots of cells treated with (a) negative control (Roswell Park Memorial Institute Medium only), (b) *Colocasia gigantea* extract (CGE) (1000 μg/ml), (c) gold nanoparticles (AuNPs) (100 μg/ml), and (d) a mixture of AuNPs (50 μg/ml) and CGE (250 μg/ml)

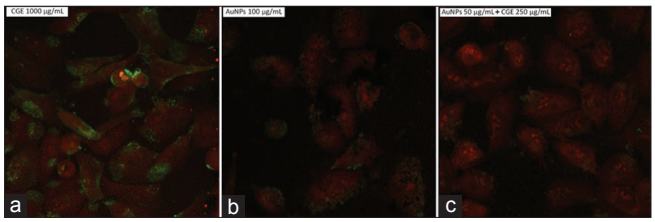

Figure 5: Confocal images of melanoma cells treated with (a) *Colocasia gigantea* extract (CGE) (1000 μg/ml), (b) gold nanoparticles (AuNPs) (100 μg/ml), and (c) a mixture of AuNPs (50 μg/ml) and CGE (250 μg/ml)

cancer-fighting properties of this plant extract [5]. However, it is known that the uses of medicinal plants have some limitations, such as low bioavailability and stability [14]. The effectiveness of AuNPs for the increase of the bioavailability and stability of herbal drugs has been proposed for many years [15]. In this study, we investigated the cancer inhibiting effects of CGE combined with AuNPs in the A375 melanoma cell line. Even though, the mechanisms of action of AuNPs combined with CGE in the increase of the bioavailability and stability of plant extract are still unknown. Our results revealed that the combination of CGE and AuNPs showed more cytotoxic damage than either the AuNPs or the CGE alone. A possible explanation for the combined effect of plant extract and AuNPs in the enhance of anticancer activity is like to be in agreement with the previous report of Manju *et al*. They performed their study on the anticancer effects of curcumin conjugated AuNPs [16]. They reported that the inhibiting activities of curcumin were increased by the AuNPs conjugation [16].

From our cell viability study, CGE was found to be cytotoxic toward A375 melanoma cells in a dose-dependent manner. This discovery presents a new discovery of another possible medicinal use of *C. gigantean,* apart from those mentioned in traditional folklore. Even though, we do not know the identity of the active compounds, it is possible that such compounds will be identified from 4,22-stigmastadiene-3-one, diazoprogesterone, 9-octadecenoic acid (Z)-, hexyl ester, and oleic acid. These isolated compounds from *C. gigantea* have shown their cytotoxic potential in the cervical cancer cell line [5]. It is also possible that the active compounds may be from the flavonoid family, since many such compounds have been previously isolated from *Colocasia esculenta*, and have been shown to display a strong antioxidant activity [17]. The cytotoxic effect of *C. esculenta* against colonic adenocarcinoma cells was reported by Brown *et al*. [18]. Ultimately, there remains work to isolate and identify the active compounds of *C. gigantea*, and examine their chemical structures to better understand their cancer inhibiting properties in melanoma cells.

A mixture of AuNPs and CGE was shown to display a cytotoxic effect against melanoma cells to a greater extent than a Mere treatment with either AuNPs or CGE alone. With regard to the stability observed with CGE -mixed AuNPs, one explanation for the enhanced cytotoxic effect is that the more stabilized CGE -mixed AuNPs may be more taken up the A375 melanoma cells. This is possibly since they remained in the appropriate size for entry for a longer time, as opposed to the agglomerating AuNPs which kept increasing in size. A possible reason for AuNPs increase of the cancer inhibiting properties of CGE may occur due to the abilities of AuNPs in drug delivery systems [2]. Previous studies have demonstrated the biological activities of AgNPs loaded with natural formulations for treating cancerous cells [19,20]. They indicated that the effect of AuNPs is an improvement of the drugs' efficacy, solubility, and bioavailability. Consequently, the effectiveness of the herbal drug in treating hepatocellular carcinoma was improved [19,20].

From the flow cytometry and confocal images analysis, it can be concluded that the major mechanism of cell death in A375 melanoma cells, treated with AuNPs, CGE and a combination of the two, is apoptosis. Apoptotic death following treatment with AuNPs and AuNPs conjugated herbal extract has been previously reported in many cancerous cell types [21,22]. This apoptotic behavior is believed to be initiated by the disruption of DNA replication during the synthesis phase. Although apoptotic death following CGE treatment has never been studied before, treatment using plant extracts from the same family as *C. gigantea*, on colonic adenocarcinoma cells was shown to have resulted in apoptotic cell death [18]. From the confocal images of melanoma cells following these different treatments, the breakdown of nuclei into smaller granules observed in the cells treated with a mixture of AuNPs and CGE has led to a postulation that some novel compounds in CGE might be conjugated to AuNPs. This possibly represents a potential system to preferably target the nuclei leading to their degradation. The nuclear fragmentation observed in the cells treated with a mixture of AuNPs and CGE might suggest a nuclear targeting role of some novel compounds in the CGE, which potentiate the nuclear damaging and apoptotic actions of AuNPs against the melanoma cells. Nonetheless, there remains future work to confirm if CGE-mixed AuNPs actually favorably target nucleus, and to identify the active compounds involved.

CONCLUSION

In this study, we have investigated the use of AuNPs to enhance the activity of the oriental herb extract, *C. gigantea,* on the A375 melanoma cell line. Our study has explored for the first time, the integration of crude extracts of *C. gigantea* and AuNPs in amplifying the cytotoxic effect against melanoma cells. In future, the novel compounds in CGE which are responsible for the enhancement of such cytotoxic activity will have to be isolated and identified. Further investigation into the mechanism providing the enhanced cytotoxic effect will also be necessary before real cancer therapies could be developed using the mixture of AuNPs and CGE. Finally, our successful demonstration of the nuclear degrading effect of A375 cells, treated with a mixture of AuNPs and CGE, may also exemplify the intrinsic role of CGE as a natural nuclear targeting carrier for AuNPs. This could pave way for the future developments in the area of targeted drug delivery.

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