

ORIGINAL ARTICLE

***In vitro* antitumor activity of guttiferone-A in human breast cancer cells is mediated via apoptosis, mitochondrial mediated oxidative stress and reactive oxygen species production**

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Summary

Purpose: Breast cancer is the second most frequently diagnosed cancer and is considered as the main cause of cancer related death in females. It is estimated that about one-third of women with breast cancer develop metastases and eventually die of this disease. The main treatment options for breast cancer include surgical interventions followed by chemotherapy, hormonotherapy or radiation. However, the side effects associated with the treatment of breast cancer negatively affects the quality of patient's life. In the present study a plant-derived compound, guttiferone-A, was evaluated for its anticancer activity against MCF-7 breast cancer cell line.

Methods: MTT assay was used to evaluate the cytotoxic effects while phase contrast microscopy was used to assess the effects of the compound on cell morphology. Effects on reactive oxygen species (ROS) and mitochondrial

membrane potential were evaluated by flow cytometric analysis.

Results: It was observed that guttiferone-A reduced the cell viability of MCF-7 cancer cells in a dose-dependent manner. The IC₅₀ for guttiferone-A was found to be 15 μM against MCF-7 cells. Moreover, guttiferone-A induced the production of high levels of ROS and caused significant reduction in the mitochondrial membrane potential. Additionally, guttiferone-A also induced apoptosis in MCF-7 cancer cells in a dose-dependent manner.

Conclusion: Taken together, we conclude that guttiferone-A is a potential anticancer molecule and may prove to be a lead molecule in cancer drug discovery.

Key words: apoptosis, breast cancer, guttiferone-A, reactive oxygen species

Introduction

Cancer is characterized by a swift and unregulated growth of cells followed by local invasion and ultimately distant metastases [1,2]. Despite recent advancements in medicine and technology, treatment of cancer still remains below expectations. Cancer is considered as a major public health problem both in developed and developing countries and its incidence is growing with the passing of time [3,4]. Among different types of cancers, breast cancer is the second most widespread cancer and is considered as the principal cause of cancer-related death in women [5]. It is estimated that about 1/3 of

women with breast cancer develop metastases and eventually die of this disease. The main treatment options for breast cancer include surgical interventions followed by chemotherapy, hormonotherapy or radiotherapy. However, the side effects associated with the treatment of breast cancer negatively affect the quality of patient's life [5]. In this regard there is an urgent need of new and efficient treatment options for cancer in general and breast cancer in particular.

Natural products derived from plants have gained tremendous importance in the recent past

due to their lower toxic effects. The present study aimed to evaluate the anticancer activity of a plant-derived compound, guttiferone-A (GA) (Figure 1). GA is a polyisoprenylated benzophenone derivative firstly extracted from *Symphonia globulifera* and later it was also isolated from several other plant species such as *Garcinia aristata* [6]. GA has been reported to possess tremendous pharmacological properties including anticancer [7] and anti-HIV [6]. The present study was therefore designed to evaluate the *in vitro* antitumor activity of guttiferone-A against human breast cancer cells.

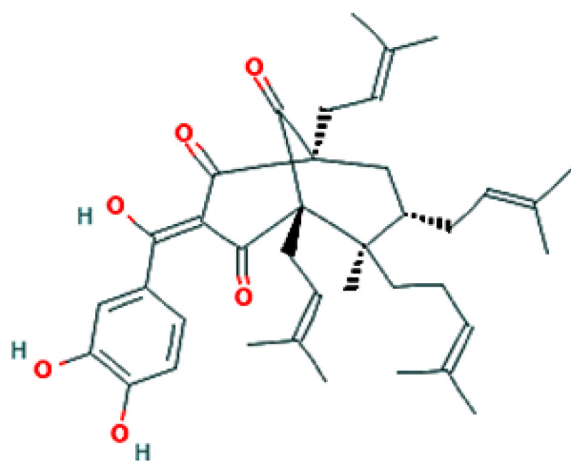


Figure 1. Chemical structure of Guttiferone-A.

Methods

Chemicals reagents and cell culture

A number of chemicals and reagents were used in the present study and included (i) Guttiferone-A, RNase A, Triton X-100 and dimethyl sulfoxide (DMSO) obtained from Sigma-Aldrich Co. (St. Louis, MO, USA); and the fluorescent probes DCFH-DA, DiOC6, 4'-6-diamidino-2-phenylindole (DAPI), RPMI-1640 medium, L-glutamine and antibiotics obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). The human breast cancer cell line MCF-7 was procured from Cancer Research Institute of Beijing, China, and was maintained in RPMI-1640 medium (Gibco-Invitrogen) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/mL), streptomycin (100 mg/mL), and 10 % fetal bovine serum (FBS).

Cell cytotoxicity assay

In brief, MCF-7 cells were cultured at a density of 2×10^6 cells/ml in a 96-well plate for 24 hrs before using them in the experiment. The cells were then treated with 0, 7.5, 15 and 30 μ M of GA for 48 hrs. Subsequently, MTT was added into each well and then again cultured for 3 hrs before the supernatant was discarded. The formazan crystals formed were dissolved in DMSO and the absorbance values were assessed with Automated Microplate Reader (Bio-Tek, VT, USA) at 570 nm. Cytotoxicity was presented as the concentration of GA inhibiting cell growth by 50% (IC_{50} value).

Determination of ROS and MMP

For the determination of ROS and MMP, MCF-7 cells were seeded in a 6-well plate at a density of 2×10^5 cells/well and kept for 24 hrs; Then, cells were cultured with 0, 7.5, 15 and 30 μ M of GA and incubated for 72 hrs at 37°C in 5% CO₂ and 95% air. Afterwards, the treated cells from all samples were harvested and washed twice with PBS. The cells were then re-suspended in 500 μ l of DCFH-DA (10 μ M) for ROS and DiOC6 (1 μ mol/l) for evaluation of MMP at 37°C for 30 min in the dark. The samples were then assessed immediately using flow cytometer as described previously [8].

DAPI staining and flow cytometric analysis of cell apoptosis

MCF-7 breast cancer cells were seeded at a density of 2×10^5 cells/well in 6-well plates and were exposed to 0, 7.5, 15 and 30 μ M of GA for 48 hrs. The cells were then stained with DAPI for detection of apoptosis and annexin-V/propidium iodide (PI) staining for estimation of apoptotic cell populations. Afterwards, the sample was studied and photographs were taken under fluorescence microscopy as previously reported [8].

Protein expression by Western blot analysis

GA-exposed cells were washed twice in ice-cold PBS and then extracted with a RIPA buffer each comprising of a mixture of 5% phosphate inhibitor and protease inhibitor. The cell lysates were centrifuged at 15,000 rpm for 15 min. The protein concentrations were thereafter determined by using Bio-Rad protein assay. The proteins' samples were then subjected to SDS-PAGE and shifted to nitrocellulose membranes. The membranes were then probed with specific antibodies at 4°C overnight, followed by washing with buffered saline and incubation with the appropriate secondary antibody for 1 hr. The bands were then photographed using an ECL chemiluminescent detection kit (Perkin Elmer Cetus, Foster City, CA, USA).

Statistics

All experiments were carried out in triplicate and the results were expressed as mean \pm standard deviation (SD). Results were considered significant at $p < 0.05$. One way ANOVA and Tukey's test were used for statistical analysis with the help of GraphPad prism 7 software.

Results

GA exerts growth inhibitory effects on MCF-7 breast cancer cells

For evaluation of anti-proliferative activity of GA, MCF-7 cells were treated with different concentrations of GA ranging from 0 to 30 μ M. The results of MTT assay revealed that GA exerts potent anti-proliferative effect on MCF-7 cells and reduces the cell viability in a concentration-dependent manner (Figure 2A). The IC_{50} of GA was found to be 15 μ M. Microscopic study also revealed dose-dependent reduction in the viable cancer cells (Figure 2B).

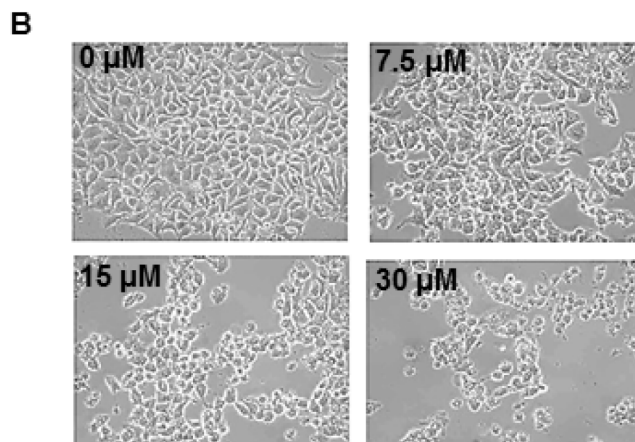
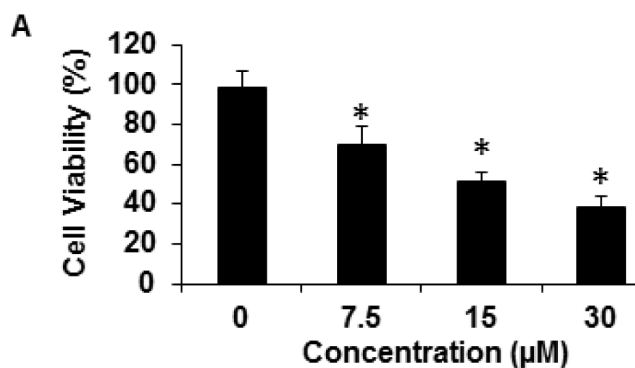


Figure 2. Effect of indicated doses of GA on (A) cell viability of MCF-7 breast cancer cells. (B) Microscopic images showing reduction in the number MCF-7 cancer cells with increase in the dose of GA. GA caused significant morphological changes on MC-7 cells. All experiments were carried out in triplicate and expressed as mean \pm SD. * $p < 0.05$.

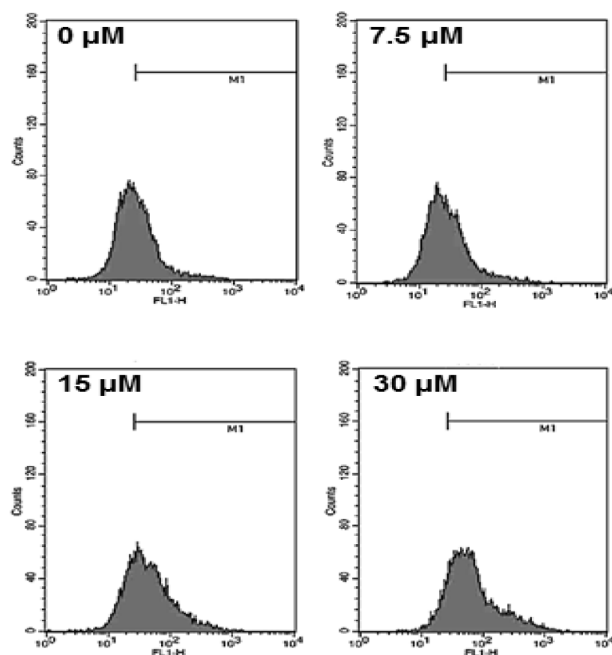


Figure 3. Effect of indicated doses of GA on reactive oxygen species production determined by flow cytometry. The images are representatives of three biological experiments.

GA causes ROS production and mitochondrial membrane potential (MMP) reduction in MCF-7 breast cancer cells

Since several anticancer drugs exert their anti-proliferative effects through production of ROS, we attempted to evaluate the effect of GA on ROS status of the MCF-7 breast cancer cells. The MCF-7 cells were treated with different concentrations of GA ranging from 7.5 to 30 μ M for 48 hrs. The results indicated that intracellular ROS levels of treated cells increased up to 117 % as compared to untreated cells (Figure 3). Therefore, GA is an effective molecule for activating ROS production in MCF-7 cells. In continuation, we examined whether GA decreases the MMP in MCF-7 cells and observed that GA-treated MCF-7 cells displayed a considerable reduction in MMP in a dose-dependent manner. The MMP reduced up to 48.5% at 30 μ M of GA as compared to untreated control (Figure 4).

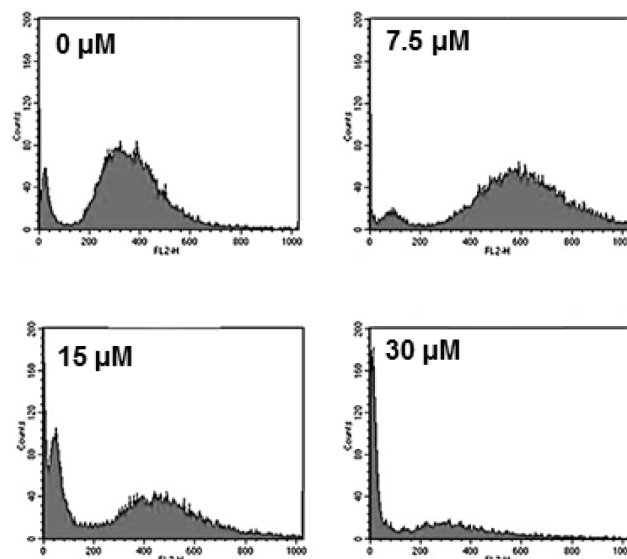


Figure 4. Effect of indicated doses of GA on mitochondrial membrane potential determined by flow cytometry. The images are representatives of three biological experiments. GA caused production of ROS in a concentration-dependent manner.

GA induces apoptosis in MCF-7 breast cancer cells

To investigate if GA induces apoptosis in MCF-7 breast cancer cells, the cells were exposed to GA at various concentrations ranging from 7.5 to 30 μ M. The results indicated that GA prompted apoptosis in MCF-7 cells as evident from DAPI. Untreated cells exhibited uniformly bright nuclei and treated cells showed apoptotic bodies (Figure 5). These results indicate that GA induces apoptotic death in a concentration-dependent manner. To confirm apoptosis, annexin V/PI staining was carried out which showed that the apoptotic

population increased 5.2%, 16.40%, and 26.25% compared to untreated control (1.9%) and the apoptotic cell populations increased in a concentration-dependent manner (Figure 6). These results clearly indicate that GA induces apoptosis in a dose-dependent manner.

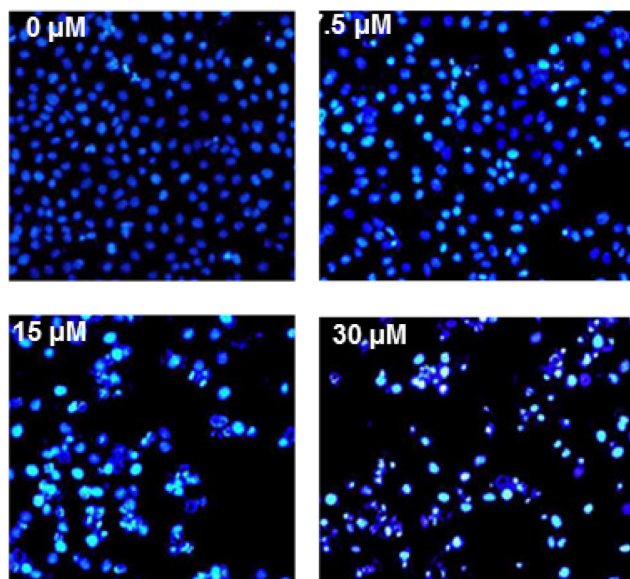


Figure 5. Effect of indicated doses of GA on induction of MCF-7 cell apoptosis as detected by DAPI staining, determined by flow cytometry. The images are representatives of three biological experiments. It was observed that GA induced apoptosis in MC-7 cells in a concentration-dependent manner.

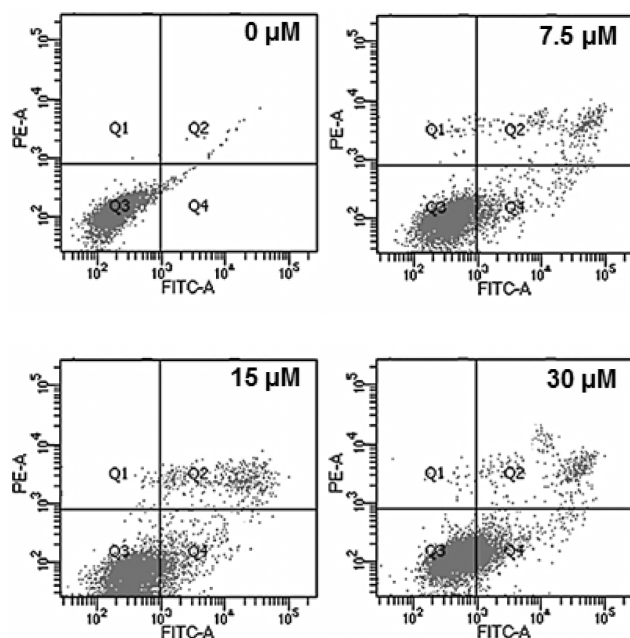


Figure 6. Estimation of apoptotic MCF-7 cell populations at indicated doses of GA by annexin V/PI and followed by flow cytometry. The images are representatives of three biological experiments. The results showed that the apoptotic cell populations increased in a concentration-dependent manner.

GA alters the Bax/Bcl-2 ratio in MCF-7 breast cancer cells

Bax and Bcl-2 are chief marker proteins for apoptosis and GA administration resulted in enhanced upregulation of Bax (pro-apoptotic protein) and inhibition of Bcl-2 expression (antiapoptotic protein) causing an apparent increase in the Bax/Bcl-2 ratio in a dose-dependent manner (Figure 7).

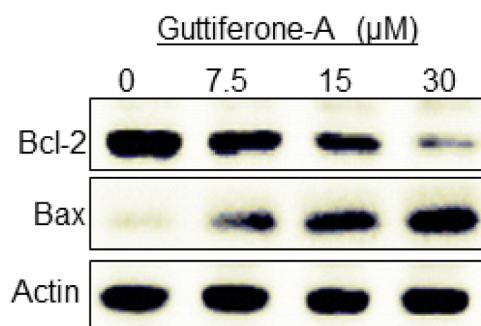


Figure 7. Effect of indicated doses of GA on the Bax/Bcl-2 protein expression as determined by western blotting. The images are representatives of three biological experiments. It was observed that the expression of Bax increased and the expression of Bcl-2 decreased in a concentration-dependent manner.

Discussion

GA is a polyisoprenylated benzophenone derivative mainly isolated from the roots of *S. globulifera* and *G. aristata* [6]. Previous studies have reported several pharmacological activities of GA which include but are not limited to anti-cancer [7] and anti-HIV [6]. In the present study GA was evaluated for anticancer activity against MCF-7 breast cancer cell line and it was found that it caused significant reduction in the viability of MCF-7 breast cancer cells. The IC_{50} of GA was found to be 15 μ M at the incubation period of 48 hrs. The antiproliferative activity of GA is of high significance as previous studies have reported that molecules with the capacity to inhibit cell growth and proliferation may prove potential lead molecules for cancer treatment [9-11]. GA administration caused significant increase the production of ROS which was implicated in the induction of apoptosis. It should be noted that previous studies have indicated that several anticancer drugs exert their anti-proliferative activities through the production of high amounts of ROS [12]. Moreover, our results are in concordance with earlier studies wherein GA has been shown to stimulate enormous amount of ROS in cancer cells [13]. ROS generation is directly linked to MMP [14] and in our study it was observed that GA significantly reduced the MMP. Additionally, GA treat-

ment prompted apoptosis in MCF-7 breast cancer as evidenced from DAPI staining. Annexin V/IP staining followed by flow cytometer analysis further confirmed that GA increased the apoptotic cell populations in a dose-dependent manner. It is well established that apoptosis induction is one of the important mechanisms by which several anticancer drugs exert their effects [15]. Bax/Bcl-2 ratio is an important indicator of apoptosis. It was observed that GA administration resulted in enhanced upregulation of Bax which is a pro-apoptotic protein. Simultaneously, GA also caused significant inhibition of antiapoptotic protein Bcl-

2 expression causing an apparent incremental increase in the Bax/Bcl-2 ratio (Figure 7).

In conclusion, the present investigation provides essential information that ethanolic GA exhibits potent anticancer activity against MCF-7 cells. The results of the present study encourage us to carry out more studies on GA and extend the investigation of this molecule to other cell lines.

Conflict of interests

The authors declare no conflict of interests.

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