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Full Length Research Paper

Induction of apoptosis in human breast cancer cell line MCF-7 by phytochemicals from *Gmelina asiatica*

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Currently, breast cancer is the leading cause of cancer-related death in women. Therefore, there is an urgent need to develop alternative therapeutic measures against this deadly disease. Many components from dietary or medicinal plants have been identified that possess substantial chemopreventive properties. India has unique plant varieties yet to be studied for anticancer components. Therefore, cytotoxicity activity and the mechanism of cell death exhibited by the extracts prepared from *Gmelina asiatica*, in human breast cancer MCF-7cells was investigated. The MCF-7 cells were seeded in 96-well culture plates in the presence and absence of different concentrations of extracts of *G. asiatica* to determine their anticancer effects using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Mechanism of cell death was evaluated by chromatin condensation using Hoest staining and morphological changes with the use of a contrast microscope. Plant extracts of *G. asiatica* were observed to induce apoptosis of MCF-7 cells as evidenced by MTT-cell proliferation assay, cell-morphological changes and chromatin condensation. The cytotoxicity of the chloroform extract was greater than other extracts of *G. asiatica*. The results of the present investigation is the first report on the potential anticancer activity of *G. asiatica* extracts and its possible mechanism of action on cancer cell proliferation in breast cancer MCF-7 cell lines.

Key words: Breast cancer, MCF-7 cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole assay, apoptosis, *Gmelina asiatica.*

INTRODUCTION

Breast cancer is the second most prevalent cancer in the world today next to lung cancer (Parkin et al., 2001) and is a major public health problem in developing countries like India. Every year, 75,000 new cases of breast cancer are reported in India. In future, this figure may further increase due to factors like environmental pollution, food habits, consumption of genetically modified food stuffs among others. The mortality due to cancer is a challenge similar to that posed by HIV/AIDS. Breast cancer is becoming more common among urban women than in rural women and it might be due to the aging of the population and increase in age–specific incidents (Sen et al., 2002). Breast cancer is both genetically and histopathologically heterogeneous and the mechanism(s) underlying breast cancer development remains unclear (Hedenfalk et al., 2002). The development of breast cancer involves several *genes* and they remain either activated or inactivated in order to promote malignancy (Ingrasson, 2001). A major limitation with the present cancer chemotherapy is the serious deficiency of active drugs for the curative therapy of tumors (Valeriote et al., 2002; Kinghorn et al., 2003). For thousands of years, natural products have played an important role throughout the world in the treatment and prevention of human diseases (Chin et al., 2006). Over 60% of the currently used anticancer agents are derived in one way or other from natural sources (Cragg and Newman, 2003; Balunas and Kinghorn, 2005; Cragg and David, 2005). The search for anti-cancer agents from plant sources

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Abbreviations: DMEM, Dulbecco's modified eagle's medium; **FBS,** fetal bovine serum; **GA,** *Gmelina asiatica*; **MTT,** 3-(4-5 dimethylthiozol-2-yl) 2-5 diphenyl-tetrazolium bromide.

started in the 1950s through the discovery and developpment of the vinca alkaloids, vincristine, and the isolation of the cytotoxic podophyllotoxins (Reddy et al., 2003; Tsuda et al., 2004; Srivastava et al., 2005; Pezzuto, 1997).

The chemotherapeutic drugs including etoposide, camptothecin, vincristine, *cis*-platinum, cyclophos-phamide, paclitaxel (Taxol), 5-fluorouracil and doxorubicin have been observed to induce apoptosis in cancer cells (Tan et al., 2005; Kaufmann and Earnshaw, 2000; Johnstone RW, Ruefli, 2002). Among them, agents that alter the cell cycle have been of particular interest, since cell cycle regulation is the basic mechanism underlying cell fate, that is, proliferation, differentiation or death (Dobashi and Takehana, 2003). Thus, uncontrolled cell proliferation is one of the main hallmarks of cancer, and tumor cell damages in *genes* that are directly involved in the regulation of the cell cycle (Garrett, 2001; Tachibana et al., 2005; Sandal, 2002).

Negative cell growth is also an important aspect of maintaining normal tissue homeostasis. This regulation involves the suppression of cell proliferation, as well as the induction of cell death (Symonds et al., 1994). In cancer therapy, one approach to suppress tumor growth is by activating the apoptotic machinery in the cell (Fan et al., 1998). Apoptosis is the ability of a cell to induce self-destruction by the activation of an intrinsic cellular suicide program when the cells are no longer needed or when they are seriously damaged. Evidence that has been obtained during the last few years is enough to establish that a large majority of cancer chemotherapy agent's affect tumor cell by killing *in vivo* and *in vitro* through launching the mechanisms of apoptosis (Hannun, 1997). Morphologically, apoptosis is characterized by the appearance of membrane blebbing, cell shrinkage, chromatin condensation, DNA cleavage, and the fragmentation of the cell into membrane-bound apoptotic bodies (Kerr et al., 1972).

Gmelina asiatica L. (*Verbenaceae*) popularly known as *Nilakkumil* in Tamil and *Gopabhandra* in Sanskrit, is a large straggling shrub found in South India. Its roots are used for the treatment of gonorrhoea, catarrh of the bladder, rheumatism and for purification of the blood (Kirtikar and Basu, 1984). Since this plant is claimed to be useful in the treatment of rheumatism, it is said to possess anti-inflammatory action (Syed et al., 1997). The root of the plant also has potent hypoglycaemic activity (Kasivisvanathet al., 2005). The aerial parts of *G. asiatica* (GA) have antimicrobial activity (Sudhakar et al., 2006). The ethanolic extract of *G. asiatica* is found to possess anticancer activity in p 388 lymphocytic leukaemia in mice (Dhawan et al., 1980).

The present study aimed to isolate and evaluate the compound responsible for cytotoxic and anticancer effects of *G. asiatica* extract. This study sheds light on the mechanism of induction of apoptotic activity induced by the active principles of *G. asiatica* in breast cancer cell line MCF-7.

MATERIALS AND METHODS

Preparation of extract

Aerial parts of the *G. asiatica* were collected from Therkkumalai Estate, Courtallam Hills, Western Ghats, Tamil Nadu, India and identified by Chelladurai, Research Officer, Central Council for Research in Ayurveda and Siddha, Palayamkottai. Voucher specimens were prepared and preserved in the Department of Pharmacognosy, KM college of pharmacy, Madurai for further reference (Voucher specimen no. KMCP/GA/23).

The dried and powdered aerial parts of the plant (500 g) was subjected to subsequent extraction in a Soxhlet apparatus using petroleum ether, chloroform, ethyl acetate and ethanol. The solvents from the extracts were removed by distillation under reduced pressure at 45 - 50^{P} to get GA extracts viz., GA1 (petroleum ether extract), GA2 (chloroform extract), GA3 (ethyl acetate extract), GA4 (ethanolic extract). GA1, GA2, GA3 and GA4 were used to evaluate their mechanism of action in cancer cell line MCF-7.

Chemicals and reagents

The extracts were dissolved in DMSO to get 200 µg.ml^{-1} stock solutions and stored at -20°C. Further dilutions were made in complete Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Hoechst 33342 and MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide were procured from Sigma (St. Louis, MO). FBS, DEMEM, antibiotics and trypsin-ethylenediaminetetraacetic acid (EDTA) were from Inutrogen.

Cell culture

Human breast cancer cell line, MCF-7, was obtained from the National Centre for Cell Sciences, Pune. The cells were maintained in DMEM containing 10% FBS, supplemented with additional glutamine (0.03%) and 100 µg.ml^{-1} benzyl penicillin, 100 U.ml^{-1} streptomycin and 2.5 µg.ml^{-1} amphotericin. Cells were allowed to grow in tissue culture flasks (Corning, USA) and were kept in CO_2 incubator at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For experimental purpose, cells from exponentially growing culture were used. All experiments were repeated three times.

Cell viability assay

Cell viability assays were carried out as described by Suresh et al. (2007). Briefly, cells were seeded at a density of 3 - 10^{4}cells/well into 24-well plates. After 24 h, GA extracts were added to the medium at various concentrations and incubated for 24 or 48 h as indicated. At the end of the incubation, 3-(4-5 dimethylthiozol-2-yl) 2-5 diphenyl-tetrazolium bromide (MTT) (2 mg.ml^{-1}) per well was added, and the formazan crystals formed were solubilized in acidified isopropanol after aspirating the medium. The extent of MTT reduction was measured spectrophotometrically at 570 nm and the cell survival was expressed as percentage over the untreated control.

Cell morphology studies

The effect of various extracts of *G. asiatica* on cell morphology of MCF-7 cells was investigated. MCF-7 cells grown in a 6 well plates were treated with 200 µg.ml^{-1} of different extracts of *G. asiatica* at 37°C for 24 h. Morphological changes occurring in the cells were

observed under phase – contrast microscope and photographed using CCD camera attached to the TE 2000E microscope (Nikon).

Chromatin condensation and apoptosis measurement

One of the prominent changes of apoptosis is the condensation of chromatin brought by the activated caspases. For analyzing chromatin condensation by Hoechst 33342 staining, the cells were grown on 96 well plates, after indicated treatment, were stained with 0.5 ug.ml^{-1} of Hoechst 33342 for 10 min and viewed under UV filter sets using Nikon fluorescent microscope (TE2000E). The images were captured with CCD camera and analysed using Image pro software. Apoptotic nucleus with condensed chromatin was scored in percentage from 200 - 300 cells/sample at least by two investigators.

RESULTS

Effect of GA on cell viability

The effects of various extracts of *G. asiatica* on the growth of MCF-7 cells were examined by the MTT assay. Chloroform extract (GA2) at the dose of 50, 100, 200 µg.ml^{-1} induced marked cytotoxicity as evidenced from the MTT assay in a concentration dependent manner and the percentage viability of cells were estimated as 70.25, 58.4, and 38.9 respectively. The petroleum ether, ethyl acetate and alcoholic extracts did not show notable cell death even at 200 µg concentration and the results are shown in Figure 1.

Cell morphology studies

Under phase-contrast microscope, the untreated MCF-7 cells exhibited typical growth patterns and a smooth, flattened morphology with normal nuclei (Figure 2a). When treated with GA2, the MCF-7 cells exhibited condensed chromatin, apoptotic morphological changes with cytoplasmic blebbing and detachment from the surface (Figure 2c). Most GA2-treatment cells showed apoptotic bodies with cytoplasmic condensation indicating apoptosis like changes. These observations provide evidence that an apoptotic pathway is triggered with the GA2 treatment in breast cancer cell line. GA1, GA3 and GA4 failed to produce significant morphological changes. The results are shown in Figure 2.

G. asiatica induced cytotoxocity was mediated by chromatin condensation

Chromatin condensation is one of the hallmarks of apoptosis. In order to show that the cytotoxicity of *G. asiatica* is mediated by the induction of apoptosis, chromatin condensation was analyzed in MCF-7 cells by Hoest staining. MCF-7 cells were treated with *G. asiatica* extracts. At the end of the incubation period, the cells were stained with Hoechst 33342 dye and the cells were observed under the microscope for chromatin condensation. The nucleus of GA2 treated cells showed condensed chromatin, which was strongly bound with fluorescent dyes allowing non apoptotic cells to be discriminated from apoptotic ones. The results were in complete agreement with reports of Darzynkiewicz et al. (1994). The representative images of chromatin condensation and percentage positive cells are shown in Figure 3. Apoptotic cells showed clear difference from non-apoptotic cells as the former are bright and their nuclei were condensed. GA1, GA3 and GA4 did not induce notable changes in nuclear morphology of MCF-7 cells.

DISCUSSION

Cancer is the second leading cause of death worldwide. Conventional therapies cause serious side effects and at best, merely extend the patient's lifespan by a few years. Cancer control may therefore benefit from the potential that resides in alternative therapies. There is thus, an increasing demand to use alternative concepts or approaches for the prevention of cancer. There is an increasing realization that chemotherapeutic agents act primarily by inducing cancer cell death through the mechanism of apoptosis (Lowe and Lin, 2000). However, there are many cancers that are intrinsically resistant to apoptosis, making it vital to develop novel drugs for combination chemotherapy.

Breast cancer is the most common type of cancer affecting women. It is the number 2 killer (after lung cancer) of women aged 35 - 54. Approximately, 20% of these cases occur in women under 30 years of age, and 70% in women over 50 years of age (Holmes et al., 2001). Most of the breast cancers are known to be resistant to currently used chemotherapeutics because of mutational inactivation of apoptotic machinery. Breast cancers often lack caspase 3 that renders them resistant to most antitumour agents. The breast cancer cell line MCF-7 is one of the important cell line models with deficiency of functional caspase 3 and are interestingly resistant to most drugs. This cell line model was used in the present study to identify potential anti cancer activity of *G. asiatica*.

A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damages to normal cells. This ideal situation is achievable by inducing apoptosis in cancer cells. Chemopreventive agents comprise diverse groups of compounds with different mechanisms of action with ultimate ability to induce apoptosis. Understanding the modes of action of these compounds should provide useful information for their possible applications in cancer prevention and perhaps in cancer therapy (Taraphdar et al., 2001; David, 2004).

Among the different extracts evaluated for cytotoxicity in

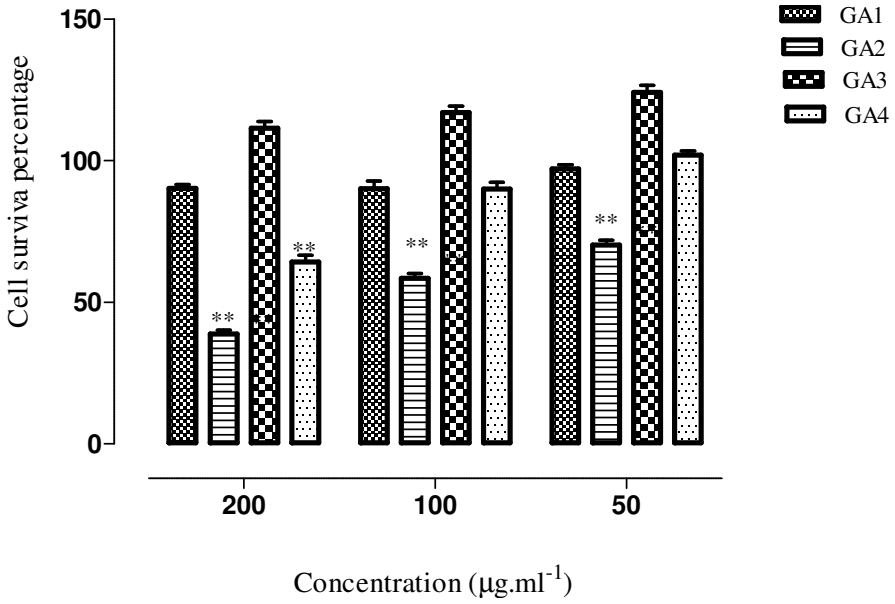

Figure 1. Effect of *Gmelina asiatica* on cell viability. MCF-7 cells were treated with 50 - 200 µg of GA1, GA2, GA3 and GA4 for 48 h. Cytotoxicity was analyzed by MTT assay. The percentage cell death was calculated from the control. The statistical analysis was carried out using one way ANOVA. Values are mean ± S.E.M, n =3. ** $p<0.01$ compared to control.

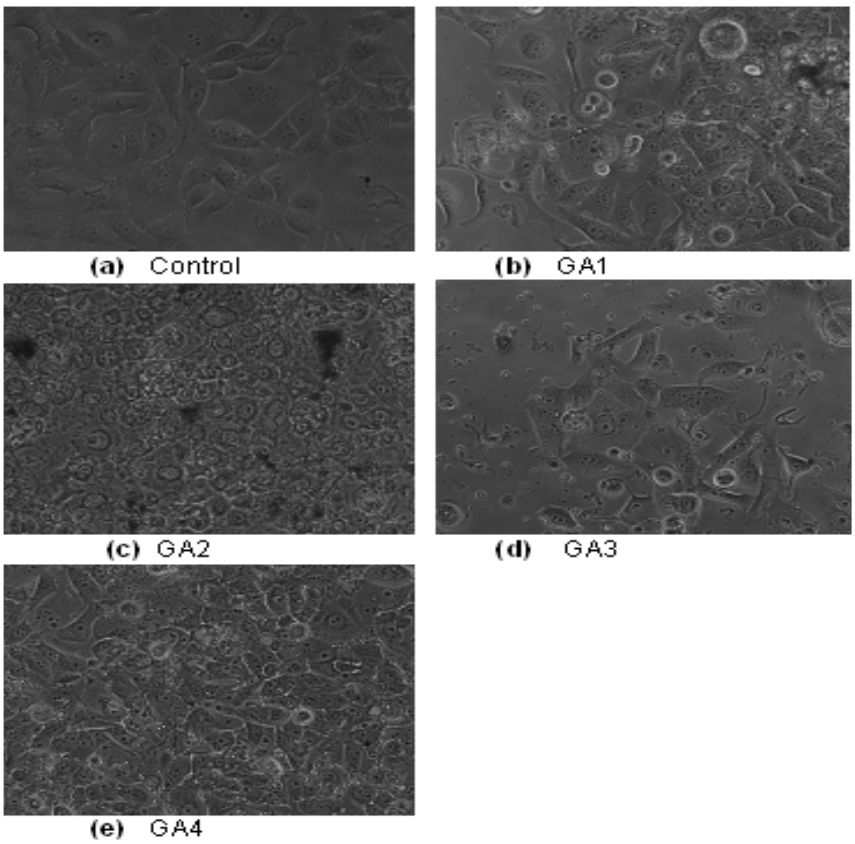

Figure 2. Morphological characteristics of MCF-7 cells visualized with a phase-contrast microscope. (A) Control cells, (B) GA1 treated cells, (C) GA2 treated cells, (D) GA3 treated cells, (D) GA4 treated cells.

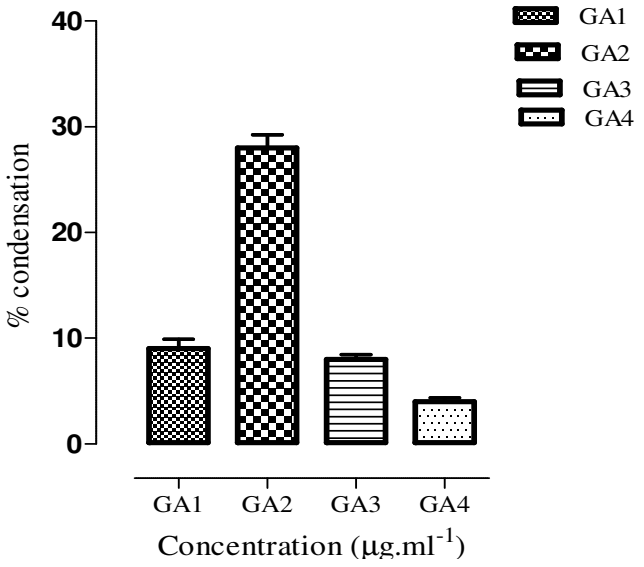

Figure 3. Effect of *G. asiatica* on chromatin condensation. Cells with condensed and fragmented nuclei were counted in five different fields among a total of 200 cells. The mean values of triplicate samples are expressed in percentage ± S.E.M.

MCF-7 cells, the chloroform extract emerge as the most efficient preparation in inducing cytotoxicity. Further studies confirmed that, the cytotoxicity potential is closely associated with chromatin condensation, one of the well known markers for apoptosis. 50 µgm.ml^{-1} of chloroform extract significantly increased percentage of cells with condensed nuclei compared to other extracts. The loss of chromatin integrity is often induced by activated caspases.It is well known that in apoptosis, the earliest recognized morphological changes are compaction and segregation of the nuclear chromatin, with the result of chromatin margination and condensation of the cytoplasm (Kerr et al., 1972). Progression of the condensation is accompanied by convolution of the nuclear and cell outlines followed by breaking up of the nucleus into discrete fragments and by budding of the cell as a whole to produce membrane-bounded apoptotic bodies.

Caspases are important cysteine proteases activated during apoptosis. The prominent execution of caspases (caspases 3, 6 and 7) are capable of targeting large number of structural and functional proteins between the cells. It is interesting to note that hence, the MCF-7 cells used in the current study are inherently deficient for functional caspase-3 because of mutation activation. Several previous studies have shown that this cell line is resistant to more conventional antitumour agents due to lack of caspase-3. However, the chloroform extract significantly induced chromatin condensation in MCF-7 cells despite the absence of caspases. This study suggests that some other caspases are activated by the extract that culminates to chromatin condensation. Further studies are needed to substantiate the important alternate caspases targeted by the extract. The possible role of caspase independent cell death also cannot be ruled out.

Overall, the present study substantiate that, the chloroform extract of *G. asiatica* has potential anticancer activity in caspase 3 deficient breast cancer cell line MCF-7. This anticancer activity is associated with apoptotic changes like chromatin condensation. Currently, it is not very clear which of the novel bioactive molecules is responsible for the apoptotic activity. Studies are ongoing to identify the particular bioactive molecule.

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