Induction of caspase dependent apoptosis by-Mangostin in breast cancer cells

Navya Atluri1, Vasavi thirumalananadhuni1, Uma Maheswari Devi Palempalli*

*Corresponding author:
Uma Maheswari Devi Palempalli
1Department of Applied Microbiology, Sri PadmavatiMahilaVisvavidyalayam (S.P. Women's University), Tirupati-517502, Andhra Pradesh, India.

A b s t r a c t
Apoptotic potential of -Mangostin (98-99.99% HPLC purified) was evaluated in vitro using MDA-MB-231 human breast cancer cell line as a model system. The nucleosomal DNA gel electrophoresis was performed to delineate the mechanism of -Mangostin treated MDA-MB-231 cells death. The dose-dependent oligonucleosomal fragmentation of DNA was observed in -Mangostin treated cells. Flow cytometry was performed to confirm the apoptotic potential of -Mangostin (10µM) on MDA-MB-231 cells. The control cells showed only 1.85% of hypodiploid DNA and the percentage increased to 13.70% on exposure of cells to -Mangostin. Caspase-3, a critical regulator of cell death, was detected in -Mangostin treated MDA-MB-231 cells by Immunocytochemical analysis. These results demonstrated the expression of caspase-3 in MDA-MB-231 cells exposed to 10µM of -Mangostin. The results suggest that -Mangostin induced cell death was by the activation of cell death pathway particularly mediated by caspase-3.

Keywords: -Mangostin, Apoptosis, Extrinsic pathway, Caspase-3, DNA fragmentation.

Introduction
Apoptosis or programmed cell death is a biological process by which cells undergo death to control cell proliferation. Apoptosis provides a conceptual frame work to link cancer genetics with cancer therapy [1, 2]. The apoptotic process may proceeds either through the extrinsic / death receptor mediated pathway or the intrinsic /mitochondrial pathway [3]. The extrinsic pathway involves the engagement of specific death receptor from TNF –R family and result in the formation of death inducing signaling complex (DISC). This complex promotes activation of initiator caspase–8 [4]. Once activated, caspase-8 induces downstream caspases by proteolytic cleavage of their zymogens like Caspase–3, caspase–6 and caspase–7 and thus amplifying the caspase signal to bring apoptosis [5]. Caspase-3 has been considered as the principal effector caspase in the apoptotic process. The activated form of Caspase-3, cleaves several substrates and results in DNA fragmentation, nuclear condensation, membrane disruption and cell shrinkage [6]. On other hand, inhibition of caspase–3 activity blocks the cell apoptosis and induces further developments in tumor formation by altering the dynamic balance between apoptosis and proliferation [7].

Natural compounds are now gaining more pharmacological attention as many unexplored plant products are showing a wide range of activities like anti-inflammatory and anti-cancer [8,9].Xanthones are plantpolyphenolic compounds and have been known to exert biological and pharmacological activities. Recent phytochemical studies revealed that these xanthones exhibit a variety of biological activities such as antioxidant and anti-inflammatory effects [10]. -Mangostin, a major xanthone derivative, isolated from the pericarp of Mangosteen belongs to Garcinia mangostana. Keeping the importance of -Mangostin as antioxidant [11] and anti-inflammatory agent [12], the present study is designed to understand its role in induction of apoptosis and molecular mechanism underlying apoptosis process.

Materials
Mangostin (98-99.99% HPLC purified, ChromaDex, LGC Promochem India Pvt. Ltd); MDA-MB-231 cell line (National Centre for Cell Science, Pune, India); DMEM, FBS, Trizol (GIBCO Laboratories); DMSO (HiMedia Laboratories); LPS, and other chemicals used in present study were of analytical grade.

Cell Proliferation Assay
Effect of -Mangostin on the Proliferation of cells was assessed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [13]. The MDA-MB-231 cells (1x10⁴ cells / well) were seeded in 0.2mL of DMEM in a 96-well plate and incubated at 37 C for 24h in 5% CO₂ atmosphere. After the confluence of cells, different concentrations of -Mangostin (1, 2.5, 5, 10.15 and 20µM) and 0.1% DMSO (control) were added, incubated for 24h. To the treated cells 0.02mL of MTT (5mg/mL in PBS) was added and the incubation was extended for an additional 4h. After incubation, the formazan crystals were dissolved with DMSO (0.15ml/well) and the percentage of cell proliferation was measured.

DNA fragmentation analysis
The treated MDA-MB-231 cells were trypsinized and pelleted by centrifugation for genomic DNA isolation. The pelleted cells were incubated for 30min. with 0.5ml of ice cold lysis buffer (20mM Tris-HCl, pH 7.4, 10mM EDTA and 0.5% Triton X-100) and centrifuged at 4°C. The supernatant was collected and RNase A (10µg/ml) was added and incubated at 37 C for 1h, followed by Proteinase K

This work is licensed under a Creative Commons Attribution 3.0 License.
(20µg/ml) treatment for 2h at 60 C. An equal volume of pure phenol was added and gently agitated for 30min and subjected to centrifugation at 10,000rpm for 5min. The upper aqueous phase was carefully harvested and an equal volume of 1:1 mixture of phenol and chloroform was added and centrifuged for 5min. at 14,000rpm and DNA precipitated with double thevolume of absolute ethanol. The obtained pellet was washed with 70% ethanol, air dried, suspended in 50µl of TE buffer (10mM Tris-HCl, pH 7.4, 1mM EDTA). The DNA was electrophoretically resolved on a 1.8% agarose gel in 1x TAE buffer and stained with ethidium bromide (0.5µg/ml) for visualization.

**Quantification of apoptosis**

Flow cytometric analysis was performed to quantify the DNA content by using propidium iodide staining. In brief, MDA-MB-231 cells (2x10^5 cells / well) were seeded in 6-well plates containing DMEM. The confluent cells were treated with -Mangostin at a concentration of 10µM and 0.1% DMSO (control) for 24h at 37 C. Then, cells were washed with PBS, harvested, fixed with ice cold 70% ethanol (2.5ml) at 4 C for 30min. and rinsed with 0.01M PBS (pH 7.4). The cell pellets were suspended in 0.1% Triton X-100; 0.08N HCl and 0.146M NaCl followed by RNase A (10µg/ml) treatment at 37 C for 10min. The cells were stained in absence of light with PI solution (50µg/ml of Propidium Iodide, 1% Triton X-100, 0.9% NaCl) by incubation at room temperature for 30min. Then fluorescence of PI stained MDA-MB-231 cells was measured at 488nm at a flow rate of 1000-1200 cells/sec using FACSCALIBUR Flow Cytometer [14].

**Detection of Caspase-3 by immunocytochemical analysis**

Caspase activation is one of the most specific indicators of the apoptotic process and the detection of caspase-3 could, therefore, be a valuable and specific tool for indicating apoptosis. Immunostaining was utilized to analyze the caspase-3 expression in -Mangostin treated MDA-MB-231 cells [15]. In brief, the treated cells were permeabilized, washed and blocked with 0.5ml of 1% BSA and the cells were incubated with the primary antibody i.e., rabbit anti-caspase-3 (1:500) for 2h and secondary antibody, anti-rabbit (1:5000) for 30min. and later with peroxidase substrate 3, 3'-diaminobenzidine (DAB) for 5min. The cells were washed and counterstained with hematoxylin for 5min. at room temperature. After counterstaining, the cells were washed and covered with DPX mounting solution and observed under light microscopy.

**Results and Discussion**

In the present study, the effective role of -Mangostin on growth inhibition and apoptosis induction in highly invasive and metastatic human breast cancer MDA-MB-231 cell line was determined by performing cell proliferation and apoptosis analysis. Mitochondrial respiration, the representative of cell viability, was determined by the mitochondrial-dependent reduction of MTT to formazan. MTT, a yellow tetrazole, is reduced to purple formazan in living cells. MDA-MB-231 cells were incubated with different concentrations (1 to 20µM) of -Mangostin for 24h and assessed by MTT assay to determine the anti-proliferative activity of -Mangostin. We observed that more formazan crystals were formed by control (0.1% DMSO) cells than in cells treated with 10µM of -Mangostin due to mitochondrial dehydrogenase activity. A significant decrease in cell proliferation with increasing concentrations of -Mangostin was noticed at 24h. The number of viable breast cancer cells was decreased below the initial number after 24h at 10µM of -Mangostin concentration. The MDA-MB-231 cells are significantly sensitive to -Mangostin with an IC50 of 10µM as determined by cell viability assay. The percentage of cell proliferation of MDA-MB-231 cells reduced to 47.6% and 3.4% respectively with 10µM and 20µM of -Mangostin (Figure1). Thus, -Mangostin displayed a strong anti-proliferative activity on MDA-MB-231 breast cancer cells. Studies on prenylated xanthones of (β, γ-Mangostin) showed the anti-proliferative effect in DLD-1 cells [16]. It is generally believed that the balance between proliferation and apoptosis influences the response of tumors to cytotoxic treatment [17].

In order to demarcate the mechanism of cell death mediated by -Mangostin, DNA fragmentation analysis was performed, which is the hallmark of apoptosis. MDA-MB-231 cells treated with different concentrations (2, 5, 10,15 and 20µM) of -Mangostin for 24h indicated the generation of oligonucleosomal fragments of DNA on 1.8% agarose gel. The degree of nuclear DNA fragmentation is directly proportional to the increasing concentrations of -Mangostin (Figure2).

The induction of apoptosis in cells treated with -Mangostin was further confirmed by Flow cytometric analysis. The control cells showed prominent G1 phase, followed by S and G2/M phases (Figure3A). The control cells showed only 1.85% of hypodiploid DNA and the percentage was increased to 13.70% on exposure of cells to 10µM of -Mangostin (Figure3). Thus, MDA-MB-231 cells in the presence of -Mangostin showed significant apoptotic peak to the left of G1/G0 peak as compared to control cells(Figure-3B). Our study reveals that the anti-proliferative effect of -Mangostin on MDA-MB-231 cells was primarily through the induction of apoptosis.

Apoptosis can be triggered by the diversity of extracellular and intracellular factors [18]. The signal transduction and execution of apoptotic mechanism require activation of a series of caspases (cysteiny1 aspartate-specific protease) [19]. Caspase-3 has been considered as the principal effector caspase and its activation stimulates the cleavage of several substrates and results in the formation of characteristic features of apoptosis, such as DNA ladder formation [20] and chromatin condensation [21]. Caspase-3 is a critical regulator of cell death during execution phase of cells undergoing apoptosis. It is indispensable for the cleavage of a large number of proteins and leads to morphological changes in cells such as DNA fragmentation and nuclear collapse all along the apoptosis process. Immunocytostaining for caspase-3 protein expression was performed and developed using DAB substrate kit.
Then cells were counterstained with hematoxylin to show nuclear staining (blue). The results showed the expression of caspase-3 in -Mangostin (10μM) treated cells, as a marker of apoptosis (Figure-4). Yunjie et al., (22) reported that the suppression of apoptosis was partly due to inhibition of caspase-3 activity suggesting that overexpression of COX-2 interferes with caspase pathway.

Figure 1. The inhibition of MDA-MB-231 cells proliferation from 0.1% DMSO (C) to 1, 2.5, 5, 10, 15 and 20μM of -Mangostin treatment determined by MTT assay after 24h. Each bar represents the data from triplicate determinants and * indicates significant difference in comparison to control (p < 0.01).

Figure 2. DNA fragmentation analysis of -Mangostin treated MDA-MB-231 cells. After 24h treatment with or without different concentrations of -Mangostin, DNA was isolated and analysed on 1.8% agarose gel. Lane M: DNA marker, Lane C: 0.1% DMSO treated control cells, Lane ‘1’ to ‘5’ are the cells treated with different concentrations (2.5, 5, 10, 15 and 20 μM) of -Mangostin.
Figure 3: Flow cytometric analysis was performed to determine the apoptotic effect of -Mangostin on MDA-MB-231 cells. The cells were exposed to -Mangostin (10μM) and 0.1% DMSO for 24h. Then the cells were harvested, fixed and stained with propidium iodide, and the DNA content was analyzed by Flow cytometry. The DNA histogram of (A) Control cells (0.1% DMSO) and (B) 10μM -Mangostin treated cells after 24h.
Conclusion

The present findings clearly demonstrates the therapeutic importance of γ-Mangostin based on the antiproliferative activity dependent on caspase mediated apoptotic pathway.

Acknowledgement

We gratefully acknowledge the support by DST-SERC programme (SR/SO/HS/007/2008) for providing the financial assistance to carry out this investigation.

References


