Original Research Article

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

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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 cytometrywas performed to confirm the apoptotic potential of -Mangostin ($10\mu M$) 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 $10\mu M$ 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 thecell 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 andanti-inflammatory effects [10]. -Mangostin, a major xanthone derivative, isolated from the pericarp of Mangosteen belongs to *Garciniamangostana.* Keeping the importance of -Mangostin as

antioxidant [11] andanti-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 ($1x10^4$ cells / well) were seededin0.2mL of DMEM ina 96-well plate and incubated at 37 C for 24h in 5% CO_2 atmosphere. After the confluence of cells, different concentrations of -Mangostin (1, 2.5, 5, 10,15 and $20\mu M$) 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^oC. The supernatant was collected and RNase A ($10\mu g/ml$) was added and incubated at 37 C for 1h, followed by Proteinase K

(20µg/ml) treatment for 2h at 60 C. An equal volume of pure phenol was added and gently agitated for 30min.andsubjectedto 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 pelletwas 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 1.8% agarose gel in 1x TAE buffer and stained withethidium 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 488ηm at a flow rate of 1000-1200 cells/secusing FACS-CALIBUR Flow Cytometer [14].

Detection ofCaspase-3 by immunocyto chemical analysis

Caspase activation is one of the most specific indicators of the apoptotic process andthe 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 andsecondary antibody, anti-rabbit (1:5000) for 30min. and later with peroxidase substrate 3, 3'-diaminonbenzidine (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 IC_{50} 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- and 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, 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 G_1 phase, followed by S and G_2/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 G_1/G_0 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 (cysteinyl 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 feataures 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 proces. 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 over-expression of COX-2 interferes with caspase pathway.
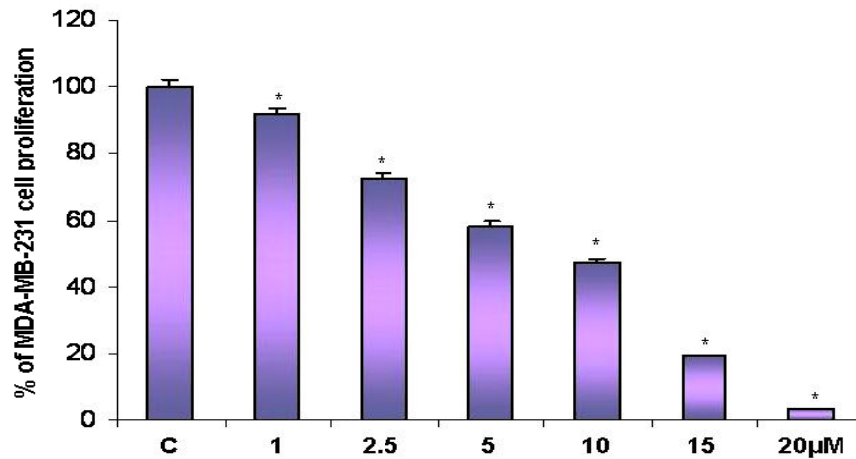

Figure1. The inhibition of MDA-MB-231 cells proliferation from 0.1% DMSO (C) to 1, 2.5, 5, 10,15 and 20µM of -Mangostin treatment determined by MTT assay after 24h. Each bar represents the data from triplicate determinants and * indicates significant difference in comparison to control (p < 0.01).
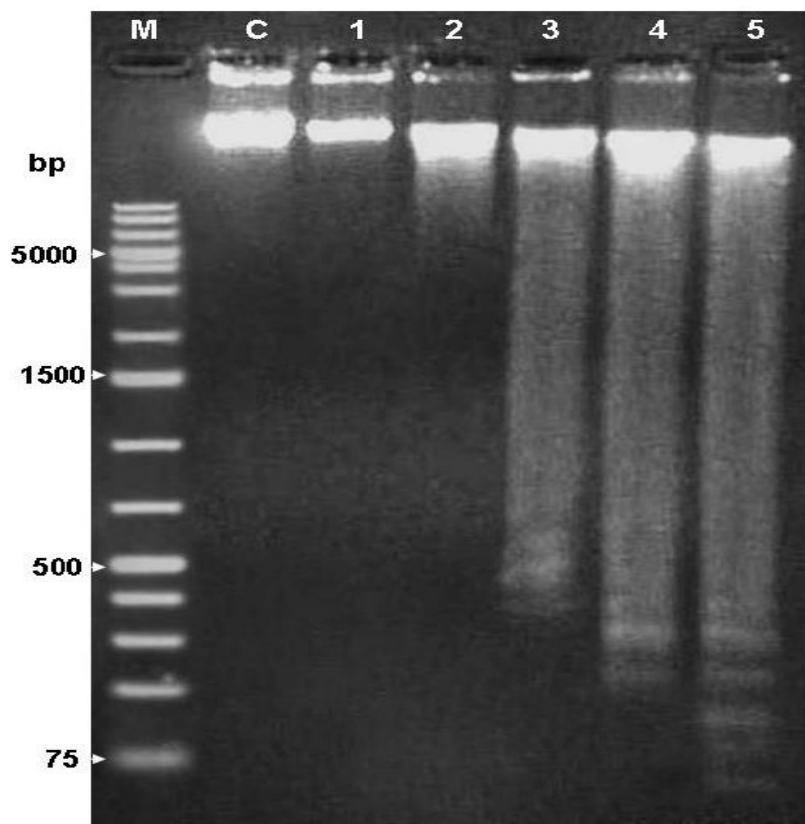

Figure 2. DNA fragmentation analysis of -Mangostin treated MDA-MB-231 cells. After 24h treatment with or without different concentrations of -Mangostin, DNA was isolated and analysed on 1.8% agarose gel. Lane M: DNA marker, Lane C: 0.1% DMSO treated control cells, Lane '1' to '5' arethe cells treated with different concentrations (2.5, 5, 10, 15 and 20 µM) of -Mangostin.
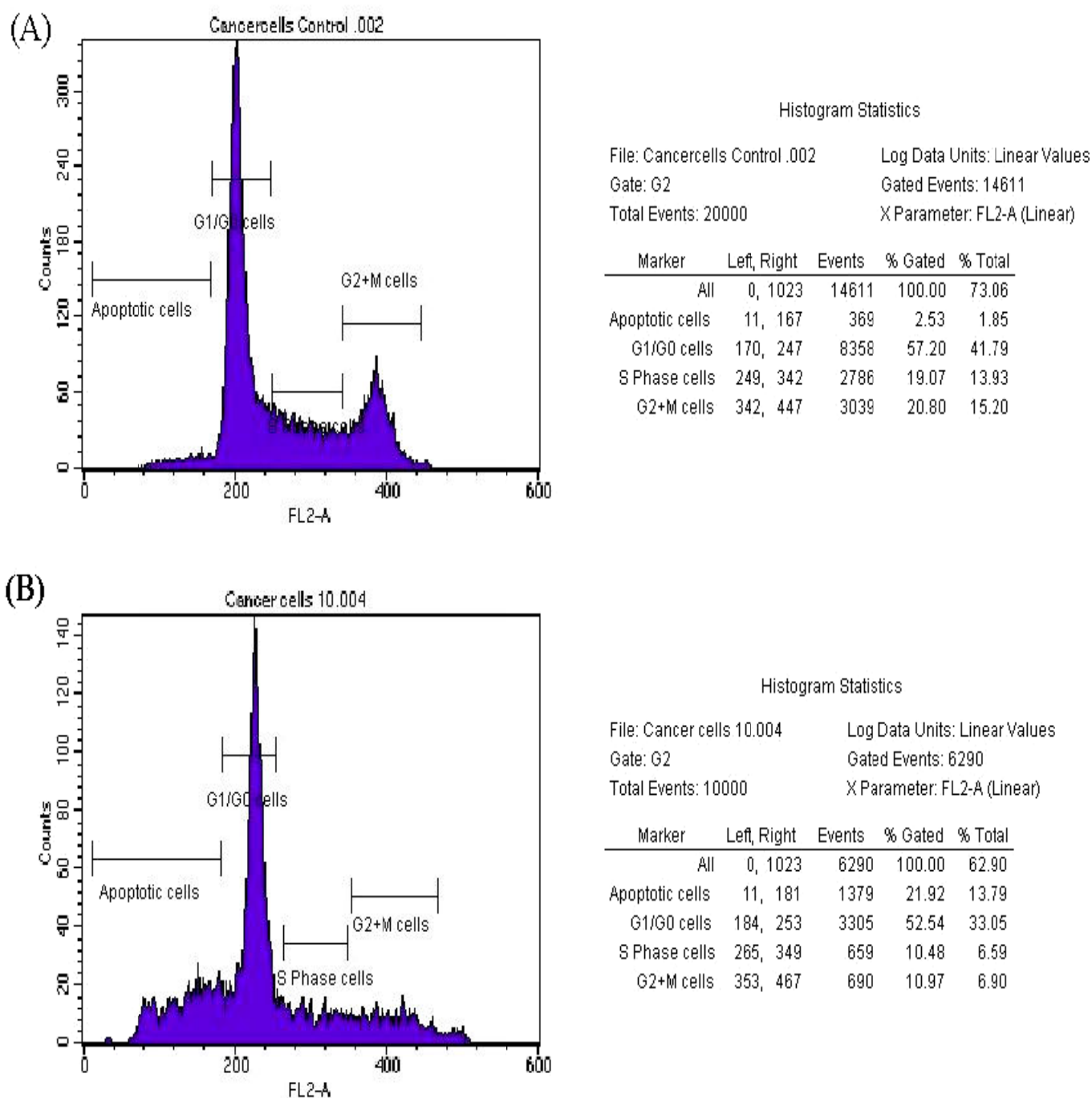
(A)

Cancercells Control .002

G1/G0 cells

Apoptotic cells

G2+M cells

S Phase cells

FL2-A

Histogram Statistics

File: Cancercells Control .002   Log Data Units: Linear Values
Gate: G2   Gated Events: 14611
Total Events: 20000   X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total
All	0, 1023	14611	100.00	73.06
Apoptotic cells	11, 167	369	2.53	1.85
G1/G0 cells	170, 247	8358	57.20	41.79
S Phase cells	249, 342	2786	19.07	13.93
G2+M cells	342, 447	3039	20.80	15.20

(B)

Cancer cells 10.004

G1/G0 cells

Apoptotic cells

G2+M cells

S Phase cells

FL2-A

Histogram Statistics

File: Cancer cells 10.004   Log Data Units: Linear Values
Gate: G2   Gated Events: 6290
Total Events: 10000   X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total
All	0, 1023	6290	100.00	62.90
Apoptotic cells	11, 181	1379	21.92	13.79
G1/G0 cells	184, 253	3305	52.54	33.05
S Phase cells	265, 349	659	10.48	6.59
G2+M cells	353, 467	690	10.97	6.90

Figure3: Flow cytometric analysis was performed to determine the apoptotic effect of -Mangostin on MDA-MB-231 cells. The cells were exposedto -Mangostin (10μM) and 0.1% DMSO for 24h. Then thecells 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.
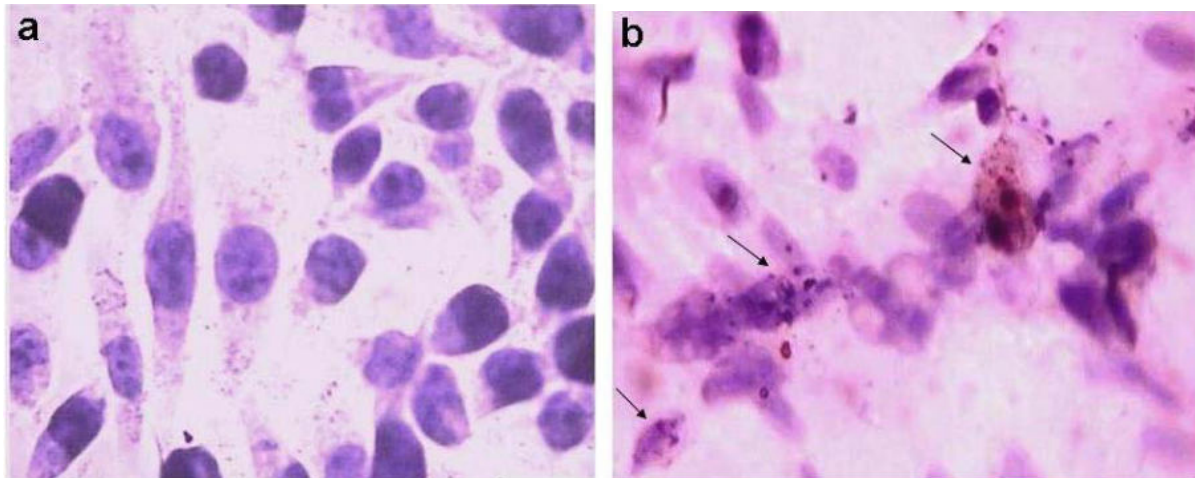
Figure 4: Immunocytochemical staining for detecting caspase-3 expression in MDA-MB-231 cells. a) Control cells (0.1% DMSO) and b) 10µM - Mangostin treated cells after 24h. Arrows shows the expression of caspase-3 as a marker of apoptosis in -Mangostin treated cells. Pictures were taken with a 40x objective under light microscope.

Conclusion

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

Acknowledgement

References

[1]. Chen YC, Gudrun P, Wang TL, Morin PJ, Riseberg B, Kristensen GB, Yu A, Davidson B, Shih IM. Apolipoprotein E is required for cell proliferation and survival in ovarian cancer. Cancer Res. 2005;65:331–337.

[2]. Garg, A D, Nowis D, Golab J, Vandenabeele, P, Krysko DV, Agostinis P. Immunogenic cell death, DAMPs and anticancer therapeutics: an emerging amalgamation. *Biochim Biophys Acta*. 2010;1805:53–71.

[3]. Garrison SP, Phillips DC, Jeffers JR, Chipuk JE, Parsons MJ, Rehg JE. Genetically defining the mechanism of Puma- and Bim-induced apoptosis. *Cell Death Differ*. 2012;19: 642–649.

[4]. Walsh CM, Luhrs KA, Arechiga AF. The "fuzzy logic" of the death-inducing signaling complex in lymphocytes. *J ClinImmunol*. 2003; 23: 333–353.

[5]. Muzio MJ. Ni P. Feng, Dixit VM. IRAK/Pelle family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science*.1997; 278: 1612-1615.

[6]. Lee SH, Meng XW, Flatten KS, Loegering DA, Kaufmann SH. Phosphatidylserine exposure during apoptosis reflects bidirectional trafficking between plasma membrane and cytoplasm. *Cell Death Differ*.2013; 20: 64–76.

[7]. Choi YE, Butterworth M, Malladi S, Duckett CS, Cohen GM, Bratton SB. The E3 ubiquitin ligase cIAP1 binds and ubiquitinates caspase-3 and -7 via unique mechanisms at distinct steps in their processing. *J Biol Chem*. 2009; 284: 12772–12782.

[8]. Chang J. Medicinal herbs: drugs or dietary supplements? *Biochem Pharmacol*. 2000;59:211-219.

[9]. Raghav SK, Gupta B, Shrivastava A. Das HR. Inhibition of lipopolysaccharide-inducible nitric oxide synthase and IL-1β through suppression of NF-κBactivation by 3-(1'-1'-dimethyl-allyl)- 6-hydroxy-7-methoxy-coumarin isolated from *Rutagraveolens* L. *Eur J Pharmacol*. 2007; 560:69-80.

[10]. Nakagawa Y, Iinuma M, Naoe T, Nozawa Y. Akao Y. Characterized mechanism of -mangostin-induced cell death: caspase-independent apoptosis with release of endonuclease-G from mitochondria and increased miR-143 expression in human colorectal cancer DLD-1 cells. *Bioorg Med Chem*. 2007; 15: 5620-5628.

[11]. Navya A, Nanda Kumar Y, Hari Prasad O, Santhrani T. Uma Maheswari Devi P. Anti-inflammatory and Antioxidant Potential of –Mangostin. Current Trends in Biotechnology and Pharmacy.2012;6(3):356-363.

[12]. Navya A, Nanda Kumar Y. Hari Prasad O., Santhrani T. Uma Maheswari Devi P. *In vivo* and *in silico* Analysis Divulges the Anti- inflammatory Activity of A-Mangostin. International Journal of Applied Biotechnology and Biochemistry.2012; 1:69-80.

[13]. Borenfreund E, Babich H. Martin AN. Comparisons of two *in vitro* cytotoxicty assays-the neutral red (NR) and tetrazolium MTT tests. *Toxicol In vitro*.1988; 2: 1-6.

[14]. Ayumi S, Hironori F, Hisae O, Kyoko I. Yasushi O. -Mangostin Induces Ca^{2+}-ATPase Dependent Apoptosis via Mitochondrial Pathway in PC12 Cells. *JPharmacol Sci.* 2004; 95:33-40.

[15]. Lorette CJ. Immunocytochemistry *Methods and Protocols*.2nd ed. Human Publishing;1999.p. 1322.

[16]. Matsumoto K, Akao Y, Ohguchi K, Ito T, Tanaka T, Iinuma M, Nozawa Y.Xanthones induce cell-cycle arrest and apoptosis in human colon cancer DLD-1 cells. *Bioorg Med Chem*. 2005; 13: 6064-6069.

[17]. Lewis JS, Meeke K, Osipo C, Ross E.A, Kidawi N, Li T, Bell E, Chandel NS, Jordan VC. Intrinsic mechanism of estradiol induced apoptosis in breast cancer cells resistant to estrogen deprivation. *J Nat. Cancer Inst*. 2005; 97: 1746-1759.

[18]. Evan G, Littlewood T. A Matter of Life and Cell Death. *Science*.1998; 281:1317-1324.

[19]. Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science*.1998; 281:1312-1316.

[20]. Sakahira H, Enari M, and Nagata S. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature*.1998; 391: 96-99.

[21]. Sahara S, Aoto M, Eguchi Y. Imamoto N, Yoneda Y, Tsujimoto Y. Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation. *Nature*.1999; 401: 168-173.

[22]. Yunjie S, Xi MT, Elizabeth H, Tien KM, Frank AS. Cyclooxygenase-2 Overexpression Reduces Apoptotic Susceptibility by Inhibiting the Cytochrome *c*-dependent Apoptotic Pathway in Human Colon Cancer Cells. *Cancer Res.* 2002; 62: 6323-6328.