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Original Research Article

Effect of A-Mangostin on Anti-Proliferative and Apoptosis Potential against Colorectal Cancer Cells

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Abstract:

Background: α-mangostin, a natural xanthonoid has been reported as anticancer compound against multiple cancer of human origin. The studied was to investigate the anti-cancer activity and its molecular/cellular mechanism in colorectal cancer cell line HCT 116.

Methods: We have performed different methods MTT, FACS and ROS for the anticancer effect of α-mangostin.

Results: We found that, α-mangostin substantially decreased cell viability in time and dosage dependent manner with an IC50 value of 13.34 µM ± 1.86, 9.29 µM ± 1.92 and 4.57 µM ± 0.83 at 24 h, 48 h and 72 h respectively. Furthermore, it was found that α-mangostin significantly decreased the colony formation and cells migratory ability as well. Flow cytometry analysis confirmed that α-mangostin significantly held the progression of HCT 116 cells in G_2/G1 phase of cell cycle and influenced the cells to undergo apoptosis by promoting ROS generation, and decreasing mitochondrial membrane potential and elevated early and late apoptotic cells by increased level of pro-apoptotic Bax, p53 and decreased level of anti-apoptotic BCL-2 genes. RT-PCR analysis also revealed that α-mangostin regulated the expression levels of Bax, p53 and BCL-2 a key apoptotic genes.

Conclusion: These results indicated that α-mangostin could be effectively decrease the growth and proliferation of colorectal cancer cells through regulating the expression of Bax, p53 and BCL-2 leading to apoptosis.

Keywords: α-mangostin, Colorectal Cancer, Cell viability, Apoptosis, P53.

Introduction

Globally, colorectal cancer is second most occurring cancer wild wide after lung cancer and one the major leading cause of cancer related death. [1]. In present era, colorectal cancer incident and motility have found to be increased fast in growing countries [2]. It is expected that the incident of colorectal cancer can be rise upto 60%. [3]. Colorectal cancer is caused by a progressive accumulation of genetic and epigenetic alterations, resulting in significant genomic instability. The bioactive constituent from medicinal plants around the world used as promising source against cancer related aliments is about 70% [4].

Firstly, in 1950 medicinal plant constituent i.e., vinca alkaloid was investigated as anticancer constituent. Then, other medicinal plants constituent come into picture i.e., Vinblatin, Toaxol, irinotecan camtothecin, Vincristine molecules itself and derivative/analog are now a day in clinical use as a anticancer agents. [5]. In order to having established efficacy and safety, natural product specially from plant source and their engineered derivatives has attracted scientist from worldwide towards development of suitable drug against the cancer and can be expected as excellent well as cheap sources of novel anticancer molecules.

The study compound, α-Mangostin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a quinonoid constituent isolated from mangosteen, a tropical fruit native to southeast Asia [6]. From past, the α-Mangostin was used as promising constituents against the skin infection disease, digestion stabilizer, wound infection, suppuration and chronic ulcerd chronic ulcers [7–9]. α-Mangostin has been also documented asan anticancer/anti-proliferative molecule against multiple cancer. One of interesting studies documented that the α-mangostin significantly inhibited tumor growth in mammary cancer mouse models [10]. It has also been shown to induce apoptosis *via* the mitochondrial pathway and inhibited the PI3K/Akt signalling pathway in the human breast cancer cell line MDA-MB231 [11]. In

human mitochondrial-mediated apoptosis, α-Mangostin activates caspase-8 by decreasing the levels of phosphorylated extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and Akt. By inducing mitochondrial dysfunction, α-Mangostin has been reported to have a potent cytotoxicity effect against both COLO 205 human colon cancer cells and human leukaemia HL60 cells [12,13]. α-mangostin was recently found to induce apoptosis and cell cycle arrest in prostate cancer, as well as inhibit tumor growth in Athymic nude mice [14]. Furthermore, it has been shown to reported as inducer of autophagic cell death in glioblastoma cells *via* AMPK activation and inhibits mTORC1 activity [15].

Apoptosis induction in cancer cells is a valuable treatment strategy because the suppression of apoptotic machinery is a hallmark of cancer development. Apoptosis is basically a program cell death and can be disrupted by mutation in particular genes which lead to development of cancer [16]. A significant reduction in apoptosis has been observed in some transgenic mice with a p53 gene mutation. Bax, and caspase9 disruption can promote oncogenic transformation and tumour development [17,18]. Apoptosis-related events could lead to the development of potent and specific therapeutics. In present study, we have demonstrated that α-mangostin may inhibit cell proliferation and induce apoptosis in human colorectal cancer cells HCT 116 cells *via*Bax, Bcl-2 and p53 dependent apoptotic signaling pathways. We found that α-mangostin affected the expression levels of p53,Baxand BCL-2 in colorectal cancer cells by influencing the cell cycle, mitochondrial membrane potential, ROS levels, nuclear morphology as well as progression of apoptosis.

2.0 Materials and Methods:

α-mangostin were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), antibiotic, and trypsin were purchased from Himedia India and Fetal Bovine Serum (FBS) from Gibco, South America. Dimethyl sulfoxide (DMSO) was purchased from CDH laboratory chemical (India). All other chemicals were used of high purity and water also used in experiments was Millipore grade.

2.1Cell line Maintenance

Human colorectal cancer cell line, HCT 116 was obtained from the NCCS, Pune India. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma), at 37°C in a humidified atmosphere with 5% CO2. Cells were passaged every 2-3 days in order to maintain exponential growth.

2.2 Cell viability with the MTT Assay

The MTT test was used to investigate the effect of α-mangostin on the cytotoxicity of HCT 116colorectal cancer cell lines [19] . Briefly, $1x10^4$(Depends on study time point) cells in 96 well plate were seeded and treated with different concentrations of α-mangostin for 24, 48 and72 hours. After completion of incubation period, cells were further incubated with MTT solution for 4 hours at 37ºC in incubator. Following solubilization with DMSO, the number of viable cells was directly related to the amount of formazan produced. Using a 96-well ELISA reader, the absorbance of the colored product was measured at 570 nm. The trials were carried out three times.

2.3 Clonogenic Assay

This assay examined the individual cell in a population for its potential to divide indefinitely and finally formation colonies [20]. α-mangostin treated and un-treated HCT116 cells allow for 13 days in culture medium having 10% FBS under the appropriate culture condition. After that cells were washed and fixed with 4% paraformaldehyde followed by staining with 0.3% crystal violet solution and further image were captured using inverted containing camera.

2.4 Cell Migration Assay

For cell migration assay, briefly cells (1×10^6) were plated in 12-well flat bottom plate. After getting about 90% cells confluency, the sharp wound was created by using sterile 200UL Tips in both untreated an α-mangostin treated cells groups. Following marking the observation wound area, the same region was viewed and captured under the inverted microscope after different time points. Each cells group's wound area was calculated with image j software.

2.5 Cell Cycle Analyses

Briefly, HCT 116 cells were cultured in 6 well plate and waited until for 60-70% confluency. Then, cells were treated with calculated IC_{50} concentration from MTT assay of α-mangostin for 24 hours for examine the influence of α-mangostin on the cell cycle. After that, the cells were stained with propidium iodide (PI), as reported previously [22]. Flow cytometry was used to examine 10,000 events using an excitation wavelength of 488 nm and an emission wavelength of 610 nm. Flow cytometry was per-formed within 25-30 min using BD FACS ARIA III flow cytometer (BD Biosciences. San Jose, CA USA).

2.6 Annexin V/Propidium Iodide Apoptosis Assay and DAPI staining for apoptosis detection:

The assay was performed by using BD FITC Annexin V Apoptosis Detection kit (BD Biosciences, San Diego, CA). According to the

manufacturer's instructions, cells were grown until 60-70% confluent and treated with α-mangostinIC50 calculated from MTT assay for 24 h. Furthers, cells were washed twice with cold PBS, trypsinized and resuspended in binding buffer. Cell count at rate of 1×10^5 cells/mL were stained with Annexin V FITC and propidium iodide for 15 min in the dark. Flow cytometry was performed within 25-30 min using BD FACS ARIA III flow cytometer (BD Biosciences. San Jose, CA USA). [23]

2.7 DAPI staining

For DAPI staining, HCT 116 cancer cells were cultured in 6-well flat bottom plates for 24 hour. After attachment to the surface, cells were treated with IC_{50} concentration calculated from MTT assay and then washed and fixed with 4% paraformaldehyde for 10 minutes and then treated with DAPI for 30 minutes at 37ºC in dark. After that, the cells were rinsed with PBS and analyzed using Nikon fluorescent microscope (Tokyo, Japan). Florescent intensity of each group was calculated suing image J software.

2.8 Mitochondrial membrane potential detection using TMRM

The mitochondrial membrane potential of HCT 116 cells was determined by using TMRM as discussed in [24]. Both the treated and untreated cells were stained with TMRM for 45 minutes at 37ºC, and then washed with PBS buffer for 5 minutes. After washing, the cells were examined under a fluorescent microscope for red fluorescence. Florescent intensity of each group was calculated suing image J software.

2.9 Reactive oxygen species level detection by DCFDA

The Reactive oxygen species(ROS) levels of HCT 116 cells was determined by using DCFDA. After treatment with α-mangostin, both treated and untreated cells were stained with DCFDA for 30 minutes at 37^0C, then washed with PBS buffer for 5 minutes [25]. Samples were immediately examined under a fluorescent microscope for green fluorescence. Florescent intensity of each group was calculated suing image J software.

2.10 Real Time PCR for expression analysis

After the incubation period of α-mangostin, cells were collected, washed with PBS, and RNA was extracted using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 1 μg RNA was utilized to generate cDNA using a cDNA synthesis kit (BioRad Laboratories, Inc.) according to the manufacturer's instructions. Following that, qRT-PCR was carried out in the ABI Quant studio5 using SYBRGreen dye (BioRad Laboratories). Thermocycling conditions were as follows: 95ºC for 5 minutes, followed by 40 cycles at 95ºC for 30 seconds, 58ºC for 30 seconds, and 60ºC for 30 seconds. For the experiment, GAPDH gene was taken as an internal control. The results were analyzed using the 2-ΔΔCq method. Table I indicate lists the primers that were utilized.

Table I: List of primers used for reverse transcription quantitative PCR.

Primer name Primer sequences (5'→3')
BaxF: GCCCTTTTGCTTCAGGGGATG
R: CAGCTGCCACTCGGAAAAAG
p53 F: CCAGCAGCTCCTACACCGGC
R: AAACCGTAGCTGCCCTG
BCL-2 F: CTGCACCTGACGCCCTTCACC
R:CACATGACCCCACCGAACTCAAAGA
F, forward; R, reverse.

2.11Statistical Analysis

All statistical analyses were done using Graph Pad Prism v 7.0. Differences between groups were analyzed either by paired or unpaired t-test, one-way ANOVA, or two-way ANOVA as applicable with appropriate post hoc tests as indicated. All the data are represented as mean±SEM of at least three replicates unless otherwise stated. P values corresponding to $p \leq 0.05$ were considered statistically significant. Statistical significance was assumed for p-values <0.05: *p< 0.05, **p< 0.01, ***p< 0.001.

3.0 Results:

3.1 α-mangostin decreased cell viability of HCT 116Colorectal cancer cells

MTT assay was employed to determine cell viability of Colorectal cancer cell line for 24h 48h and 72 h. α-mangostin was found to be able to significantly kill the cells of colorectal cancer cell lines in time and dose dependent manner. The IC_{50} values for 24 h, 48h and 72 hour were found to be13.34 μM ± 1.86, 9.29 μM ± 1.92, 4.57 μM ± 0.83respectively (Figure 1A) with significant value p<0.001. Cells death was also detected at lower doses, however, it was found that consistently at increasing concentration with increased cell death. Cell morphology was also found to changein case of treatments group and drastic change is cell

morphology was found to observe as to increase in drug concentration that was capture under inverted microscope (Figure 1B).

3.2 α-mangostin hindered the colony formation ability of HCT 116colorectal cancer cells

After getting positive results from cell viability assay, we, further, analyzed the effect of α-mangostin oncolony formation ability of HCT 116 cells. We found a significant low colony formation in case of α-mangostin treated HCT 116cells in compared to control group of cells. Our finding showed that in control group, more than 800 colonies were formed, however, in treated groups, it was found that only 30 colonies were formed (Figure 2A and 2B). Colony formation results confirmed that α-mangostin significantly reduce the dividing potential of HCT 116 cancer cell line as compared to control group.

3.3 α-mangostinhindered the cell migration ability of HCT 116 cells colorectal cancer cells:

After analyzing the effect of α-mangostinon colony formation and anti-proliferative effect of α-mangostin, then study was switch to assess thecells migratory ability of HCT 116colorectal cancer cells. The cells migration potential of studied HCT 116 cells was found to be significantly decreased in 24 h and 48 h (Figure 3A and 3B). At the zero-hour control cells group demonstrated the 71.54%±1.31 and α-mangostin cell group showed 69.452%±2.533 wound area. After 24 hours control cells group showed the 50.77172852%±2.685 wound area, however, α-mangostin cell group showed the 69.266%±2.2031. Similarly 48 hours results demonstrated the control with 26.711%±2.468549507 wound area, however, α-mangostin cell group showed the 64.240±%2.146.Results was indicated the effective efficacies of α-mangostin toward the studied HCT 116 colorectal cell line.

3.4 α-mangostin arrested cells in G2/M phase of cell cycle in HCT 116colorectal cancer cells:

We found thatα-mangostin treatment to inhibit cells growth, colony formation inhibition and inhibition of cell migration in HCT 116 cells. Further, we therefore, switch to next level of experiment to examine the effect of α-mangostinon cell cycle. Our results exhibited that the α-mangostin treated cells arrested in G2/M phase of cell cycle. Cell cycle comparative analysis results showed that cells were arrested in G2/M phase more 12.67% ± 0.66 to 20.73 % ± 1.38 in treated group as compared to untreated group (Figure 4A and 4B).

3.5. Annexin V assay for apoptosis detection in HCT 116 cells:

The FACS data showed that the increased Annexin V positive cells in case of α-mangostin treated HCT 116 cells. Further, it observed the significant differences encountered between the α-mangostin and control groups, with apoptotic cells increasing from 0.4 % ± 0.2 to 72.3 % ± 1.43 % ± and live cells and decreased from 98.8 % ± 0.3 in control group to 25.7 % ±1.6 in α-mangostin (Figure 5A and 5B).

3.6 α-mangostin developed changes in nuclear morphology and chromosomal condensation

DAPI staining showed altered nuclear morphology in α-mangostin treated HCT 116 as compared to normal control group i.e., without treatment.α-mangostin treatment caused nuclear morphological changes including nuclear distortion and nuclear shrinkage, nuclear condensation with nuclear degradation. Finding from DAPI assay indicated the α-mangostin act as apoptosis inducer (figure 6 A and B).

3.7 α-mangostin increased ROS production in colorectal cancer cells:

Our finding from ROS,α-mangostin treated cells showed the increased ROS production in colorectal cancer cells significantly within 5uM and 10uM concentration at 24 h incubation compared with untreated control group (Figure 7A). ROS relative florescence levels were found to be increased in 19.6% when cells incubated with concentration of 5μM, however, at 10 μM concentration 26.6% as compared to control relative florescence level 8.07%.Reactive oxygen Species level elevation by anticancer agents is known to be linked with Apoptosis (Figure 7B)

3.8 α-mangostin decreased the mitochondrial membrane potential in HCT 116 colorectal cancer cells:

α-mangostin treated cells results showed the decrease of mitochondrial membrane potential ascompared to untreated control group at 24 h (Figure 7A and7B). The depolarization of cancer cells was observed to be significantly as the relative florescence intensity decreased from in control group 19.22%to 8.53%at concentration of 5μM&2.52 %at the concentration of 10 μMfor α-mangostin treatments. The depolarization of membrane potential is a potential contributor in programed cell death contributing towards to apoptotic effect of α-mangostin in colorectal cancer.

3.9. Expression analysis of Apoptotic and anti-apoptotic genes through qRT-PCR:

To support the current study's findings, qRT-PCR analysis was performed for the expression of crucial apoptosisrelated genes. qRT-PCR results showed that α-mangostin treated demonstrated the increased level of expression of p53 and bax, however, decreased level of bcl-2 gene expression. The α-mangostin treated group has considerably higher levels of expression of the pro-apoptotic gene Bax

and the tumor suppressor gene p53 (Figure 8). As a result of these findings, apoptosis gene expression was found much greater in cells treated withα-mangostin in HCT116 cells.

Figure Legends

Figure 1: A) **Cytotoxic effect of α-mangostin on HCT 116 cells *in vitro***: Cells were treated with α -mangostin (3.9 to 125μM) for 24h, 48 h and 72h respectively. Error bars indicate the standard error of the mean from three independent experiments. B) Effect of α-mangostin was seen in HCT 116 cells comparison to untreated cells. α-mangostin treated cells showed the morphology changes as compared to un-treated control capture under the inverted microscope.

Figure 2 (A)Effect of A-mangostin on colony formation: Culture dishes with stained colonies of a representative experiment. **(B)** Error bars indicate the standard error of the mean from three independent experiments.

Figure 3 (A and B) **Effect of α -mangostin on wound healing assay:** HCT 116 cancer cell lines were grown to monolayer with full confluency further was wounded the line in both control and treated groups. The images of the wound healing were captured/documented at different time intervals with a magnification of 20×10 fold. Statistical significance was assumed for p-values <0.05: *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 4 (A and B) Effect of α-mangostin against cell cycle progression: Cell progression was restricted in G2/M stage of cell cycle by α-mangostin treatment. Error bars indicate the standard error of the mean from three independent experiments.

Figure 5 (A and B): Detection of apoptosis by Annexin V FITC and Propidium Iodide staining after treatment with α-mangostin**(A)** Dot plots showing apoptosis of HCT 116 cells in response to exposure to α-mangostin**(B)** Percentage of cell population of live and Apoptotic cells. Error bars indicate the standard error of the mean from three independent experiments.

Figure 6 (A) Fluorescence microscopic images of untreated control and α-mangostin treated HCT 116 cancer cells. Control cells were with intact nucleus whereas treated cells showed nuclei distortion and shrinkage as

signs of apoptosis by DAPI staining. **(B)** Error bars indicate the standard error of the mean from three independent experiments.

Figure 7 (A and B) Fluorescent microscopy by DCFDA and Mitochondrial membrane potential detection by TMRM: α-mangostin treated cells showed increase in green florescence which is due to increase (DCF) of ROS levels as compared to untreated cells. In case of untreated cells TMRM stained mitochondria showed intact mitochondrial membrane potential in comparison to α-mangostin treated groups where the mitochondria were less stained due to damage to mitochondria. Error bars indicate the standard error of the mean from three independent experiments. Statistical significance was assumed for p-values <0.05: *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 8. Expression of apoptotic markers. α-mangostin induced apoptosis was confirmed by detecting the upregulation of apoptotic genes Bax, p53 and BCL-2 in both treated and untreated HCT116 cells. (T1 = Treated HCT116 cells with 5 μM, T2 = Treated HCT116 cells with 10 μM)

4.0 Discussion:

There is urgent need of new technology and innovative chemo-preventive in order to enhance the efficiency of current cancer therapy. Plants are sources of naturally occurring phytochemicals and important factor for development of new and effective medicine and can be used to cure cancer [26].

Various chemotherapeutic agents such as oxaliplatin, leucovorin and irinotecan are commonly used to show a severe adverse effect [27]. These photochemical frequently affect the molecular pathways that have been linked to cancer development and progression. Antioxidant reported as major player to minimize the cancer progression. Increased level of antioxidant status can lead to inactivation of carcinogen, proliferation inhibition, induction of cell cycle arrest and apoptosis [28].

Previously,α-mangostin was found to be effective against multiple types of cancer cell lines in dose and time dependent manner including breast, lung and others cancers as well. α-mangostin showed cytotoxicity effect in HCT 116 colorectal cancer cells with IC_{50} values 13.34 μM ± 1.86, 9.29 μM ± 1.92, and 4.57 μM ± 0.83 in 24 h, 48 h and 72 h respectively. Acquiring metastatic property is major problem by which cancer cells move easy, the α-mangostin act as suppressor of metastatic property has been documented in against several cancer cells [10].Our study also supported that α-mangostin restricted the migration of cells. At the zero-hour control cells group demonstrated the 71.54%±1.31 and α-mangostin cell group showed 69.452%±2.533 wound area. After 24 hours control cells group showed the 50.77172852%±2.685 wound area, however, α-mangostin cell group showed the 69.266%±2.2031. Similarly, 48 hours results demonstrated the control with 26.711% ±2.468549507 wound area, however, α-mangostin cell group showed the 64.240±%2.146. The antimigratory effect was found significantly effective in both 24 h and 48 h.

Cell cycle is controlling mechanism that regulated by series of event/protein and cell cycle control the mechanism of cell proliferation/cell division/cell duplication. The dysregulation of mechanism of regulated cell cycle progression can be lead to unregulated/aberrant cell division which further lead to be cancer. There is a huge of therapeutic agents were reported to use to arrest the cell cycle at a specific check point in order to control the dysregulated cell division [29,30].

There is series of genes/protein involve in regulation of program cell death and maintaining tissue growth and homeostasis as well. Apoptosis is program cell death, it helps in maintaining the tissue hemostasis. If there is any regulation occurs in apoptotic pathway can be lead to irregulation in program cell death. In case of normal circumstance, it was found that apoptosis pathway is activated, however, in case of cancer the suppression of apoptosis has been reported [31,32]. It is linked with number of morphological alterations in cells, including chromatin condensation, DNA fragmentation, and nuclear blebbing. DAPI is a fluorescent dye that has been shown to enter damage cell pores and stain fragmented nuclear DNA. The quantity of chromatin condensation is shown by the intensity of the DAPI stain. Normal and healthy cells with no chromatin condensation are shown by the light and uniform DAPI stain, whereas condensed chromatin is represented by the brighter stain [33,34]. Apoptosis is characterized by condensed chromatin [35]. α-mangostin could induce apoptosis in colorectal cancer cells line HCT 116 investigated by FACS and DAPI staining. We found that it increased the apoptotic cell population as compared to control group by DAPI staining. It revealed that chromosomal deformities and increase in fluorescence in treated cells lead to the apoptotic. Our data on the apoptotic effects of α-mangostin in colorectal cancer cells support the well-investigated properties of α-mangostin as a strong anti-cancer candidate. Cell cycle arrest and depolarization of mitochondrial membrane potential were showed the characteristic future of anticancer drugs. α-mangostin has shown a cell cycle arrest properties and decrease in mitochondrial membrane potential in colorectal cancer. We also found this property to be true as HCT 116 cells were restricted in G2/M phase of cell cycle.

It is well establishing fact that the expression of BCl-2 has found to be reported high in case of malignancies, however, the expression of Bax and p53 have been reported low. Bax is key player of mitochondrial dependent apoptosis pathway that regulates the downstream pathway genes/proteins i.e., cytochrome c, caspases and lead to apoptosis[35]. There is number of genes expression at transcriptional level have been identified that regulated by p53. The transactivation of bax by p53 have been reported. The activated bax can be translocated from cytosol to mitochondrial membrane and trigger the released of mitochondrial cytochrome cand activate the caspase-9 occur and followed by activation/release of caspase-3, 6 and 7. In normal cells, it was found to report that the expression of bax and p53 high, however, the expression of Bcl-2 found to be reported low [36].

Conclusion:

Our finding with α-mangostin treated cells demonstrated the similar pattern of results, in which treated cells high expression of bax and p53, however, low expression of Bcl-2.

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