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INDUCTION OF APOPTOSIS BY XANTHONES FROM *GARCINIA MANGOSTANA* IN HUMAN BREAST AND LARYNGEAL CARCINOMA CELL LINES

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ABSTRACT

The present study is an attempt to evaluate the standardization of crude mangosteen and γ – mangostin from *Garcinia mangostana* on MCF-7 (breast cancer cell line) and Hep2 (laryngeal cancer cell line). The study includes cell doubling time, ED_{50} analysis (growth inhibition), cell proliferation assay and assessment of cell cytotoxicity by dye exclusion assay. The result of the study showed that the cell doubling time was found to be 72 hours for MCF -7 cells and 48 hours for Hep2 cells. Growth inhibition assay for MCF-7 and Hep2 showed 2.5 and 5.25µg (crude mangosteen) and 0.85 and 1.75µg (γ – mangostin) respectively. The mitochondrial survival and cell cytotoxicity was significantly less when compared to control groups.

KEY WORDS

Garcinia mangostana, Apoptosis, Cell proliferation, Cytotoxicity, Cell lines.

INTRODUCTION

Breast cancer is the second most common cancer in women. In India, risk of breast cancer is higher in the urban areas than in the rural areas. International Agency for Research on Cancer estimated 80,000 women in India were affected in 2002. Average incidence in women varies from 22 - 28 / lakh in urban areas to 6 / lakh in rural areas[1]. Breast cancer is very rare below 30 years of age. The incidence rises steeply with advancing age up to about 50 years, thereafter, incidence still increases more slowly with increase in age[2]. Breast cancer incidence in different states of India accounts for 25%, 21%, 18%, 17%, 13% and 6% in respective of Delhi, Mumbai, Chennai, Bangalore, Calcutta and Rural areas. Same way squamous cell carcinoma of the head and neck is one of the most common cancer world wide, with incidence of more than 30 per 1,00,000 population in India. The ratio among men and women are 3:1. Over 95% of laryngeal cancer arise from the lining of the throat and are thus called squamous cell carcinoma[3].Cell lines are widely used in many aspects of laboratory research particularly as *in vitro* models in cancer research. They have a number of advantages; for instance, they are easy to handle and represent an unlimited self – replicating source that can be grown in almost infinite quantities. In addition, they exhibit a relatively high degree of homogeneity and are

easily replaced from frozen stocks if lost through contamination. However some disadvantages too. Cell lines are prone to genotypic and phenotypic drift during their continual culture. This is particularly common in the more frequently used cell lines, especially those that have been deposited in cell banks for many years. Subpopulations may arise and cause phenotypic changes over time by the selection of specific, more rapidly growing clones within a population[4].

The most commonly used breast cancer cell line, namely MCF – 7 of various laboratories had many discrepancies such as variation in cell growth rate, changes in hormone receptor content, karyotype and clonogenicity, despite the cells appearing morphologically identical[5]. The present study was aimed to evaluate the growth rate of MCF – 7, cell lines provide a useful *in vitro* model to study the efficacy of plant extract on these cell lines. The resistance of cancer cells to multiply chemotherapeutic agents poses a major problem in the successful treatment of breast cancer. Whether drug resistance is due to changes induced in the drug – exposed tumor cells or represents the selective growth of one or more drug – resistant clones present in the initial tumor remains controversial[6].

The fruit hull of the mangosteen (*Garcinia mangostana)* has been used as a traditional medicine for the treatment of skin infections, wounds, and diarrhea in South East Asia[7]. The hull contains various derivatives including α - mangostin and γ - mangostin which has anti inflammatory[8], hypolipidemic[9] and anti - bacterial activities on *Staphylococcus aureus*, *Shigella dysentriae, Shigella flexineri, Escherichia coli, Vibrio chlorae* and *Helicobacter pylori*[10]. The pericarp of *Garcinia mangostana* was reported to be the source of mangostin, tannin, xanthone, chrysanthemin, garcinone, gartanin, vitamin B_1, B_2, C and other bioactive substances[11].

Thus the development of new therapeutic approach to breast cancer remains one of the most challenging areas in cancer research. Many tropical plants have interesting biological activities with potential therapeutic applications. The present investigation was undertaken to evaluate the effective dose analysis, cell viability and cell proliferation assay of crude mangosteen and γ – mangostin from *Garcinia mangostana* on MCF – 7 and Hep2 cell lines.

MATERIALS AND METHODS

Preparation of Mangosteen extract: Concentrations of mangosteen were ranging from 1 – 14 µg and γ – mangostin 0. 5 to 4 µg were dissolved in 0.1 % DMSO (Sigma, St. Louis, USA) in culture medium and filtered through 0.2 µm filter. The filtrate was stored at 4 ℃ for further analysis.

Cell lines and culture: MCF – 7, and Hep2 were obtained from National Centre for Cell Science, Pune, and cells were cultured in Dulbeco's Modified Essential Medium (Gibco, India) supplemented with 10 % heat – inactivated fetal bovine serum (Gibco, India), 3 % L - Glutamine, 100 IU / ml Penicillin (Himedia, India) and 100 µg / ml Streptomycin (Himedia, India). The cells were incubated at 37 ℃ in a humidified atmosphere with 5 % CO_2.

Analysis of cell doubling time: A total of 4 x 10^4 cells / well were seeded in a 24 well plate with 1 ml of 10 % Dulbeco's Modified Essential Medium. The plate was incubated at 37 ℃ in a humidified CO_2 chamber for 7 days. The plate was observed under inverted microscope for microbial contamination, turbidity and pH change. These wells were washed with phosphate buffered saline and trypsinized with trypsin EDTA. Cells were mixed with 0.1 % trypan blue and counted within 2 minutes using a haemocytometer. Likewise up to 7 days, the cells were observed and counted for the growth rate.

ED_{50} analysis of crude mangosteen and γ-mangostin: Cells were seeded in the

concentration of 4 x 10^4 cells / well in a 24 well plate and it was incubated at 37 ℃ in a 5% humidified CO_2 chamber for 72 hours for MCF-7 and 48 hours for Hep2 cells. Various concentrations of crude mangosteen ranging from (1µg, 2µg, 4µg, 6µg, 8µg, 10µg, 12µg, and 14µg / ml) and γ – mangostin (ranging from 0.5µg, 1µg, 1.5µg, 2.0µg , 2.5µg 3.0µg, 3.5µg and 4.0µg) to the wells. 4 wells / concentration were used for the analysis. Then the cells were incubated for an additional 24 hours. After 24 hours, cells were washed with phosphate buffered saline and trypsinized. Cells were mixed with 0.1% trypan blue and counted using haemocytometer. The ED_{50} was derived from the following equation.

Total number of live cells in 4 corners = X
Total number of live cells / ml = X / 4 * dilution factor * 10^4
= Z cells / ml

***Cell Proliferation Assay*:** A total of 1 x 10^4 cells / well were seeded in a 96 well plate and incubated at 37 ℃ in a 5% humidified CO_2 chamber for 72 hours for MCF-7 cells and 48 hours for Hep2 cells. After incubation of respective period, ED_{50} concentration of the crude mangosteen and γ-mangostin were added to the wells. Cells with 0.1% DMSO served as a solvent control and cells without any addition were considered as test control. The cells were incubated for an additional 24 hours. After 24 hours of incubation, 1mg/ml of MTT [3-(4, 5-dimethylthiazol – 2 –yl) -2, 5 – diphenyl tetrazolium bromide], in phosphate buffered saline was added to each well and incubated for 4 hours at 37℃. The formazan crystal was dissolved in DMSO and the optical density was measured at 590 nm in a UV-Spectrophotometer[12].

The growth inhibition was determined by

Growth inhibition (%) = control OD– sample OD/control OD* 100

Cell Cytotoxicity assay: MCF-7 and Hep2 cells were seeded in a 96 well plate at the same concentration as above at 37 ℃ in a 5% humidified CO_2 chamber for respective hours of incubation. 2.5 µg concentration of crude mangosteen and 0.85 µg of γ – mangostin were added to 4 wells. Likewise 5.25 and 1.75µg for Hep2 were added and four wells served as control (without drug) and 0.1% DMSO was added to other four wells, which was served as solvent control. Then the cells were incubated for an additional 24 hours, after 24 hours of incubation, cells were washed with phosphate buffered saline and trypsinized. Viable cell counting was performed by enumerating cells with trypan blue and counted using haemocytometer. The percentage of viable cells (%) was calculated by the method of Kummalue *et al*[13].

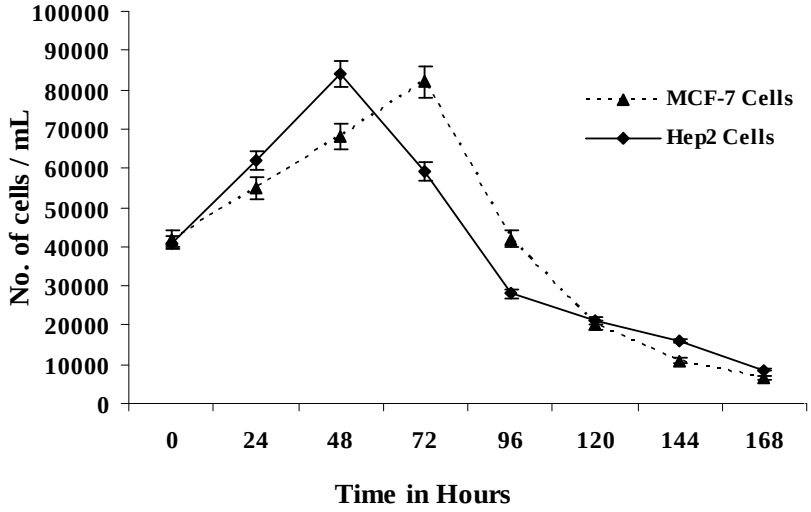
[Total cells – Dead cells / Total cells] * 100

Statistical Analysis: All the experiments were performed in tetrads. The data for cell proliferation and cell viability assay were expressed as mean ± SD. *p* value less than 0.05 were considered significant.

RESULTS

Cell doubling time : The cell doubling time was found to be 72 hours to double it self in cell number for MCF-7 and 48 hours for Hep2 cells (Fig.1), provided 5% CO_2 and 10% DMEM at 37℃.

Fig. 1.
Cell doubling time for MCF – 7 & Hep2 cells



Growth inhibition by crude mangosteen and γ – mangostin : The growth inhibition of the crude mangosteen (Fig. 2) and γ – mangostin (Fig. 3) on human breast and laryngeal cancer cells is shown.

Fig. 2.
Growth inhibition by crude mangosteen on MCF 7 & Hep2 cells

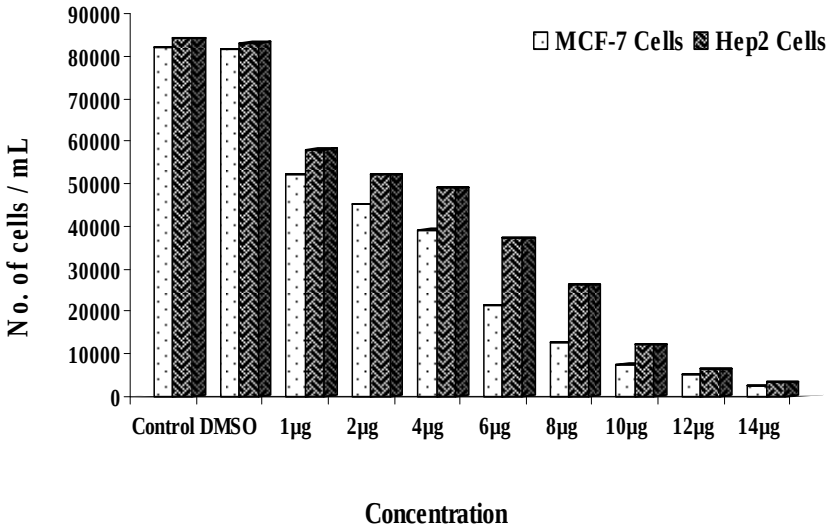
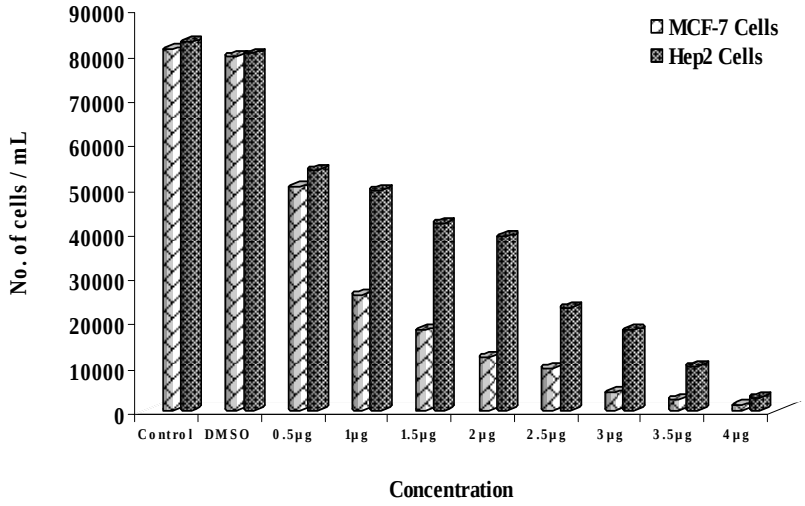


Fig 3.
Growth inhibition by γ - mangostin on MCF – 7 & Hep2 cells



ED_{50} (50% inhibition of cancer cell growth) analysis was done with the concentration range from 1 to 14 µg for MCF – 7 and Hep2 cell lines. ED_{50} values of 2.5µg and 5.25 µg showed the 50% inhibition on MCF – 7 and Hep2 cell lines respectively for crude mangosteen. Concentrations of γ – mangostin with the range from 0.5 to 4.0 µg were studied on both the cell lines. ED_{50} values of 0.85µg and 1.75 µg were obtained for MCF – 7 and Hep2 cell lines respectively. Therefore, this concentration was used to investigate the further study on the effects of this herbal extract on these cell lines.

Effect of crude mangosteen and γ – mangostin on cell proliferation:

Cell Proliferation assay on crude and γ - mangostin on MCF– 7 and Hep2 cells

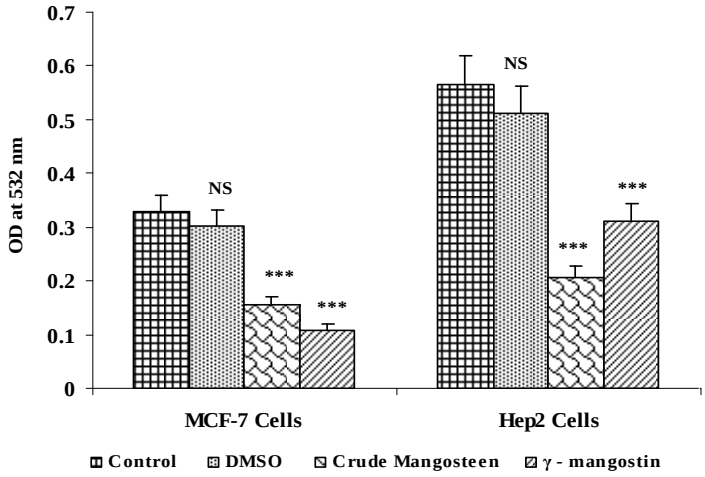


Figure 4

Comparison of DMSO, crude mangosteen, γ-mangostin groups with control group and statistically significant values as given *** p<0.001; NS- non significant

Effect of crude mangosteen and γ – mangostin on cell proliferation was measured using MTT, which is shown in fig. 4. After treatment with both the drugs, the mitochondrial survival was significantly reduced when compared to control (p<0.001). There was no significant changes were observed on DMSO treated control on these cell lines.

Effect of crude mangosteen and γ – mangostin on cell viability:

Effect of crude mangosteen and γ – mangostin on cell viability

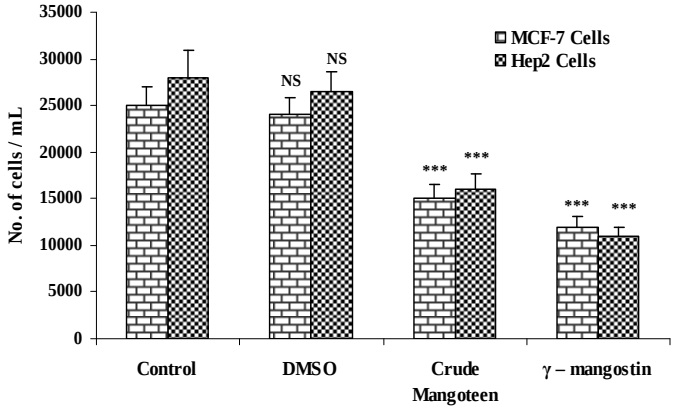


Figure 5

Comparison of DMSO, crude mangosteen, γ-mangostin groups with control group and statistically significant values as given *** p<0.001; NS- non significant

Cytotoxicity effect of crude mangosteen and γ – mangostin on MCF – 7 and Hep2 cells were performed and shown in fig. 5. The cells were treated with effective concentrations of crude mangosteen, 2.5 µg / ml for MCF – 7 and 5.25 µg / ml for Hep2 cells were showed a significant change (p<0.001) in cell number when compared to control. 0.85 and 1.75 µg / mL concentration of γ – mangostin showed a significant decrease (p< 0.001) in cell viability in both the cell lines. No significant change in cell viability was observed in DMSO treated group when compared with control.

DISCUSSION

Gracinia mangostana contains a variety of secondary metabolites, such as oxygenated and prenylated xanthones. These xanthones have various biological functions like anti – inflammatory[14] and anti – bacterial[15] were reported. The inhibitory growth activity of the treated cells acted in a dose dependent manner which was confirmed by the determination of minimum inhibitory concentration on these cell lines. Percentage cytotoxicity was measured using trypan blue exclusion assay which showed a gradual decrease in cell number by drug toxicity due to the components present in the *Gracinia mangostana* induced apoptosis[16]. Anti – proliferate activity was measured using mitochondrial survival rate which demonstrated that the active components of the plant extract may play an important role in cell killing[17]. Though the breast cancer therapy which has usually multimodality treatment is in advance, herbal drugs play an important role by killing of cancer cells and increasing survival rates along with

improve in quality of life of patients[18]. The results of this study confirmed that the MTT assay to be a fast, simple, inexpensive, and accurate method for the determination of cell densities in the cell cultures[19].

Therefore, the development and search for novel and effective anti cancer agents have become very important issues[1]. To date, many toxic agents including natural products isolated from plant sources have been investigated for the discovery of the potential novel anticancer drugs. Higher plants have long been shown to be excellent and reliable sources for the development of novel anti cancer drugs. However, the property of this plant, especially its anti - cancer activity, has not yet been investigated in epidermoid and adenocarcinoma together. Hence, this prompted us to investigate the inhibitory growth effect of this plant on MCF – 7 and Hep2 cell lines. Further analysis is necessary to prove its anti - cancer activity in various cell lines with more parameters.

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