Antimetastatic Activity of Garcinone E

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Abstract

Objective: Oral cancer presents as a devastating type of malignancy. It is predominant in populations with high use of alcohol and various forms of tobacco as well as poor diets with low intake of fruits and vegetables. The present study focused on the potential of Garcinone E to inhibit HSC-4 oral cancer cell proliferation, migration and invasion. Methods: MTT and colony forming assays were performed to study antiproliferative effects of Garcinone E. Hoechst staining was used to determine levels of apoptosis, with cell invasion and scratch assays conducted for migration and invasion characteristics. The levels of MMPs and cytokines were quantified in Garcinone E treated cells by ELISA. Results: Garcinone E inhibited the proliferation and colony forming potential of HSC-4 cells. It also suppressed migration and invasion with inhibition of MMP-2 and MMP-9 expression. Moreover, it elevated IL-2 and reduced IL-6 expression in HSC-4 cells. Conclusion: Our results demonstrate for the first time that Garcinone E might inhibit metastasis of an oral cancer cell line by blocking invasion, migration and MMP production.

Keywords: Garcinone E- oral cancer- MMPs- invasion- wound healing assay- interleukine-6

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Introduction

Cancer remains a complex disease and a major health issue to the society. Oral cancer is a subtype of head and neck cancer. It is a broad term that includes various malignancies include cancer of the lip, floor of mouth, buccal mucosa, gingiva, palate or in the tongue (Pablo et al., 2015). It is considered as the sixth most common malignancy worldwide with significant recurrence and frequent metastasizes to cervical lymph nodes (Okura et al., 2009; Chang et al., 2016). Classical cancer treatments rely on surgery, radiation and chemotherapy. Majority of the treatment approaches has adverse side effects and causes many serious health issues (Mondal et al., 2015). The treatments are often failed to prevent disease progression due to metastasis. Metastasis is the process of disseminating cells from the primary site into secondary site. It is a multistep complex process involving detachment of cells from primary site, enter into circulation, adhesion in the inner membrane of blood vessels, extravasation, colony formation and finally angiogenesis (Steeg, 2016; Turajlic and Swanton, 2016). All steps in the metastatic cascade must be completed for successful manifestation of metastasis. It is well documented that each of the events represent ideal target for antimetastatic therapy (Stoletov et al., 2014). Modern technology has developed sophisticated treatment modalities but the side effect as well as the development of resistant cell type reduced the survival rate in cancer (Arruebo et al., 2011; Housman et al., 2014). Hence more efficient and less toxic therapeutic approaches are needed. Studies have revealed that consumption of fruits and vegetables rich in phytochemicals may reduce the risk of development and/or progression of tumor (Steinmetz and Potter, 1996; Kundu et al., 2014; Turati et al., 2015, Key, 2011; He et al., 2017). It can also be given as adjuvant therapy along with radiation and chemotherapy to lower the treatment induced adverse effects. Research has been conducted by several group of scientist all over the world to exploit the potential of natural compounds to defeat cancer and some of them are in use and many more yet to be explored.

Garcinia mangostana is a tropical tree with exotic, round, purple color fruit. It is quite popular for its snow-white, juicy, delicious arilst. It received great attention as a nutritional therapeutics due to rich source of pharmacologically relevant molecules called xanthones. Xanthones exhibits antibacterial, antioxidant, antiinflammtory activities (Zarena and Sankar, 2009). Garcinone E, one of the xanthone derivatives present in Garcinia mangostana. Ho et al., reported for the first time that Garcinone E induced cytotoxicity in different cancer cell lines but its mechanism is yet to be explored. (Ho et al., 2002). Recent study indicates that Garcinone E could induce apoptosis and inhibit invasion in cervical cancer cell progression (Xu et al., 2017). No study has
been conducted to exploit the effect of Garcinone E on oral cancer cells. In the current study we have evaluated the effect of Garcinone E on metastasis of human oral squamous cell carcinoma cell line (HSC-4).

Materials and Methods

Chemicals

Dulbecco’s Modified Eagle’s Medium (DMEM), antibiotic and antimycotic solution and Hoechst 33342 were obtained from Sigma (USA). Foetal Bovine Serum (FBS) was purchased from GIBCO laboratories (Grand Island, NY). MTT was purchased from Himedia Laboratories (India). Cytokine ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, USA). Garcinone E was purchased from Wuhan Chem Faces Biochemical Co Ltd. (Hubei, China). All other reagents and chemicals used were of the highest purity grade.

Cell Culture

HSC-4 cells line was kindly provided by Dr. Tessy (Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum, Kerala, India). Cells were maintained in high glucose DMEM supplemented with 10% FBS and 1% antibiotic and antimycotic solution and incubated in a humidified atmosphere with 5% CO₂ at 37°C. Exponentially growing cells were used for experiments.

Cytotoxicity assay

The cytotoxic activity of the Garcinone E was determined using MTT assay (Romijn et al., 1988). Briefly, HSC-4 cells were seeded (5x10⁴ in 200µl) in 96 well plate in triplicates and incubated for 24h and then treated with different concentrations of Garcinone E (0.5, 2, 4, 6, 8 and 10 µM). Control wells were cultured in DMEM without Garcinone E. Cells treated with DMSO (Dimethyl sulfoxide) was kept as vehicle control. After 20h of treatment, 100 µl of the medium was removed and replaced with 100 µl DMEM containing 20 µl of MTT solution (5mg/ml). Cells were incubated again at 37°C in CO₂ for 4h. Following incubation, 100µl DMSO was added to each well and the colour intensity was measured at 570 nm using a micro plate reader (Epoch Microplate Reader, BioTek Instruments, Winooski, VT, USA). All the experiments were done in triplicates and the data were represented as percentage of cell viability with respect to untreated cells. It is calculated using the equation:

Percentage of cell viability = Mean OD of Test/ Mean OD of Control X 100

Colony forming assay

Effect of Garcinone E on colony formation ability of HSC-4 cells was performed using clonogenic survival assays (Franken et al., 2006). Cells were pretreated with different concentrations of Garcinone E (2.4, 4.8 and 6.25 µM) for 24h, trypsinized and the viable cells were plated at a density of 2,000 cells/60mm dish. Cells were allowed to grow for additional 10 days and colonies were fixed, stained with 0.5% crystal violet and counted. DMSO (0.1%) treated cells were kept as vehicle control. All treatments were performed in triplicate and results expressed as mean ± SD.

Hoechst 33,342 staining

Hoechst staining is performed to study the morphological changes in the cells (Cariddi et al., 2015). HSC-4 cells were seeded into 96-well plates at a cell density of 4 x10³ cells per well, cultured overnight, and treated with Garcinone E for 24h. After incubation, cells were washed with PBS and 10 µM of Hoechst 33,342 was added and incubated again for 15 min in the dark and morphological changes were observed using fluorescent microscope.

Adhesion assay

Cell adhesion assay was performed in 96 well microtiter plate coated with Type I collagen (20 µg/ml) (Ho et al., 2011). Cells were seeded at a density of 4x10⁴ (200µl) and incubated in the absence and presence of different concentrations of Garcinone E (2.4, 4.8 and 6.25 µM) for 5h in 5% CO₂ incubator. At the end of the incubation period, cells were washed gently with PBS to remove unattached cells. The cells were stained using 0.5% crystal violet and photographed using inverted microscope. Cells then lysed with solution of 0.1N HCl in methanol and absorbance was measured at 550 nm in a micro plate reader. The number of attached cells was determined from the absorbance. The assays were performed in triplicate wells. The data were expressed as percentage of adhesion compared with control.

Wound healing assay

The effect of Garcinone E on cell migration was tested by wound healing assay (Liang et al., 2007). HSC-4 cells were cultured in 96 well plates (5x10⁴ cells/well) and allowed to form a confluent monolayer. After 24h of incubation, scratch or wounds were created by scrapping the monolayer cells using sterile microtip (200µl). Subsequent to wounding, the cells were gently washed with serum free medium to remove dislodged cells. The cells were again incubated in fresh medium in control dishes and with fresh medium and different concentrations of Garcinone E (2.4, 4.8 and 6.2 µM) in treated dishes. The cells were observed at different time intervals (0h, 24h and 48h) after scrapping. The migration of cells was analyzed by the decrease in the distance between wounded edges using inverted microscope and photographed. The percentage of wound closure was calculated using the formula:

Percentage wound closure = Area of initial wound at time t0- Area of wound at time t1/ Area of initial wound at time t0 x 100

Invasion assay

Cell invasion assay was performed in cell culture inserts with collagen coated membrane. Briefly, cell suspensions containing, 5x10⁴ cells were suspended in serum free DMEM and seeded into the upper chamber and different concentrations of Garcinone E (2.4, 4.8 and 6.25 µM) was added. DMEM containing 20% FBS was...
added in the lower chamber and incubated for 24h. After incubation period non invading cells on the upper surface of the membrane were removed with a cotton swab and invading cells on the lower surface of membrane were fixed with methanol, stained using 0.5% crystal violet and photographed. The number of cells were counted and expressed as % of inhibition in the invasion compared to control. Three independent experiments were performed.

Estimation of Interleukin-IL-6 (IL-6)

The detection of IL-6 in cell culture supernatant after Garcinone E treatment (2.4, 4.8 and 6.25 µM) was performed by ELISA according to the manufacturer instructions. Human IL-6 was diluted (500-31.25pg/ml) and used as a standard to establish the standard curve. Average results of 3 independent experiments were compared by measuring the optical density at 450 nm.

Estimation of Interleukin-2 (IL-2)

The detection of IL-2 concentrations in cell culture supernatant of Garcinone E treated cells were performed by ELISA according to the manufacturer instructions. Recombinant human IL-2 was diluted (4,000–250pg/mL) and used to establish the standard curve. Average results from 3 independent experiments were compared by measuring the optical density at 450 nm.

MMP 2 and MMP 9 profile in culture supernatant of HSC-4 cells

The concentration of MMP 2 and MMP 9 in the supernatants of the cells treated with different concentrations of Garcinone E (2.4, 4.8 and 6.25 µM) was determined by enzyme linked immunosorbent assay (ELISA) as described by the manufacturer. Average results from 3 independent experiments were compared by measuring the optical density at 450 nm.

Statistical analysis

Statistical analysis of data was performed by using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test. The results are presented as Mean ± S.D. P < 0.05 was considered statistically significant.

Results

Anti-proliferative effects of Garcinone E

MTT assay was performed to evaluate the anti-proliferative activity of Garcinone E. The result of the experiment is given in the Figure 1. Concentration depended decrease in the cell viability was observed when the HSC-4 cells were treated with different concentrations of Garcinone E for 24h. Garcinone E at a concentration of 0.5µM produced 97.96±5.00% viable cells and at 10 µM, percentage cell viability was 11.78±1.63. The percentage of viable cells in DMSO treated cells was 99.95±5.69. IC 50 value was calculated as 4.8µM.

Effect of Garcinone E on colony formation of HSC-4 cells

Clonogenic assay is used to check ability of cells to grow and form colony. In the colony formation assay, Garcinone E effectively inhibited the colony formation in dose dependent manner. It was observed that at 2.4 µM, there was no significant change in number of colonies formed when compared to untreated cells but when the cells were treated with high concentrations significant inhibition was observed. Garcinone E at 4.8 µM and 6.25

Figure 1. Effect of Garcinone E on Cell Viability was Assessed by MTT Assay. Cells were treated with different concentrations of (0.5, 2.4, 6 and 10 µM) for 24h. After incubation, absorbance was measured at 570nm and viability was calculated. Results are expressed as percentage of viable cells. Data are mean ± SD values of three independent experiments, ***P<0.001.

Figure 2. Effect of Garcinone E on colony formation by clonogenic assay: HSC-4 cells were treated with different concentrations of Garcinone E for 24h and cells were cultured for 10 days and photographed. (a) Untreated control (b) DMSO treated control (c) Garcinone E 2.4 µM (d) Garcinone E 4.8 µM (e) Garcinone E 6.25 µM. The numbers of colonies were counted (f). Results are mean ± SD (n = 3). ***p < 0.001
µM produced 8.5±2.6 and 1.83±1.47 colonies respectively (Figure 2). Along with decrease in the number of colonies, there was decrease in the size of colonies as compared to control.

Effect of Garcinone E on apoptosis of HSC-4 cells

After treatment of 24h, HSC-4 cells were observed for morphology changes under inverted microscope (Figure 3). Garcinone E treated HSC-4 cells showed uneven staining and apoptotic morphology. However, the control cells were uniformly stained and showed normal nucleus without any type of condensation and fragmentation. This indicates that Garcinone E could induce apoptosis.

Table 1. Effect of Garcinone E on Migration of HSC-4 Cells. Confluent monolayers of HSC-4 cells were scratched, rinsed to remove debris, and incubated in the absence or presence of different concentration of Garcinone E for 48h. Wounded area were photographed at different intervals and measured by imagej software. Table showing the % wound closure. Results are mean ± SD (n = 3). ***p < 0.001.
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Effect of Garcinone E on adhesion of HSC-4 cells

The results of the adhesion analysis is presented in Figure 4. No significant changes in the adhesion property of HSC-4 cells were observed when they were exposed to Garcinone E. This indicates that Garcinone E is not interfering with the adhesion potential of oral cancer cells.

Effect of Garcinone E on migration of HSC-4 cells

In wound healing assay active cell migration was observed in untreated control cells in which the width of the wound narrowed at 24h and completely closed over 48h period. The results were shown in the Figure 5. Treatment with Garcinone E found to be significantly inhibited the migration of HSC-4 cells in a concentration dependent manner. When the cells were treated with Garcinone E (2.4 μM) 58.67±3.06% wound closure was observed at 24h and at 48h wound completely closed. At a concentration of 4.8 μM, HSC-4 cells showed 17.14 ±1.70% (24h) and 24.76±1.98% (48h) of wound closure when compared to time t0. Treatment with 6.25 μM of Garcinone E produced 0% wound closure at 24h and 4.90±0.84% wound closure at 48h (Table 1). The results indicate the antimigratory potential of Garcinone E against HSC-4 cells.

Effect of Garcinone E on invasion of HSC-4 cells

The result of the invasion assay is given in the Figure 6. Garcinone E significantly inhibited the invasion of HSC-4 cells as indicated by the decreased number of cell present on the lower side of the membrane compared to control cells. When the cells were exposed to 2.4 μM of Garcinone E, 15.14±0.27 % inhibition of cell invasion was observed. Treatment with 4.8 μM and 6.25 μM showed 53.48±1.5% and 90.61±3.87% inhibition respectively. Cells treated with DMSO showed similar pattern of invasion compared to untreated control cells. These results

Figure 7. IL-6 Profile in Culture Supernatant of HSC-4 Cells. Control cells were grown in growth media without any treatment. Experimental cells were exposed to different concentration of Garcinone E (2.4, 4.8 and 6.25 μM) for 24h. After incubation, culture supernatant was collected, centrifuged and IL-6 level was analysed using ELISA Kit. Results were expressed as % of inhibition. Data were represented as means ± SD. ***p < 0.001

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Figure 8. IL-2 profile in culture supernatant of HSC-4 cells. Control cells were grown in without any treatment. Experimental cells were exposed to different concentration of Garcinone E (2.4, 4.8 and 6.25 μM) for 24h. After incubation, culture supernatant was collected, centrifuged and IL-6 level was analyzed using ELISA Kit. Results were expressed as % increase in IL-2 level. Data were represented as means ± SD of 3 independent experiments. ***p < 0.001.
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μM: 0.472±0.001ng/ml) compared to control. MMP 9 level was further decreased in a concentration dependent manner (4.8 μM: 0.639±0.001ng/ml and 6.25 μM: 0.608±0.02 ng/ml) compared to control.

Effect of Garcinone E on MMP 2 and MMP 9 Level in Culture Supernatant of HSC 4 Cells. Control cells were grown without any treatment. Experimental cells were exposed to different concentration of Garcinone E (2.4, 4.8 and 6.25 μM) for 24h. After incubation, culture supernatant was collected, centrifuged and MMP 2 and MMP 9 level was analyzed using ELISA Kit. Results were expressed as % of inhibition. Data were represented as means ± SD of 3 independent experiments. ***p < 0.001.

The concentrations of MMP 2 and MMP 9 in the supernatant was reduced when they were exposed to different Garcinone E. When the cells exposed to 2.4μM the level was increased only to 19.6±0.03%, while at 4.8 and 6.25 μM of Garcinone E the percentage of increase was 54.12±1.03% and 84.46±2.10% respectively.

Effect of Garcinone E on IL-6 and IL-2 in HSC 4 cells

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Figure 9. Effect of Garcinone E on MMP 2 and MMP 9

The concentrations of MMP 2 and MMP 9 in the culture supernatant were found to be reduced when they were treated Garcinone E. The results are presented in the Figure 9. HSC-4 cells showed reduced level of MMP 2 and MMP 9 level was further decreased in a concentration dependent manner (4.8 μM: 0.639±0.001ng/ml and 6.25 μM: 0.472±0.001ng/ml) compared to control.

Discussion

Cancer invasion and metastasis is responsible for progression of tumor into advanced stage (Steeg, 2016). It is well documented that dietary measures can prevent the development of cancer (Guan, 2015). Mangosteen is one of the popular tropical fruit rich in pharmacologically relevant compounds (Ovalle-Magallanes et al., 2017). It has been a part of the traditional medicine of many countries (Ibrahim et al., 2016). In the present study we analysed the antiproliferative and antimetastatic activity of Garcinone E in oral cancer cell line HSC-4. It was found that Garcinone E could inhibit the proliferation of HSC-4 cell in a dose dependent manner. Similarly, less number of colonies produced in the Garcinone E treated cells revealed the inhibitory effect of Garcinone E on colony forming potential of HSC-4 cells.

Apoptosis is a kind of programmed cell death and evasion of apoptosis is a prominent hallmark of cancer (Hassan et al., 2014; Wong, 2011). During the apoptotic process, cells display distinct apoptotic morphology: membrane blebbing, breakdown of chromatin and nuclear condensation. It is triggered by activation of caspase proteins (George et al., 2017; Kerr et al., 1972). The result of Hoechst staining showed that Garcinone E induced apoptotic specific morphological changes and chromatin condensation in oral cancer cells indicates the proapoptotic effect of Garcinone E.

Next we analysed the effect of Garcinone E in various steps in metastatic cascade. Aberrant cell motility is a fundamental characteristic of cancer cell (Talmadge and Fildel, 2010). It is orchestrated by sequence of processes involving cytoskeletal modifications, changes in cell-substrate adhesive properties that lead to relocation from the primary site into new area. (Lu et al., 2012; Paul et al., 2017). Our study revealed that Garcinone E treated cells inhibited the migration of tumor cells towards the scratched area demonstrate the antimigatory potential of the compound.

Tumor cell invasion has been directly linked to advancement of tumor; in particular during metastasis (Lambert et al., 2017; Krakholm et al., 2015). Combined action of different molecules including growth factor and cytokines are required for invasion and formation of tumor in the new loci. Extra Cellular Matrix (ECM) acting as a physical scaffold for cell movement, medium of cell signal communication and plays critical role in cancer cell invasion (Frantz et al., 2010). Degradation of ECM is central event in metastasis and supports the continued expansion of the tumor mass. Collagen is considered to be the major component in ECM and the degradation of the same is an important factor in invasion. The degradation of ECM is mediated by the family of endopeptidases, matrix metalloproteinases (MMPs), also known as matrixins (Madsen and Bugge, 2015). Tumor cell expresses various proteases including MMPs to create a path for invasion (Shay et al., 2015). Experimental studies have shown that cancer therapeutics designed to target proteases are effective in blocking tumour progression (Catchart et al., 2015; Khan and Mukhtar, 2010). Studies have revealed that down regulation of these proteins by α-mangostin...
inhibited the invasion and metastasis of prostate tumor cells (Hung et al., 2009). The present study showed remarkable reduction in the number of cells invaded through the collagen coated polycarbonate membranes compared to control proved the antiprotease activity of Garcinone E. The decreased level of MMP 2 and MMP 9 in the Garcinone E treated HSC-4 cells positively correlate with observed antiinvasive activity of Garcinone E. This demonstrates that Garcinone E induced antiinvasive activity in oral cancer cells by inhibiting MMP 2 and MMP 9.

Inflammation is a prime factor during progression of tumor (Shalapour and Karin, 2015). Tumor cell itself and cells in the microenviroment secrete inflammatory molecule such as cytokines to modulate the tumor cell proliferation. IL-6 is a one of the important cytokine that is upregulated in almost all tumors and involved in tumor associated inflammation. It also protect tumor cell from oxidative stress and DNA damages which facilitate tumor progression (Kumari et al., 2016). IL-6 is considered to be a potent target molecule for cancer therapy (Guo et al., 2012). In this study we found that Garcinone E treated cells showed significantly reduced level of IL-6 in the culture supernatant indicates antinflammatory potential of Garcinone E that contribute towards the inhibition of metastasis.

Interleukin-2 (IL-2) signals are essential for lymphocyte differentiation and produce pleiotropic effects on immune system (Valle-Mendiola et al., 2016). It plays a critical role in the activation of immune system that could eradicate or suppress the tumor progression (Jiang et al., 2016). The elevated level of IL-2 in Garcinone E treated HSC-4 cells suggest its immunostimulating activity and directly contribute to the antimitastatic potential of Garcinone E.

In conclusion the results of the current investigation revealed that Garcinone E inhibited the proliferation, colony formation, migration and invasion of HSC-4 cells. It could stimulate the immunomodulatory cytokine IL-2 and inhibited the inflammatory cytokine IL-6 in HSC-4 cells, demonstrate the immunomodulatory and anti-inflammatory activity that support the anticancer property of the compound. Moreover, the observed antimigratory and antinvasive potential indicate the antimitastatic property of Garcinone E against HSC-4. Similarly the reduced level of MMP 2 and MMP 9 in the Garcinone E treated cells proved the mechanism of the antiinvasive activity Garcinone E. To best of our knowledge this is the first report of antimitastatic activity of Garcinone E on oral cancer. This study identified that Garcinone E is novel lead anticancer compound against oral cancer treatment and scope for intensive research in the field of drug development and therapy. We are now focusing on genomic studies to unravel the molecular mechanism of action of Garcinone E in the oral cancer cells.

Conflict of Interests
The authors declare that there is no conflict of interests.

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