Evaluation of anti-epithelial-mesenchymal transition property of *Garcinia mangostana* rind extract

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**Abstract**

**Background:** Epithelial-mesenchymal transition (EMT) helps solid tumors to lose their intercellular adhesive property and drives metastasis. As mangosteen fruit is known for many beneficial effects including antimicrobial, antioxidant, and anti-tumorigenic properties and has been used widely in traditional medicine, we interrogated its possible anti-metastatic effect on MCF-7 breast cancer cells.

**Results:** We found that aqueous mangosteen rind extract (MRE) inhibited growth of MCF-7 and altered the transcript levels of ERα, ERβ, and EGFR genes. Additionally, the MRE changed the expression of important markers of EMT, E-Cadherin, N-Cadherin, Snail, and MMP-9. Moreover, MRE inhibited migration of MCF-7 cells.

**Conclusion:** The results suggest that MRE suppresses growth and inhibits epithelial-mesenchymal transition in MCF-7 cells.

**Keywords:** Mangosteen rind, Breast cancer, Epithelial-mesenchymal transition, Apoptosis

**Background**

Though cancer survival rates are increasing over the years, cancer metastasis is still the major cause of mortalities among cancer cases [1, 2]. Advanced stage of cancers invades the neighboring blood vessels and metastasizes to distant organs, which is characterized by epithelial-mesenchymal transition (EMT) [3–5]. EMT is a physiological program that is important for embryogenesis and wound healing but is also involved in cancer progression. During EMT, cell adhesion and filament proteins such as cadherins and vimentin as well as several factors like Twist, Snail, and Slug are differentially expressed [6, 7]. EMT is also facilitated by a group of extracellular matrix degrading endopeptidases called matrix metalloproteases (MMPs) [8, 9]. Various chemotherapeutic drugs are developed in the market to suppress metastasis; however, they also bring forth untoward after-effects. Alternatively, phytomedicines could provide a solution for this hitch.

Mangosteen (*Garcinia mangostana*) is one of the most popular tropical fruits of Southeast Asian origin and possesses high nutritive values. It is rich in xanthones, polyphenols, anthocyanins, catechins, vitamins, and minerals. It displays effective biological properties such as antimicrobial, anti-tumorigenic, and antioxidant properties [10]. The pericarp of mangosteen has been traditionally used in the treatment of various maladies since ancient time [11–13]. Numerous bioactive compounds (including 40 different xanthones and polyphenols) are present in mangosteen pericarp and their anti-tumorigenic properties have been studied both in vitro and in vivo. Structures of some of major xanthones previously identified are given in Additional file 1: Fig. S1. Alpha mangostin, an important xanthone, present in pericarp of mangosteen was shown to induce apoptosis in a variety of cancers [14–16]. In human breast cancer cells (MDA-MB-231), α-mangostin induced apoptosis and cell cycle arrest and also indirectly suppressed cell division through...
inhibition of fatty acid synthase [17, 18]. Though many studies have implicated the use of mangosteen extract or \( \alpha \)-mangostin on inhibition of cancer cell proliferation, its effect on EMT is barely investigated.

As breast cancer is one of the common cancers in both developing and developed nations [19], in the present work, the effect of an aqueous extract of mangosteen rind on EMT was studied using MCF-7 cells. The said mangosteen rind extract (MRE) was used in this study instead of a purified compound (e.g., \( \alpha \)-mangostin) based on the possible benefits of multi-compound synergistic effects of the extract. Moreover, aqueous form of medicinal plant extract may be more beneficial as it may mimic the physiological milieu when taken as a complementary medicine.

**Methods**

**Chemicals and reagents**

Total RNA isolation (TRI) reagent, primers, KAPA SYBR FAST qPCR Master Mix (2X) Kit, and all other reagents/chemicals used in this study were purchased from Sigma-Aldrich Pvt. Ltd. (USA). Superscript III first strand synthesis reagents were procured from Thermo Fisher Scientific, USA.

**Preparation of aqueous form of mangosteen rind extract (MRE)**

Mangosteen (Garcinia mangostana L.) fruits were collected from Burliar, Nilgiris District, Tamil Nadu, India. The plant was identified and authenticated by Centre for Advanced Studies in Botany, University of Madras, Chennai, India (No. MUBL1024). The fruits were washed with distilled water, wiped cleanly, and the fruit parts (rind, pulp and seed) were separated using a sterile blade. Of these, mangosteen rind was cut into small pieces, shade dried, ground into fine powder, and strained. Five grams of mangosteen rind powder was added to 100 ml of autoclaved distilled water and kept in an orbital shaker for 24 h at 37 °C. Then the extract was spun at 5000 rpm for 15 min. The supernatant was filtered with 0.45 μ filter, aliquoted, and stored in sterile vials at – 20 °C. A portion of filtered MRE was subjected to evaporation of its liquid in hot oven, and the resultant dry weight content was used for calculation of MRE concentration in weight by volume (μg/ml) basis.

**Phytochemical screening**

The aqueous Mangosteen extract was screened qualitatively for various phytochemicals using standard protocols [20, 21].

**Determination of total flavonoid content**

Total flavonoid content of MRE was analyzed by the method of Chang et al. [22]. In short, 250 μl of either distilled water (blank) or MRE (200 μg/ml concentration) or quercetin (standard) (0–50 μg/ml concentrations) was taken. To that, 15 μl of 5% NaNO\(_2\) solution was mixed, 15 μl of 10% aluminum chloride solution was added after 5 min and incubated for 5 min at RT. To this mixture, 100 μl of 1 M sodium hydroxide and 120 μl of water were added and mixed well. The absorbance of the color developed was read at 510 nm using ELISA plate reader (Synergy HT, Biotek, USA). The total flavonoid content of the extract was expressed as μg of quercetin equivalent/mg dry weight of extract using a standard curve.

**Determination of total phenolic content**

Total phenolic content was assessed by a standard method using gallic acid as standard [23]. To 305 μl of either distilled water (Blank) or gallic acid (standard; 0–50 μg/ml concentrations) or MRE (200 μg/ml concentration), 25 μl of Folin–ciocalteu reagent and 75 μl of 10% sodium carbonate were added. The mixture was incubated at RT for 8 min. To this, 95 μl of distilled water was added, made to 500 μl, and incubated at RT for 2 h. The absorbance of the developed blue color was read at 700 nm using an ELISA plate reader. Total phenolic content was expressed as μg of GAE (gallic acid equivalent) per mg of the dried extract by plotting a standard curve.

**Determination of DPPH radical scavenging activity**

The antioxidant effect of MRE was analyzed by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assay by the method [24]. Vitamin C (Ascorbic acid) was used as standard and ethanol was used as blank. Standards were prepared by using vitamin C at various concentrations (0–300 μg/ml). In brief, 10 μl of either standard or extract were mixed with 190 μl of 0.1 M ethanolic DPPH and incubated in dark for 30 min at room temperature. The color developed (light yellow) was read using an ELISA plate reader at 517 nm. Percentage inhibition was calculated as follows:

\[
\% \text{ inhibition} = \frac{A_{\text{control}} - A_{\text{sample}} \times 100}{A_{\text{control}}}
\]

The amount of antioxidant necessary to decrease the initial DPPH concentration by half its value is inhibitory concentration \( IC_{50} \).

**Cell culture**

MCF-7 breast cancer cell line was procured from National Centre for Cell Sciences, Pune, India. Cells were cultured in DMEM F-12 HAM medium supplemented with 10% fetal bovine serum and were maintained in 5% CO\(_2\) at 37 °C. Cells were trypsinized and passaged at 70–80% confluence.
Treatment of MCF-7 cells with MRE

After reaching 70–80% confluence, MCF-7 cells were allowed to grow overnight in serum-free medium and then treated with or without MRE at various concentrations (0–300 µg/ml) for 24 h for MTT assay. MRE concentrations of 0, 50, and 100 µg/ml were used for 24 h for other experiments.

Cell viability assay

Cell viability assay was performed using a modified colorimetric technique [25]. MCF-7 cells were starved overnight in serum-free medium. After treating cells with or without MRE (as per Methods), 300 µl of MTT solution was added to each well and incubated at 37 °C for 1 h. The purple formazan crystals were dissolved in 300 µl of dimethyl sulfoxide (DMSO) and incubated in dark for an hour. Absorbance was read at 570 nm using a Micro ELISA plate reader and cell viability was represented in percentage.

Cell morphology study under light microscopy

MCF-7 cells were treated with vehicle alone (distilled water) and MRE (50 and 100 µg/ml) for 24 h in 24-well plate. Then the media was discarded and cells were washed with 1X PBS. Then the cells were immediately viewed under light microscope (Zeiss, Japan) at 40X magnification.

Cell nuclei staining using hematoxylin

MCF-7 cells were treated with vehicle (distilled water) and MRE (50 and 100 µg/ml) for 24 h in 24-well plate. Media were removed and then cells were stained with hematoxylin staining solution for 5 min. Then the solution was removed and washed with distilled water. Cells were then observed using light microscope (Zeiss, Japan) at 40X magnification.

Acridine orange/ethidium bromide (AO/EtBr) differential staining of cells

MCF-7 cells were seeded in a 24-well plate and treated with or without MRE for 24 h after reaching 70–80% confluence. Then the medium was discarded, and after washing the cells with 1X PBS twice, 500 µl of 0.1% AO/EtBr solution was added and immediately viewed under fluorescence microscope.

Total RNA isolation and real-time PCR analysis

Total RNA of MCF-7 cells was isolated using TRIZOL reagent, and cDNA was synthesized by Superscript III first strand synthesis system. Real-time PCR was performed using KAPA SYBR fast qPCR master mix (2X) kit. The quantitative RT-PCR experiments were done according to the manufacturer's protocol for analyzing the expressions of hormone receptors (Estrogen Receptor-α, Estrogen Receptor-β, and Epidermal growth factor receptor EGFR), and epithelial-mesenchymal transition markers (EMT; E-Cadherin, N-Cadherin, Snail). List of primers are given in Table 1. Data analysis was done using the comparative CT method, and the results were expressed as fold change compared with control. GAPDH was used as an internal control.

Gelatin zymography

Activities of gelatinase (MMP-9, pro and active forms) were studied using gelatin zymography. Equal volumes of conditioned media and 2X non-reducing buffer were mixed and incubated at RT for 30 min. The protein sample was electrophoresed in 7.5% acrylamide gel containing gelatin at 80 V. The gel was washed twice (30 min each) with renaturing buffer (containing 2.5% Triton-X 100) and twice with distilled water (10 min each) followed by incubation in incubation buffer overnight at RT. Staining was done with Coomassie brilliant blue for 30 min and destaining was done using water until necessary resolution of bands was obtained and image was captured using BioRad ImageLab software (gray mode).

Scratch blot assay

MCF-7 cells were grown to 70–80% confluence and a wound was introduced by scratching with pipette tip. The cells were washed with serum-free medium twice and treated with or without MRE (0, 50, and 100 µg/ml) for 24 h. The scratches were then observed using under light microscope (Zeiss, Japan).

Table 1

| S. no | Gene   | 5′→3′ sequence               |
|-------|--------|-----------------------------|
| 1     | ER alpha | F TGGGCTTACTGACCAGCAAGCCTG  |
|       |         | R CTCATCTGGGGAGTCAAAA       |
| 2     | ER beta  | F AGACTCTCTGGTGTAAGGCA     |
|       |         | R GACAGCAGCAGAAGGTGACATC    |
| 3     | EGFR    | F CTAGATCCCGCTCCATGCC    |
|       |         | R GAGAGCAGCAGACTTTGATCT   |
| 4     | E Cadherin| F GCCGGCTGGCGTCTGAGGAA    |
|       |         | R TGACAGCAGCAGCTTCTCCGCA   |
| 5     | N Cadherin| F TCGAGATCAGGCAGGACC   |
|       |         | R TGAGGGCGGCTGCTAGTTC     |
| 6     | SNAIL   | F ATGGAGGCTGGAGCAGTC      |
|       |         | R AGCCAGGCGCAGCTGTCAGT    |
| 7     | GAPDH   | F TCAAGTGCGGGCGATGCTGGC   |
|       |         | R TGACCTTGGCCAGGGGTGCT    |

F, Forward; R, Reverse
Results
Biochemical constituents of mangosteen rind extract (MRE)

Aqueous extract of mangosteen rind (MRE) was prepared from mangosteen fruit as described in the ‘materials and methods’ section. The extract was observed to be brown in color and had liquid consistency (Fig. 1A). Yield of MRE in dry weight basis was calculated to be 0.997% (w/v). MCF-7 cells treated with varying concentrations of MRE (0, 5, 25, 50, 100, 250, and 500 µg/ml of medium) and the sensitivity of cells to MRE was tested. The MCF-7 cells showed a decrease in cell viability at higher concentrations (50 µg/ml and above) and IC_{50} was calculated as 167.23 µg/ml (Fig. 1B). Hence, further studies were performed at 0, 50, and 100 µg/ml concentrations of MRE.

Biochemical constituents of mangosteen rind extract (MRE)

Qualitative analysis of MRE showed that the MRE contained flavonoids, tannins, phenols, diterpenes, phytosterols, carbohydrates, reducing sugars, and alkaloids (Additional file 1: Fig. S2). While saponins were undetectable, mild reactivity was observed for free amino acids in MRE (Additional file 1: Fig. S2). Additionally, total amounts of flavonoids, phenolics, and antioxidant capacity possessed by MRE were assessed (Additional file 1: Fig. S3). Total flavonoid content of MRE was calculated as 56.35 ± 1.8 µg QE/mg of extract using quercetin as standard (Additional file 1: Fig. S3A). Similarly, the total phenolic content in MRE was calculated as 23.12 ± 0.7 µg GAE /mg of extract using Gallic acid as standard (Additional file 1: Fig. S3B). Free radical scavenging capacity of MRE was assessed using DPPH assay (Additional file 1:
The 50% inhibitory (scavenging) concentration (IC$_{50}$) of MRE was found to be 368.8 µg/ml, and that of ascorbic acid was calculated to be 129.8 µg/ml. Thus, though the free radical scavenging activity is lower than ascorbic acid, the antioxidant effect of MRE was seen in microgram/ml level (Additional file 1: Fig. S3C).

**Effect of MRE on viability and morphology of MCF-7 cells**

As MRE was found to reduce cell viability (Fig. 1B), MCF-7 cells treated with 0, 50, and 100 µg/ml concentrations of MRE were checked for possible morphological changes. Cells were found to show altered morphology at 50 µg/ml MRE concentration which was apparent (rounded up) at 100 µg/ml concentration (Fig. 2A). Hematoxylin-stained cell nuclei after treatment with MRE showed normal nuclear staining of untreated cells, while distorted nuclei were observed on treatment with MRE at 100 µg/ml (Fig. 2B). Acridine orange/ethidium bromide dual staining is known to clearly demarcate live cells (green fluorescence) and apoptotic cells (red fluorescence). As shown in Fig. 2C, while control cells are fully viable, MRE treatment increased the number of dead cells in a dose-dependent manner. These observations clearly indicated that MRE not only induced change in morphology, but also induced apoptotic death of MCF-7 cells.

**MRE-induced expression of hormone receptors and EMT markers**

The effect of MRE on the expression of ERα, ERβ, and EGFR that are critical for the proliferation of breast cancer cells were analyzed by real-time PCR (Fig. 3A). Treatment of MCF-7 cells with MRE gradually decreased the mRNA expression of ERα in a concentration-dependent manner while it increased the levels of ERβ. In case of EGFR, a steep decline was observed upon MRE treatment at increased concentrations. Upon analysis of the expression of epithelial-mesenchymal transition (EMT) markers, it was found that the MRE increased the mRNA expression of E-cadherin whereas it decreased the levels of N-Cadherin and SNAIL in dose-dependent manner (Fig. 3A).

**Effect of MRE on the activity of MMP-9**

As evident from gelatin zymography, activity of MMP-9 (pro and active forms) was clearly visible as thick bands in untreated cells. On the other hand, with MRE treatment
Fig. 3 Mangosteen rind extract (MRE)-induced expression of hormone receptors and EMT markers in MCF-7 cells. A Messenger RNA expression of ERα, ERβ, EGFR, and EMT markers in MCF-7 cells. MCF-7 cells were treated with MRE (0, 50, and 100 μg/ml) and the effect of MRE on gene expression were studied. Messenger-RNA expressions were analyzed using real-time PCR and the experiments were done in triplicates. ERα = Estrogen receptor alpha, ERβ = Estrogen receptor beta, EGFR = Epidermal growth factor receptor, SNAIL = Zinc finger protein SNAI1 (Snail), EMT = Epithelial-mesenchymal Transition, MMP = Matrix metalloproteases. B Effect of MRE on activity of MMP-9 in MCF-7 cells. MMP-9 activity was analyzed using Gelatin zymography. The dark bands against the light background (reversed from original image) showed the activities of pro and active MMP-9.

Fig. 4 Mangosteen rind extract (MRE)-dependent control of migration of MCF-7 cells. MCF-7 cells were treated with MRE (0, 50, and 100 μg/ml) and the effect of MRE on migration of MCF-7 cells were analyzed by scratch blot assay. Status of cell migration after 24 h of treatment were observed under light microscope at 20X magnification.
(50 and 100 µg/ml), these bands were not detected clearly indicating decreased activities of MMP-9 (Fig. 3B).

**MRE-dependent control of migration of MCF-7 cells**

Migratory ability of MCF-7 cells was assessed by scratch assay. As shown in the figure (Fig. 4), same level of clearance zones were observed in control and the MRE treated (50, 100 µg/ml) cells at the start of the assay (0 h). After 24 h, the control cells showed tendency to close the clearance zone (i.e., migration of cells to the vacant zone); however, the MRE treatment vividly prohibited the migration at 50 and 100 µg/ml treatment concentrations.

**Discussion**

As mangosteen was previously shown to be a source of several bioactive molecules [26–28], the present study was focused on evaluating the effect of rind of mangosteen on EMT of breast cancer cells. An aqueous extract of mangosteen rind (MRE) displayed the presence of flavonoids, tannins, phenols, diterpenes, phytosterols, carbohydrates, reducing sugars, and alkaloids. Our results on the overall composition of MRE are consistent with previous report, which showed the presence of 166 compounds in the chloroform epicarp extract and 105 compounds in hexane endocarp extract and absence of saponins [29].

Phenolic compounds possess rich antioxidant, anti-carcinogenic, and anti-inflammatory activities. They have important roles in the prevention as well as cure of cancers by inducing cell cycle arrest, inhibition of migration and proliferation, and suppressing cell signaling pathways. Flavonoids belong to the family of polyphenolic compounds, which also influence the biological metabolic fate. The total phenolic content (TPC) was found to be 23.12±0.7 μg GAE/ mg (2312±70 mg GAE/100 g) of extract. Consistent with our findings, another researcher showed TPC of outer pericarp of mangosteen as 2930.49±318.1 mg GAE/100 g, however, from an acetone extract [30]. In our study, total flavonoid content of MRE was calculated as 56.35±1.8 μg QE/ mg of extract (5635±180 mg QE/100 g extract). Lourith and Kanlayavattanakul [31] showed a TPC of aqueous form of mangosteen’s color extract as 1608.65±3.56 mg GAE /100 g extract and total flavonoid content as 8007.68±2.18 mg QE/ 100 g extract.

Antioxidant property of plant extracts is important so that they can be used as bioactive preparations. In our study, MRE showed IC_{50} value of 368.8 μg/ml (IC_{50} of ascorbic acid=129.8 μg/ml). In a previous study, aqueous mangosteen peel extract showed IC_{50} value of 77.45 mmol ascorbic acid equivalents/kg [32]. Thus, MRE of our study is evidently shown to have good free radical scavenging activity.

In the MTT-based cell viability assay, it was clear that MRE treatments resulted in drastic decrease in viability of MCF-7 cells (IC_{50} of 167.23 μg/ml). Further, in the cell death assay using AO/EtBr dual staining, a positive correlation between the concentration of MRE and the degree of apoptotic death was observed. Previous study showed that methanolic and ethanolic extracts of mangosteen rind had antioxidant, anti-proliferative, and apoptotic effects on SKBR3 cells [33, 34]. This clearly indicates that not only the organic extracts but also the aqueous extract of mangosteen rind leads to cytotoxicity of breast cancer cells.

Estrogen receptors (α and β) play a crucial role in the development and progression of breast cancer [35]. As about 70% of breast cancers are ER positive, we first checked the effect of MRE on estrogen receptor. In this study, MRE treatment reduced the mRNA expression of ERα, but upregulated ERβ. This finding correlates with a study wherein α-mangostin treatment of MCF-7 cells resulted in a suppression of ERα [36]. On the contrary, another researcher showed that 70% ethanolic extract of mangosteen pericarp induced MCF-7 cytotoxicity without altering ER expression [37]. It is known that ERα is expressed in most of breast cancers and hence used as a marker of survival in human breast cancers [38, 39]. The reduced viability of MCF-7 cells observed in this study could be due to decrease in the levels of ERα. On the other hand, it is known that ERβ is associated with low tumor grade [40]. This also suggests that the increased expression of ERβ observed in our study resulted in reduced invasiveness of MCF-7 cells. EGFR is commonly associated with invasion and metastasis of breast cancer [41]. A reduction in EGFR mRNA observed in the present study further adds on to the notion that MRE could suppress proliferation and aggressiveness of MCF-7 cells.

E-cadherin is considered to be tumor suppressive and used as a phenotypic marker in breast cancer and N-cadherin plays a vital role in migration, integrity and development [42]. Our results indicate that MRE represses EMT by increasing the mRNA expression of E-cadherin and decreasing N-cadherin. Previous studies have shown that α-mangostin increased E-cadherin and decreased N-cadherin and vimentin in pancreatic cancer cells [43, 44]. Snail, a transcription factor involved in EMT, was found to be overexpressed in breast cancers [45, 46]. Downregulation of Snail expression by MRE observed in the present study might be one of the mechanisms of MRE leading to suppression of EMT.
Upregulation of MMPs is commonly observed in malignant cells when compared with normal cells. In cancer cells, MMPs are involved in EMT, invasion, metastasis, and angiogenesis. As evident by our gelatin zymography, MRE treatment suppressed the activity of MMP-9 and thereby possibly hindering the invasive property of MCF-7 cells. This result is consistent with the other studies that support the downregulation of MMP-9 upon treatment with α-mangostin, a constituent of mangosteen fruit [44, 47–49]. MRE treatment also suppresses the migrating ability of MCF-7 cells, where it induces a change in morphology and death of MCF-7 cells. Thus, our findings of this study point to an anti-EMT role of MRE.

Conclusion
In conclusion, mangosteen rind extract (MRE) in aqueous form was subjected to phytochemical analyses and found to contain various phytochemicals including high levels of flavonoids. MRE induced significant morphological change and death in MCF-7 cells, which could be attributed to its differential effects of ERα and ERβ. Additionally, MRE clearly altered the expression of markers of EMT such as E-cadherin, N-cadherin, Snail, and MMP-9, thereby suppressing EMT. This suggests that the aqueous form of MRE may be considered for future development of herbal medicine against EMT and metastatic breast cancer.

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Plant authentication
The mature fruits of Garcinia mangostana L. were collected from Burlar, Nilgiris District. The plant was identified and authenticated by Centre for Advanced Studies in Botany, University of Madras, Chennai, India (No. MUBL1024).

Authors’ contributions
AV conceived and designed the experiments. SM performed the experiments, and SM and AV wrote the manuscript. Both the authors read and approved the final manuscript.

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Availability of data and materials
All data of this study are included in the main manuscript as well as in the supplementary information.

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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