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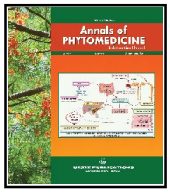

Original Article : Open Access

Inhibitory effects of *Citrus maxima* (Burm.) Merr. peel extracts and its bioactive constituent naringin on MCF-7 human breast cancer cell line: A phytochemical approach

Flama Monteiro*, Vijith Vittal Shetty, Shilpa S. Shetty***, Ranjitha Acharya*, Deepthi*** and N. Suchetha Kumari*◆**

**Department of Biochemistry, K.S.Hegde Medical Academy, Nitte (Deemed to be University), Deralakatte- 575018, Mangaluru, Karnataka, India*

***Department of Oncology, K.S.Hegde Medical Academy, Nitte (Deemed to be University), Deralakatte-575018, Mangaluru, Karnataka, India*

****Central Research Laboratory, K.S.Hegde Medical Academy, Nitte (Deemed to be University), Deralakatte-575018, Mangaluru, Karnataka, India*

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Abstract

Citrus fruits have been comprehensively utilized for their medical attributes. Outstandingly, a noteworthy fraction of chemotherapeutic drugs in skirmishing cancer originates from whole plant extracts, their specific molecules, or their synthetic analogs, integral to contemporary oncology approaches. The purpose of this investigation was to delineate the cytotoxic, antimigratory, anticlonogenic properties of peel extracts of *Citrus maxima* (Burm.) Merr. (*C.maxima*) against the MCF-7 breast cancer cell line. An enzymatic formazan-based assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide-(MTT) was conducted to evaluate the antiproliferative effects of the *C. maxima* extract peel fraction and its flavanone naringin (NAR) on MCF-7 cell line. Cell migration suppression and colonogenic impact on breast cancer cell line in lieu of antibreast cancer effects were evaluated by scratch assay and colony formation assays. The outcome suggested that peel extracts of *C.maxima* and NAR effectively inhibited breast carcinoma cell proliferation. Subsequent treatment with relative half inhibitory doses, the viability of cells declined progressively over time. Furthermore, the extracts and NAR showed a significant inhibitory effect on the colony formation ability and cell migration. The examined crude peel extracts of *C.maxima* and its flavonoid NAR warrant further comprehensive investigations to completely unravel the precise mechanism of actions responsible for their anticancer activity as potential promising candidates for future breast cancer therapeutics.

1. Introduction

Breast cancer constitutes virtually 12 per cent of the universal cancer yoke in the female population. It is a momentous health alarm affecting women, representing one in every sixth cancer related deaths (Sedeta *et al*., 2023). Previously employed anticancer drugs demonstrated considerable toxicity to carcinoma cells as well as to normal cells. Internationally, the setting up of cancer registries has incited a chase for inventive pharmaceuticals that restrictively mark cancer cells devoid of harming healthy ones. At the moment, researchers are probing novel anticancer compounds in a quest to reinforce cancer monitoring, augment the exactitude of data, and endow with insights for preventing, early discovery, diagnosing, treating, and social medical caringness for cancer patients (Lichota and Gwozdzinski, 2018).

Research points out that plant based molecules blended with anticancer medications hold noteworthy assertions for selectively eliminating tumor cells without hurting normal cells like fibroblasts. Despite the fact that various phyto compounds have demonstrated anticancer properties, contemporary medicine still faces challenges in developing efficient drugs for particular kinds of cancers (Lichota and Gwozdzinski, 2018). Phytochemicals have the capacity to thwart, defer, or even potentially alleviate cancer (Monteiro *et al*., 2023). Dietary phytochemicals constantly put forth their effects by modulating specific molecular targets and signaling pathways allied with cancer. These processes comprise enhancing antioxidant defenses, neutralizing-carcinogens, inhibiting the survival and proliferation of malignant cells, scavenging free-radicals, inducing cell cycle arrest, promoting apoptosis, and regulating tumor-invasiveness, angiogenesis, and immune responses (Monteiro *et al*., 2023).

Citrus maxima (Burm.) Merr. alternatively documented as *Citrus grandis* (L.) Osbeck, is a taxon classified within the Rutaceae botanical family. It is commonly acknowledged by vernacular names such as pomelo, pummelo, shaddock, *etc*.; indigenous to Asia, it is of late introduced to copious tropical locales and has further extended its cultivation range (Sapkota, 2023). The epidemiological authentication emphasizes a direct liaison between the consumption of dietary sources bountiful in flavonoids and low in fatty acids, notably fruits and veggies, and a dwindled vulnerability to diverse human infirmities (Nair, 2018). Up and coming evidence points to the occurrence of flavonoids like NAR being the largest part copious in *C. maxima*,

Corresponding author: Dr. N. Suchetha Kumari
Professor, Department of Biochemistry, K.S.Hegde Medical Academy, Nitte (Deemed to be University), Deralakatte-575018, Mangaluru, Karnataka, India
E-mail: kumarin@nitte.edu.in
Tel.: +91-9448451318

neohesperidin, narirutin, and hesperidin, etc. in the peel of citrus varieties, presenting a myriad of profitable effects (Ding *et al*., 2022; Sowmya *et al*., 2019). Quite a lot of investigations have explored the pharmacological attributes of extracts of *C. maxima* and its isolated elements (Pandey *et al*., 2019; Monteiro *et al.,* 2023; El-Kersh *et al.*, 2021). The swift maturation of citrus peel hinges first and foremost on its ample availability and cost-effectiveness. Contemporary pharmacological research validates the traditional uses of this plant, highlighting its effectiveness as an anti-neurologic agent. Notably, it shows promising potential in the treatment of various neurologic disorders as well as antitumor, antimicrobial, and antioxidant properties.

This current analysis sought to investigate the anticarcinogenic characteristics of the peel fraction of *C. maxima* extracts on MCF-7 human breast carcinoma cell line by employing assays for cell viability, cell migration, and colony formation.

2. Materials and Methods

2.1 Fruit specimen and method of extraction

C. maxima fruits were purchased from Sakleshpura, Karnataka, India. Following rigorous washing with tap water, pomelo peels were meticulously separated from fruit (Figure 1), sectioned into uniform pieces and subjected to desiccation in an oven set to temperatures ranging from 40 to 50°C for a period of seven days until complete dehyradation was achieved. The ensuing dried peel gist was finely powdered. To attain the aqueous peel extract, the finely powdered peel was subjected to boiling in distilled water for a time period of 20 min, then filtration using Grade 1 Whatmann filter paper. For the ethanolic extract, a Soxhlet apparatus was utilized, with ethanol employed as the extraction solvent. The extraction process was conducted at a controlled temperature of 55°C for duration of 24 h. Subsequently, after removal of solvent *via* evaporation with a rotary flash evaporator, yielding a concentrated portion of *C. maxima* peel which was then stored at 4°C for further experiments (Bhandary *et al.,* 2015).

Figure 1: *C.maxima* (Pomelo).

2.2 Identification of plant material

Dr. V. Ravishankar Rai, a Botanist and former Professor and Chairman at Department of Studies in Microbiology, University of Mysore, Mysore, India, identified and verified. *Citrus maxima* (Burm.) Merr. Interpr. Herb. Amboin.: 296 (1917).

2.3 Cell culture condition and treatment

The human estrogen receptor positive (ER+) MCF-7 breast adenocarcinoma adherent cell line was secured from National Center for Cell Science Repository Pune and conserved in Dulbecco's Modified Eagle's Medium (DMEM, Gibco), enriched with 10% fetal bovine serum (Gibco) and 1% antibiotic-antimycotic agents. MCF-7 cancer cells were cultured in a humid environment containing CO_2 (5%) @ 37°C. *C. maxima* peel extract aqueous (CMPAQ) 1 mg/ml media, *C. maxima* peel extract ethanolic (CMPE) 1000 mg, and NAR 500 mM (Sigma Aldrich) stock solutions were produced in Dimethylsulfoxide (DMSO), kept at -20°C (Roopashree *et al*., 2024).

2.4 Cell viability assay

Cytotoxicity of cancer cell line was determined by means of the MTT assay (Siddiqui *et al*., 2018). Cells (0.05 x 10^6) were seeded in tissue culture plates (24 well) with culture media for growth and then cultured in CO_2 incubator at 37°C. Following 70% confluence and subsequent 24 h fasting, cancer cells were treating with relevant IC_{50} values of *C. maxima* peel extracts, NAR, and their controls. Following treatment the cells were cultured for 24, 48, and 72 h period. Subsequently, the cells were rinsed with phosphate buffered saline (PBS) and replenished with new media. After addition of 5 mg/ml of MTT (SRL) solution (20 µl) to cells, further incubated for 4 h at 37°C. Upon removal of media, crystals of formazan were dissolved in DMSO (100 µl), and absorbance was measured at 570 nm using a multimode plate reader (Spark Tecan).

2.5 Cell migration assay

The cell scratch experiment was carried out to assess the influence of cancer cell migration inhibition (Somaida *et al.,* 2020). The cells were seeded (1×10^3) per ml in 6 well plates. After obtaining confluence within 24 h, a (200 µl) pipette tip was utilized to create a notch. Subsequently, exposed to concentrations equivalent to their respective IC_{50} values of *C. maxima* extracts peel fraction, NAR and corresponding control. The progression of scratch closure was monitored at intervals of 0, 6, 12, 24, and 48 h by means of an inverted phase contrast microscope outfitted with software Leica. Quantitative assessment of cell migration and determination of percentage of wound area were performed by means of Image J software.

The percentage of cell migration rates was determined using the **formula: Cell migration rate (%) = ($Areat_0$ – $Areat_x$)/Area t_0×100%.** The "$Areat_0$" represents the initial scarification area at 0 h; and the "$Areat_x$" represents the scarification area at 48 h (Buachan *et al*., 2020).

2.6 Colony formation assay

Cells (500 per well) were seeded in culture plate (six well) and permitted to proliferate grow for a designated time. Following this, was treatment with IC_{50} doses of *C. maxima* peel fraction and corresponding control. Upon completion of treatment, the cells be gently washed with PBS and 70% ethanol fixed, incubating at room temperature for 15 min. Subsequently, the ethanol was aspirated, and the cells were stained with 400 µl of 1% solution of crystal violet, followed by incubation for an added 15 min at room temperature. After staining, the plates were rinsed with PBS air dried. Observation and counting of colonies were performed using phase contrast microscope (Franken *et al*., 2006).

2.7 Statistical analysis

Statistical analysis was carried out using Graph Pad PRISM 8.0 software with experiments implement in threesome and expression of data as in mean ± standard deviation. To assess statistical significance, between control and treated groups, underwent ANOVA one way analysis of variance with multiple comparisons was employed. Significance levels were denoted by $*p<0.05$; $**p<0.01$;*** $p<0.001$.

3. Results

3.1 Cytotoxic effects of *C. maxima* peel extracts on MCF-7 cell viability

The MTT test was utilized to verify the viability of MCF-7 breast cancer cells following treatment with *C. maxima* peel extracts and NAR. Extracts of *C. maxima* peel (aqueous and ethanol) and NAR had IC_{50}values of 940 µg/ml, 2.05 mg/ml, and 920 µM, respectively. The treatment with appropriate drug was given for around 24 to 72 h, indicating a time dependent decrease in cell viability. A steady drop began at 24 h, reaching its greatest decline in cell viability by the seventy 2 h mark.

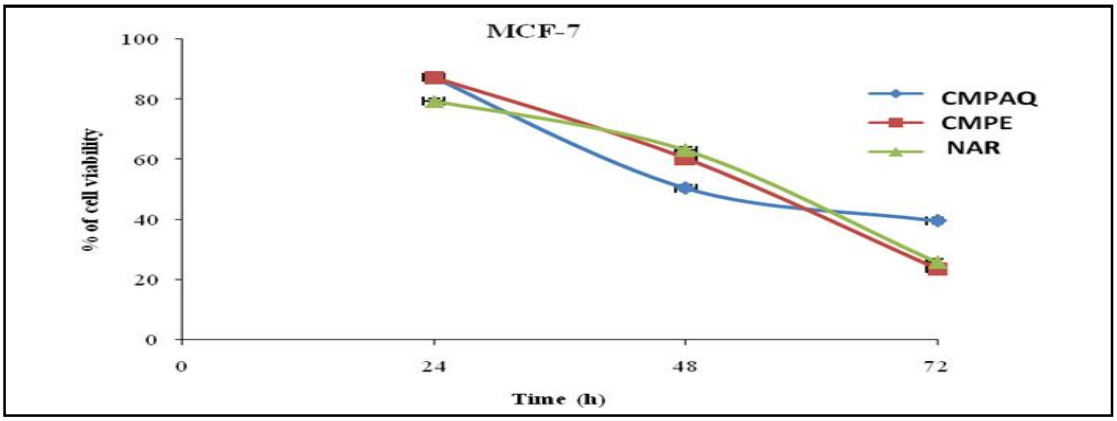
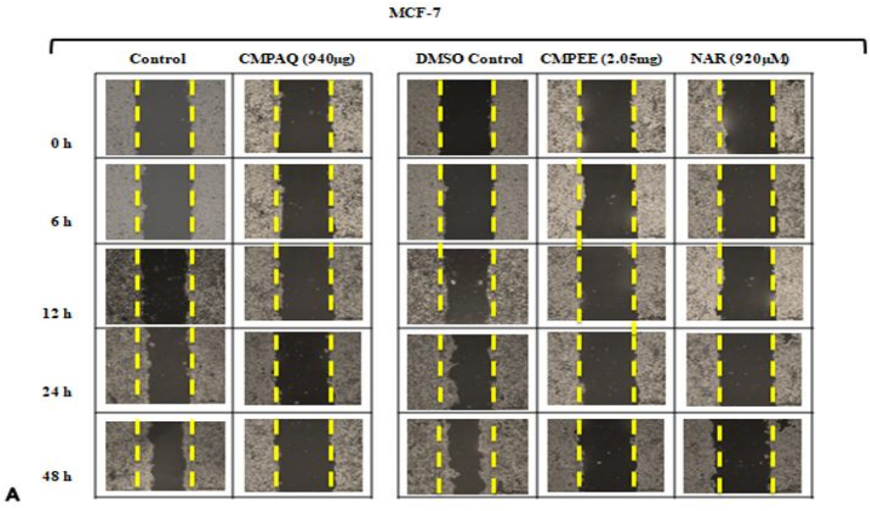


Figure 2: MTT assay exhibited a decline in viability of MCF-7 cells upon exposure to *C. maxima* peel extracts and NAR (A) over 24, 48 and 72 h intervals. Data, depiction as mean ± SD from threesome independent trails.

3.2 Impact on MCF-7 breast cancer cell migration by of *C. maxima* peel extracts

The study focused the way *C. maxima* peel fraction and NAR inhibited growth and MCF-7 cells migration capacity. Analysis was carried out at 0, 6, 12, 24, and 48 h upon dosing with each of the half maximal inhibitory doses. The examined plant extracts and NAR significant inhibited the breast cancer cell migration and growth (Figure 2) in comparison to their particular controls. Migration remained unchanged in both the untreated groups of control; however, in the treated groups the gap is partially closed after 48 h.



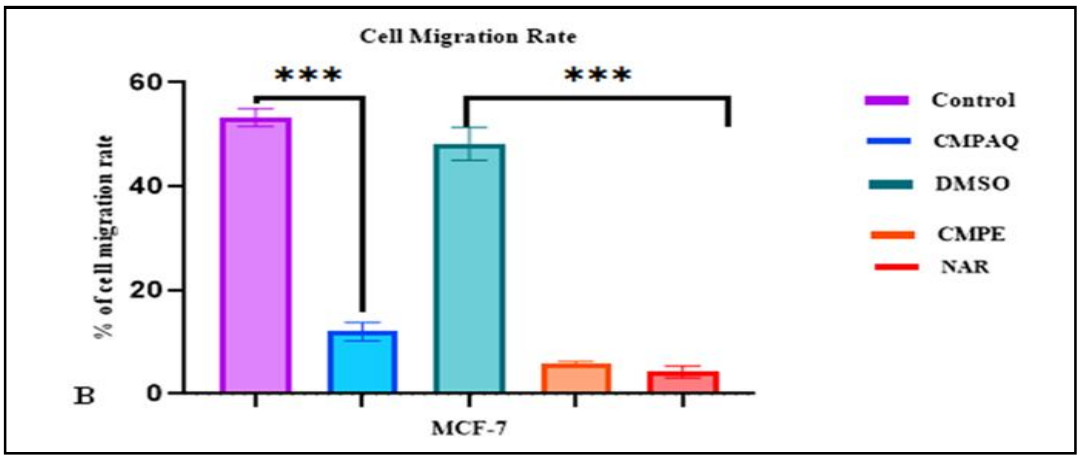
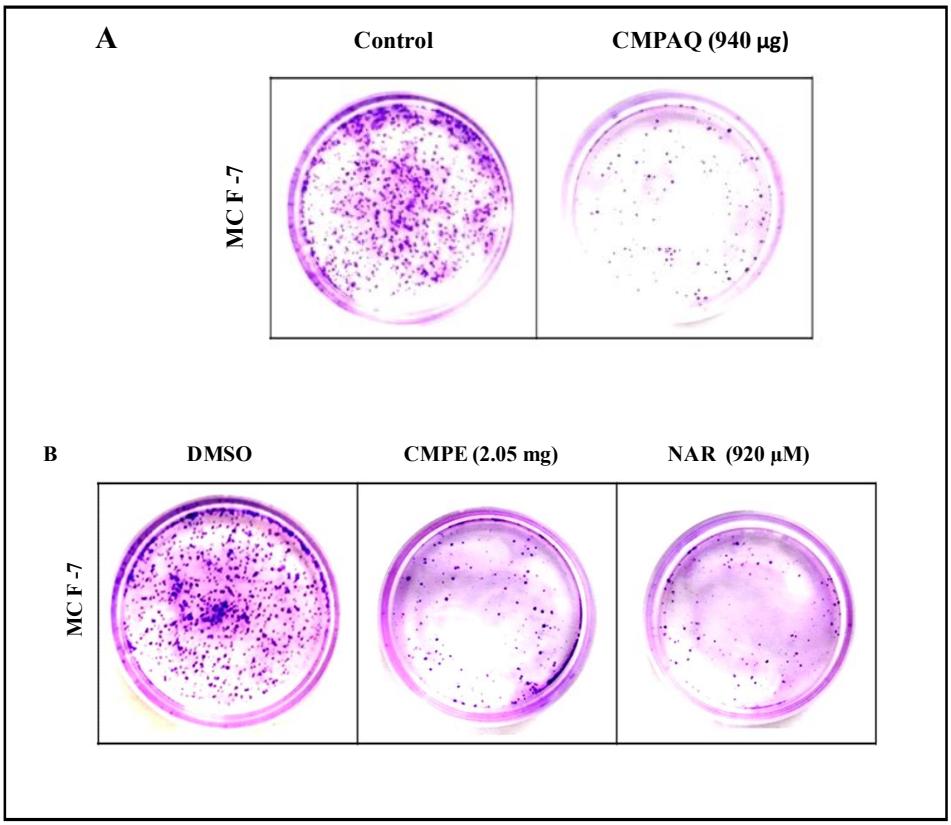


Figure 3: **Cell migration experiments investigated the impact of *C. maxima* peel extracts and flavanone NAR on MCF-7 (A) The microscopy images depict the scratch pictures (5X) and scratch area recovery following treatment with peel fraction and flavanone NAR, along with their individual controls and with their particular IC_{50} values at varying time gap from- 0-6 h, 6-12 h, 12- 24 h, and 24-48 h. (B) Bar graphs illustrate the cell migration rates that were determined by measuring the extent of wound coverage over 48 h compared to the initial wound area at 0 h. Data, presented as mean ± standard deviation from three different studies underwent (ANOVA) one way of variance. Statistical significance relative to the individual control was indicated by *, **, or *** for $p<0.05$, $p<0.01$ or $p<0.001$, respectively.**

3.3 Impact on colony formation in MCF-7 breast cancer cells by of *C. maxima* peel extracts

The colony development assay was executed utilizing MCF-7 cells in order to verify the effects of *C. maxima* peel extracts and NAR, as well as their respective controls, on development of colonies. MCF-7 cells showed a drop in colony count when treated with their corresponding IC_{50} doses in comparison to their individual controls (Figure 3). The above outcomes highlight the significant effect of the extracts and the flavanoid NAR on breast cancer cell colony development.



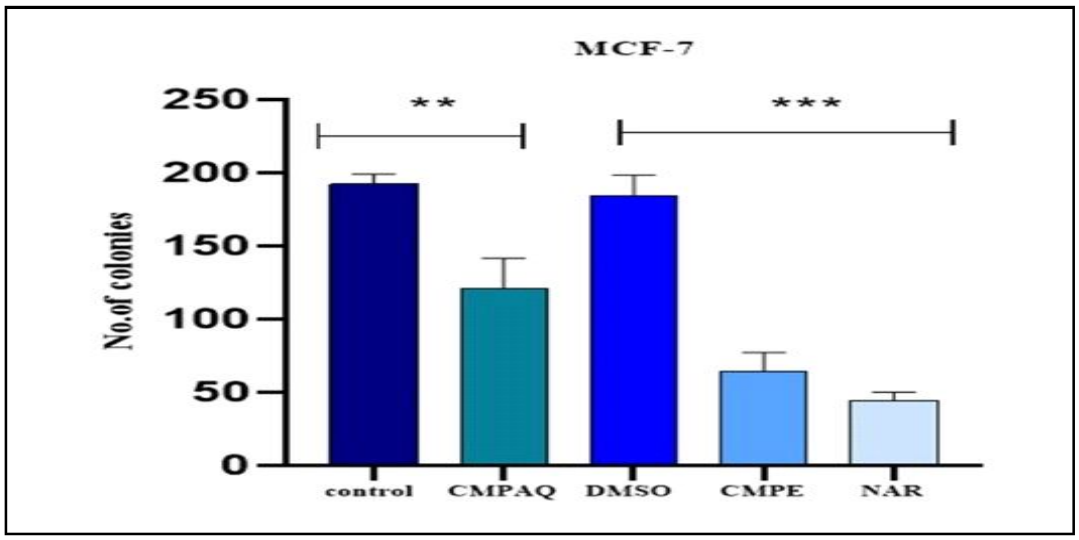


Figure 4: Colony formation assay reveals the influence of *C. maxima* peel fraction of extracts and flavanone NAR on MCF-7 (A, B): Representative figures depict cell colonies post treatment with the particular peel fraction of extracts, NAR, and their individual controls at IC_{50} concentrations efficiently suppressing development of colonies in MCF-7 cells. (C): The bar graph illustrates the percentage of colony formation relative to the control condition. Data, presented as mean ± SD from three independent experiments underwent ANOVA one way analysis of variance. Statistical significance relative to the control was indicated by *, **, or * for $p<0.05$, $p<0.01$ or $p<0.001$, respectively.**

4. Discussion

Breast cancer stands premier in incidence amongst women and is the second foremost cause of transience, accounting for 15% of deaths (Siegel *et al*., 2019). As per reported data, the foremost cancers in India encompass breast, lung, oral cavity, cervical and tongue malignancies. The recorded incidences indicate six lakhs, seventy nine thousand, four hundred and twenty one male and seven lakhs, twelve thousand, seven hundred and fifty eight female cancer subjects. Among females in India, breast and cervical cancers emerge as the predominant cancer types (Barathe *et al.,* 2022; Chhikara and Parang, 2022).

Chemotherapy is a cornerstone in the treatment of numerous cancer types, administered either independently or alongside radiotherapy. However, the long term and harsh adverse reactions associated with conventional cancer treatment have sparked fresh investigations towards herbal remedies envisioned at mitigating these undesirable outcomes (Beheshti *et al*., 2021; Mohd *et al*., 2021; Bindu *et al*., 2022).The potential of peels of citrus fruits as a reservoir of medicinal constituent remains largerly unexplored, owing to its composition compromising carotenes, essential oils, pectin and an high content of polyphenolic compounds like flavanoid, alkaloids, phenolic acids, which are secondary metabolites in plants known for their multifaceted and vital biological roles (Koolaji *et al.,* 2022; Chellammal, 2022). Multiple phytochemical compounds cause cytotoxic *via* influencing cell cycle progression and cell death, and exhibiting properties that are anti inflammatory.

Researchers have demonstrated dissimilar pharmaceutical features like antibacterial, antifungal and antitumor actions (Pandey *et al*., 2019; Monteiro *et al.,* 2023; El-Kersh *et al.,* 2021) on *C. maxima*. Our experimental data showed *in vitro* antioxidant capabilities of *C. maxima* fruit fractions including their flavanoid and content of phenol.

Our study demonstrated that the peels contain powerful phytoconstituents with promising potential for development as candidate anticancer compounds, in addition to contain antioxidant features (Monteiro *et al.,* 2022). The current examined, the cytotoxic potential of *C. maxima* aqueous and ethanolic extracts as well as NAR against MCF-7. The breast carcinoma cells viability diminished over time after treatment with *C. maxima* peel fraction of extracts and its flavanone NAR at their respective IC_{50}values, as shown by the MTT assay (Figure 2). These findings are consistent with previous research indicating the antitumor efficacy of *C. maxima* peel extracts which suppressed (MCF-7) proliferation.

El-Kersh.*et al* used Cytotoxicity assays to assess peel extracts from various citrus species and their flavonoids, including NAR against estrogen dependendent breast cancer cell lines. These demonstrated strong efficacies against cancer cell lines while causing minimal Cytotoxicity against normal melanocyte HFB4 cell line (El-Kersh *et al.* 2021).

Metastasis, or the ability of cancerous cells that enter the surrounding or distant tissues, is a multifaceted method that includes movement, cell to cell adhesion, and invasion. Antimetastatic medicines that target these systems have the potential to reduce cancer cell aggressiveness. The cancer cell migration to multiple tissues is a crucial early stage in cancer-metastasis (Majumder *et al.,* 2019).

One of the fundamental features of malignant tumors, which significantly contribute to the elevated fatality rates associated with cancer, is Cell migration, or metastasis (Siegel *et al.,* 2019). In this study focusing on cell migration, both crude peel extracts and NAR impeded the migration of MCF-7 cancer cells, resulting in diminished metastatic capability compared to controls (Figure 3).The extracts from onion peel suppressed growth, migration , of cancer cells, and also triggered apoptosis in a way that was dose-dependent

(Uttarawichien *et al*., 2021). The colonogenic experiment was conducted to assess the capability of a single cell to form a colony which shows its metastatic potency (Nakamura *et al.,* 2023). The assay (colony formation) results discovered a noteworthy reduction in the number of colonies in the treated groups in comparison to their relevant controls. The unregulated cell division and aberrant energy metabolism, which is mostly driven by Warburg effect is a distinguishing aspect of carcinoma cells.

Multiple research investigations have suggested that plant flavonoids can inhibit cancer progression by slowing the tumor growth process, increasing apoptotic pathway, halting cell cycle, and limiting carcinoma cell motility in the circulatory course of action (Kameyanda *et al.*, 2021; Kamurthy *et al.,* 2023). Antioxidant rich foods, which contain phenolic compounds and phytochemicals, can help to reduce oxidative stress and its cancer causing effects (Rao *et al.,* 2023). Citrus flavonoids, recognized for their low toxicity, are thought to influence apoptosis *via* tyrosine kinases and have anti-proliferative activities (Alaqeel, 2023).

5. Conclusion

Citrus fruits are abundant in flavonoids and along with other secondary metabolites play a significant role in reducing reactive oxygen species, in this manner lowering the risk of cancer. Based on the results of the recent studies, *C. maxima* peel extracts and NAR demonstrated robust antitumor activity against MCF-7 breast cancer cells. Furthermore, the results indicate significant restraining of breast cancer cell migration and colony formation of these extracts and NAR. Given this distinct mechanism of action, *C. maxima* peel extracts and NAR hold promise as viable options for treatment of breast cancer.

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Conflict of interest

The authors declare no conflict of interest relevant to this article.

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