**Research article**

# *In vitro* anticancer activity of *Citrus maxima* peel extract on human breast cancer cells

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## ABSTRACT

**Introduction and Aim:** The alarming rise in breast cancer cases highlights the need for a safer, effective, and more responsive chemoprevention approach for cancer treatment. In contrast to conventional chemotherapeutic medicines, which primarily function as mono-target agents, phytochemicals have been proven to inhibit cancer growth by influencing numerous processes, such as apoptosis and signaling pathways. *Citrus maxima* (Burm). Merr., frequently identified as pomelo, exhibits a wide range of biological activities, and is utilized extensively in the conventional approach to medicine, and this study focused on anti-cancer properties of *Citrus maxima* peel extracts.

**Materials and Methods:** The anti-proliferative properties of the *Citrus maxima* peel extracts and its flavonoid Naringin on the breast cancer cell line was evaluated using the MTT test. The ability of the breast cancer cells to migrate was assessed using a wound healing assay and their capacity to form colonies was assessed using a colony formation assay.

**Results:** The *Citrus maxima* crude peel extracts and Naringin inhibited the cell proliferation of the breast cancer cell line. The cell viability of breast cancer cells decreased with time after treatment with their corresponding IC50. The colony-formation capacity and migration ability of the cells was also reduced.

**Conclusion:** The examined *Citrus maxima* crude peel extracts and its flavonoid Naringin showed strong anticancer efficacy by suppressing cell proliferation and could be seen as prospective candidates for upcoming breast cancer therapeutic medications. Extensive research is also needed to fully comprehend the precise mechanism of action of extracts and its components.

**Keywords:** *Citrus maxima* peel extract*;* naringin; anticancer; cell proliferation; breast cancer.

## INTRODUCTION

Female breast cancer accounts for 6.9% of all cancer deaths and is the most diagnosed malignancy, accounting for 11.7% of all cases (1-3). Moreover, it is one of the world's greatest threats to human health. For the management of malignant illnesses, there are presently no curative therapy options accessible despite substantial research carried out over the previous few decades. Chemotherapy and radiation are two modern therapeutic techniques that have considerable systemic adverse effects, harm to growing healthy cells, structural abnormalities, behavioral illnesses, and drug-resistance, limiting their tolerability and clinical application (4). As a result, innovative, safer, and more effective selective therapy options for various cancers require immediate attention. According to growing evidence based on *in vitro* and *in vivo s*tudies, bioactive compounds derived from numerous natural sources can demonstrate chemo-preventive activity or as a co-administration in oncological therapies. Several Investigations have demonstrated the synergistic effect of natural ingredients with various chemotherapy medications (4).

Many plants are ingested for their health advantages in developed countries. At the same time, people in Asia and Africa have employed medicinal plants for thousands of years in traditional medicines, taking advantage of their therapeutic benefits (5). Polyphenolic compounds have been extracted from plants for their crucial anti-cancer properties in drug discovery and development including flavonoids, alkaloids, carotenoids etc. Low-molecular weight flavonoids are abundant in citrus fruits. Certain members of citrus species are underutilized and need to be thoroughly examined with a focus on their bioactive and therapeutic qualities (6).

*Citrus maxima* often referred to as pomelo, belong to the class *Magnoliopsida*, family *Rutaceae*, and are a highly valued species in terms of ethnomedicine. Among the phytochemicals present in this fruit, flavonoids are most often reported, and its peels contain a high number of flavanones naringin and its aglycone-naringenin (7-9).

The citrus peel flavonoids target various intracellular signaling and regulatory enzymes thus, exhibiting effective anti-cancer action such as arresting cell cycle, impeding cell-proliferation, enhancing apoptosis, preventing migration, metastasis etc., (6).

In this current investigation, cell viability, cell migration and colony-formation assays were used to evaluate the anti-cancer effectiveness of *Citrus*

*maxima* peel (aqueous and ethanolic extracts) on the human breast cancer cell line MDA-MB-231.

## MATERIALS AND METHODS

### Plant material and extraction

The fruit of *Citrus.maxima* was purchased from Sakleshpur. After the fruits were cleaned under tap water, the peel was separated, cut into pieces, and dried in an oven (40-50ºC) for seven days. A powdered dry peel was used. Aqueous-peel extract was prepared by boiling the fine peel powder in distilled-water for 20 minutes, then subjected to filtration using grade 1 Whatmann filter. Ethanolic extract was prepared using Soxhlet apparatus and 99% ethanol as solvent extraction at 55℃ for 24 hours. After the solvent was eventually evaporated using a rotary-flash evaporator, the concentrated peel extract was preserved at 4ºC until use (10).

### Cell culture condition and treatment

MDA-MB 231 human breast cancer cell line was procured from NCCS cell-repository Pune. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) with 10% fetal bovine serum (Gibco) and 100 U/ml penicillin/streptomycin in a humidified carbon dioxide incubator (5% $CO_2$) at 37ºC. Stock solutions of *Citrus maxima* aqueous extract 1mg/ml media, ethanolic extract 200mg, and Naringin 100mM (Sigma Aldrich) in DMSO were prepared and preserved (-20ºC) until use.

### Cell viability assay

MTT assay (11,12) was used to evaluate the cytotoxicity. Cells ($0.05x10^6$) were seeded in 24 well plates with respective growth media and incubated in a 5% $CO_2$ incubator at 37ºC. After attaining 70% confluency and starvation for 24hours, the cells were treated with their respective $IC_{50}$ of crude *C. maxima* extracts and Naringin extracts and controls. The treated cells were incubated for 24, 48, and 72 hours and then washed with phosphate- buffered saline (PBS and reconstituted by fresh media. 20µl of MTT 3-(4, 5-Dimethylthiazol-2yl)-2, 5-diphenyltetrozolium bromide)-5mg/ml (SRL) was added, followed by incubation for 4 hours at 37ºC. After removing media, 100 µl of DMSO was used to dissolve the formazan crystals, and absorbance at 570 nm was measured.

### Cell migration assay

The inhibition of cell migration was evaluated using the wound healing assay (13). In a six well plate ($1x10^3$) cells per ml were seeded. After 24 hours of attaining confluency, a 200 µl tip was used to make a scratch. The cells were treated with the concentration-values of $IC_{50}$ of crude extracts, Naringin and their control in a serum-free medium. The wound area was observed for 0 hrs, 6 hrs, 12 hrs, 24 hrs, and 48 hrs using an inverted phase contrast microscope equipped with Leica software. Cell migration and percentage of wound area was calculated using Image J software.

### Colony formation assay

In a six well plate, 500 cells were seeded in each well and allowed to grow for a few days. Cells were treated with $IC_{50}$ –concentrations of *C. maxima* extracts and control. Following treatment, PBS wash was given. For 15 minutes cells were incubated at room temperature after being fixed with 70% ethanol. After incubation, ethanol was discarded; cells were stained with 400µl crystal violet solution (1%) and incubated for 15minutes at room temperature. After washing with PBS, the plates were left to dry. The cells were observed by a phase contrast microscope. The colonies were counted (14).

### Statistical analysis

Graph Pad PRISM 8.0 software was used to conduct statistical analysis. Experiments were carried out three times. Data was reported as mean with SD. Using One-way ANOVA or two-way ANOVA with multiple comparisons statistical significance was calculated. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ was used to represent statistical significance.

## RESULTS

### Anti-cancer effect of *Citrus maxima* peel extracts against breast cancer cell lines

Cell viability of *Citrus maxima* peel extracts and Naringin on breast cancer cell lines was assessed using the MTT test. Aqueous and ethanolic *C. maxima* peel extract and naringin with their respective $IC_{50}$ 370µg/ml, 940 µg/ml, and 150µM were used to treat MDA-MB-231 for a period of 24 to 72 hours. It was observed that the cell viability decreased over time. A gradual decline began at 24 hours, and maximum reduction in the viability of cells was recorded at 72 hours.

### Effect of *Citrus maxima* peel extracts on migration of breast cancer cells

The inhibition effect of *Citrus maxima* peel extracts and naringin was examined on the cell proliferation and migration of breast cancer cells. It was evaluated over 0 hours, 6 hours, 12 hours, 24 hours, and 48 hours after treatment with their respective half maximal inhibitory concentration. The results showed that the examined plant extracts and Naringin (Fig.2) substantially prevented the migration and progression of MDA-MB-231 cells when compared to their respective controls. In the control without treatment and the DMSO control group, the wound healing was not affected, and the gap was almost completely closed in 48 hours.

### Effect of *Citrus maxima* peel extracts on colony formation of breast cancer cells

The colony formation assay was carried out using MDA-MB-231 to confirm the effect of *Citrus maxima*

peel extracts and Naringin along with its control on the colony development of breast cancer cells. After treatment with their $IC_{50}$, MDA-MB-231 showed a reduced number of colonies compared to their controls (Fig.3). These findings demonstrate that extracts and its flavonoid naringin significantly affect the colony forming ability of breast cancer cells.

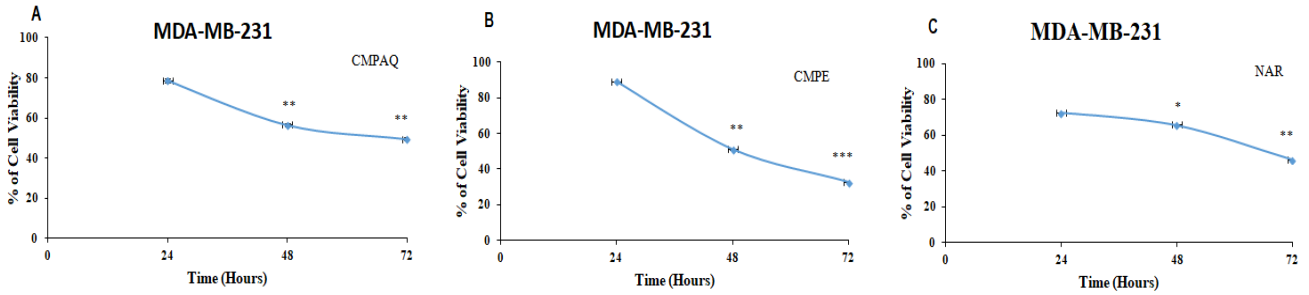


**Fig 1**: MTT assay demonstrated a reduction in cell viability of MDA-MB-231 cells after being treated with *C. maxima* peel extracts and naringin (A-C) with their $IC_{50}$ for a period of 24, 48 and 72 hours.
Values are mean±SD of three independent experiments subjected to one-way analysis of variance (ANOVA), and statistical difference from the control denoted by * and the *P<0.05, **P<0.01 or ***P<0.001 was considered statistically significant. CMPAQ-*Citrus maxima* peel aqueous extract; CMPE- *Citrus maxima* peel ethanolic extract; NAR-Naringin.

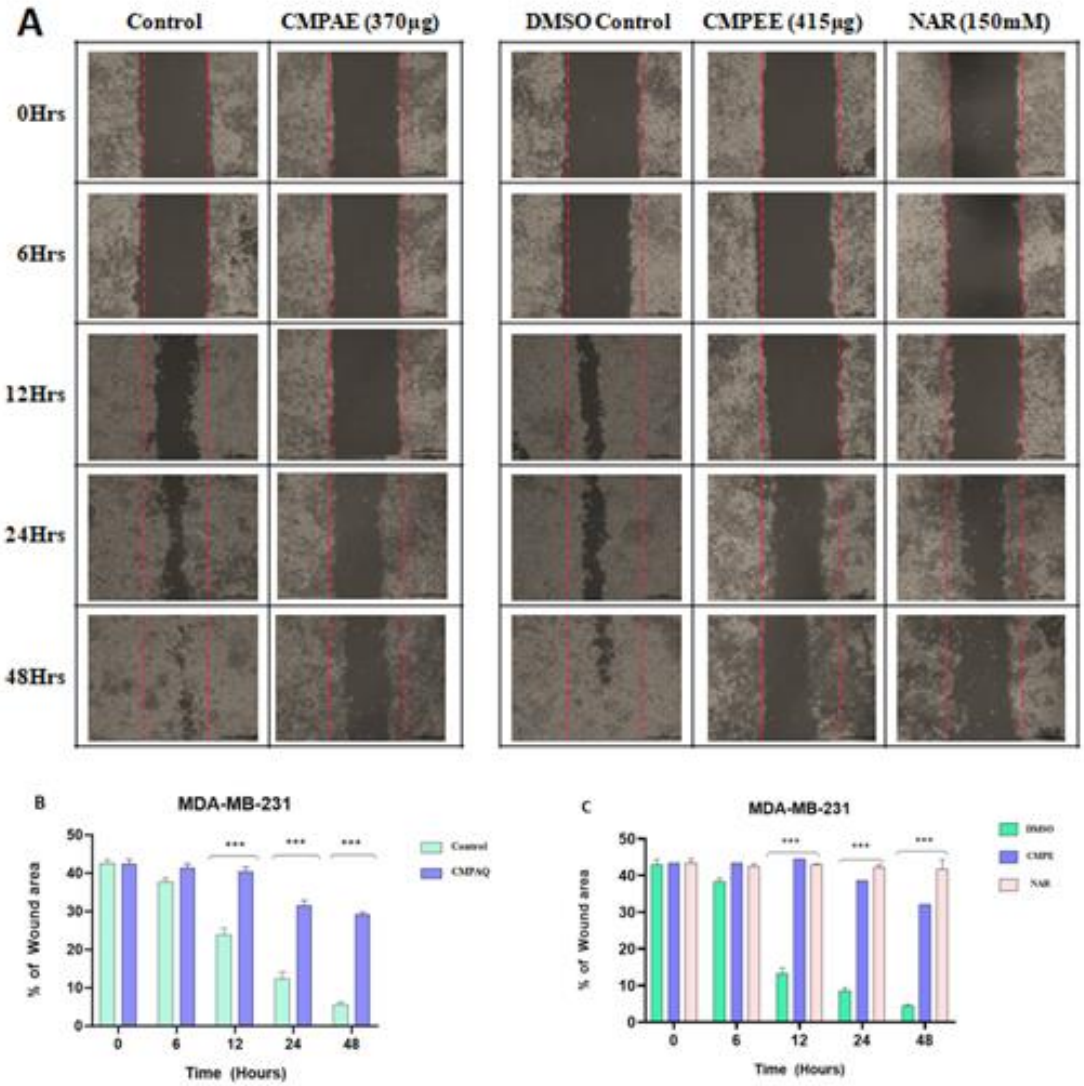


**Fig 2**: Cell migration assay shows the effect of *Citrus maxima* peel extracts and Naringin on MDA-MB-231 breast cancer cells. (A) Photomicrographs representing the images of scratch and wound area recovered after treatment with extracts and Naringin their controls with their respective $IC_{50}$ at different time intervals from 0, 6, 12, 24, and 48 hours (B) and (C) Percentage of wound area or cell migration are represented in the bar graph compared with the control. The differences from the control are indicated by * and ***P<0.001 was deemed statistically significant. Values represent mean±SD of three separate experiments that were subject to two-way analysis of variance (ANOVA). CMPAQ-*Citrus maxima* peel aqueous extract; CMPE- *Citrus maxima* peel ethanolic extract; NAR-Naringin.

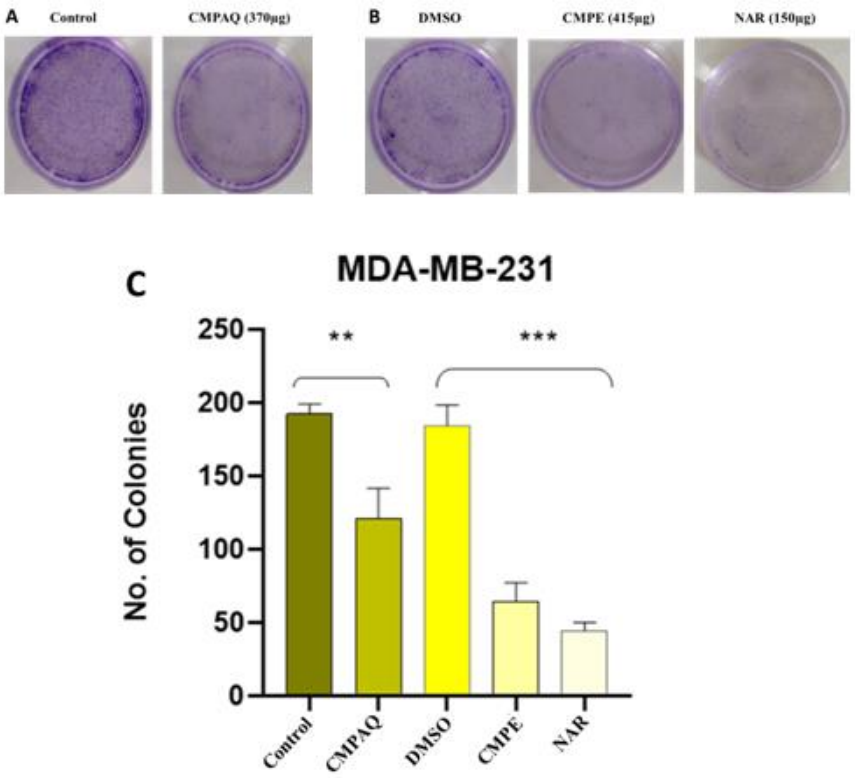


**Fig 3**: Colony formation assay shows the effect of *Citrus maxima* peel extracts and naringin on MDA-MB-231 breast cancer cells. (A, B) Representative illustrations of cell colonies after treatment with respective extracts, Naringin, and their controls at $IC_{50}$ concentrations effectively inhibited the colony development in MDA-MB-231 cells. (C) The percentage of colony formation is represented in the bar graph compared with the control.

The differences from the control are indicated by* and **P<0.01 or ***P<0.001 was deemed statistically significant. Values represent mean±SD of three separate experiments that were subject to two-way analysis of variance (ANOVA). CMPAQ-*Citrus maxima* peel aqueous extract; CMPE- *Citrus maxima* peel ethanolic extract; NAR-Naringin.

## DISCUSSION

Plant-based products are an essential source for new anti-cancer drugs that have the potential to provide long-term cancer management with minimal side effects (15). Citrus fruits like grapefruit and pomelo are rich in bioactive compounds like flavonoid which is highly concentrated in its peel fraction and have been recognized as agents in cancer treatment (6).

In our previous study, we explored the *in-vitro* antioxidant activity of *C. maxima* juice, pulp, and peel along with its flavonoid and phenolic content and found that peels contain powerful phytoconstituent that could be created as candidate compounds for an anti-cancer treatment and have the high antioxidant capacity (16).

So, we further examined the anti-carcinogenic potential of *Citrus maxima* peel extract on breast cancer cell line MDA-MB-231. The *Citrus maxima* peel extracts and its flavonoid naringin decreased the viability of breast cancer cells in a time–dependent manner when treated with their respective $IC_{50}$ and was evaluated by MTT assay (Fig.1). The results are consistent with previous findings wherein *C. maxima* peel extracts have displayed anti-cancer efficacy by inhibiting the proliferation of breast cancer cells (17). Based on Li *et al.*, findings naringin had a distinct inhibitory effect on the growth of Triple negative breast cancer cells (18).

A particular primary characteristic of malignant tumors that contribute to an increase in the fatality rate of cancer is cell-migration or metastasis (13). In this cell migration study, the crude extracts and Naringin (Fig. 2) considerably reduced the rate of cell migration in MDA-MB-231 cells when compared to their respective controls. These results might point to effective anti-cancer drugs that may prevent cancer metastasis.

In general, cancer cells have a propensity to form colonies when in contact with adjacent cells leading to cell-cell adhesion and cell motility. According to the clonogenic assay, the *C. maxima* crude extracts and Naringin significantly reduced the colony-forming ability of breast cancer cells as compared to the control (Fig.3).

Citrus peel extracts have been shown to exhibit anti-cancer action across several cell lines at varying levels of efficacy, which is swiftly connected with its chemical composition (19). The anti-cancer potential of naringin has also been studied in numerous cancer forms, such as bladder cancer (20), cancer of prostate (21), glioblastoma (22) etc., reducing proliferation,

and inhibiting ability to migrate and form colonies with efficient anti-tumor activity.

The anti-cancer effect of peel extracts from one of the study findings indicated that the combination of peel extract from orange and naringin had a greater impact on esophageal squamous cell carcinoma than the flavonoid alone (23). The cancer prevention ability of the overall citrus peel extracts with higher concentrations of its total-phenolic and total-flavonoid content is found to be higher than its extracted individual bioactive components (6).

## CONCLUSION

According to the findings of the current investigations, *C. maxima* peel extracts and its flavonoid naringin has a profound tumor prevention action against breast cancer cells-MDA-MB-231. The outcomes also showed that the extracts and Naringin significantly hindered the capacity of breast cancer cells to migrate and develop colonies. Due to this distinctive mechanism, *C. maxima* peel extracts and its compound naringin may be a more practical option in treating breast cancer patients.

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## CONFLICT OF INTEREST

No conflicts of interest declared by the authors.

## REFERENCES

1. Sung, H., Ferlay,J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A., *et al*., Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. A Cancer Journal for Clinicians.2021;71(3):209-249.
2. Nidugala, H., Prabhu, A., Avadhani, R., Ravishankar, B. *In vitro* anticancer efficacy of *Cyperus rotundus* (L.) on breast adenocarcinoma cells via the induction of DNA fragmentation and apoptosis. Biomedicine. 2023 Sep 18;43(4):1198-1202.
3. Guthigar, M., Naik, P.R. Assessment of knowledge, attitude, and practice regarding breast cancer among the women in rural Karnataka, South India. Biomedicine. 2023 Aug 30;43(4):1320-1324.
4. Kashyap, D., Tuli, H.S., Yerer, M.B., Sharma, A., Sak, K., Srivastava, S., *et al*., Natural product-based nanoformulations for cancer therapy: Opportunities and challenges. Seminars in Cancer Biology.2021; 69:5-23.
5. Greenwell, M., Rahman, P.K. Medicinal Plants: Their Use in Anticancer Treatment. International Journal of Pharmaceutical Science and Research.2015; 6(10):4103-4112.
6. Koolaji, N., Shammugasamy, B., Schindeler, A., Dong, Q., Dehghani, F., Valtchev, P. Citrus peel flavonoids as potential cancer prevention agents. Current Developments in Nutrition. 2020; 4(5): nzaa025.
7. Kumar, S., Singh, I., Kohli, D., Joshi, J., Mishra, R. Waste Pomelo (*Citrus Maxima*) Peels – A natural source of antioxidant and its utilization in peanut oil for suppressing the development of rancidity. Current Research in Nutrition and Food Science Journal.2019; 7(3):800-806.
8. Sowmya, N., N, Haraprasad., Hema, B.P. Exploring the total flavonoid content of peels of *Citrus auriantum, Citrus maxima* and *Citrus sinensis* using different solvents and HPLC- analysis of flavonones - Naringin and Naringenin in peels of *Citrus maxima*. Pharma Innovation. 2019; 8(4):12-17.
9. Cordenonsi, L.M., Sponchiado, R.M., Campanharo, S.C., Garcia, C.V., Raffin, R.P., Schapoval, E.E.S. Study of flavonoids present in pomelo (*Citrus maxima*) by DSC, UV-VIS, IR, 1H and 13C NMR and MS. Drug Analytical Research. 2017;1(1):31-37.
10. Bhandary, B. S. K., Sharmila, K.P., Kumari, S.N., Bhat, V.S. Phytochemical profile and invitro antioxidant activity of *Asparagus racemosus* root extract, isoprinosine and shatavari syrup. Journal of Harmonized Research. 2015; 3:215-223.
11. Siddiqui, F.A., Prakasam, G., Chattopadhyay, S., Rehman, A.U., Padder, R.A., Ansari, M.A, *et al*., Curcumin decreases Warburg effect in cancer cells by down-regulating pyruvate kinase M2 via mTOR-HIF1α inhibition. Scientific Reports.2018;8(1):8323.
12. Jayakar, V., Lokapur, V., Nityasree, B.R., Chalannavar, R.K., Lasrado, L.D., Shantaram, M. Optimization and green synthesis of zinc oxide nanoparticle using *Garcinia cambogia* leaf and evaluation of their antioxidant and anticancer property in kidney cancer (A498) cell lines. Biomedicine. 2021 Jul 7;41(2):206-222.
13. Somaida, A., Tariq, I., Ambreen, G., Abdelsalam, A.M., Ayoub, A.M., Wojcik, M., *et al*., Potent cytotoxicity of four cameroonian plant extracts on different cancer cell lines. Pharmaceuticals (Basel). 2020;13(11):357.
14. Franken, A.P.N., Rodermond, M.H., Stap, J., Haveman, J., Bree, V.C. Clonogenic assay of cells *in vitro*. Nature Protocols. 2006; 1:2315-2319.
15. Mukherjee, A.K., Basu, S., Sarkar, N., Ghosh, A.C. Advances in cancer therapy with plant based natural products. Current Medicinal Chemistry.2001; 8(12):1467-1486.
16. Monteiro, F., Shetty, S.S., Ranjitha, K., Shetty V.V., Shetty, D.P., Patil, P., Kumari, N.S. Phytochemical profiling, total flavonoid, total phenolic content, and *in-vitro* antioxidant evaluation of *Citrus maxima* extract. Biomedicine. 2022; 42(5):912-919.
17. Mursiti, S., Amalina, N.D., Mariantil, A. Inhibition of breast cancer cell development using *Citrus maxima* extract through increasing levels of reactive oxygen species (ROS). Journal of Physics: Conference Series. 2021;1918 :052005.
18. Li, H., Yang, B., Huang, J., Xiang, T., Yin, X., Wan, J., *et al*., Naringin inhibits growth potential of human triple-negative breast cancer cells by targeting β-catenin signaling pathway. Toxicology Letters. 2013;220(3):219-228.
19. Tajaldini, M., Samadi, F., Khosravi, A., Ghasemnejad, A., Asadi, J. Inhibition of growth and migration of esophageal squamous cell carcinoma cells by orange peel extract and naringin. Medical Laboratory Journal. 2020; 14 (2):31-35.
20. Radicchi, D., Melo, A., Lima, A. P., Almeida, T., Souza, G., da Silva, G. Naringina: potencial antitumoral *in silico* en *in vitro* en células cancerosas de vejiga. Ars Pharmaceutica.2022; 63(2):132-143.
21. Erdogan, S., Doganlar, O., Doganlar, Z.B., Turkekul, K. Naringin sensitizes human prostate cancer cells to paclitaxel therapy. Prostate International.2018;6(4):126-135.
22. Aroui, S., Fetoui, H., Kenani A. Natural dietary compound naringin inhibits glioblastoma cancer neoangiogenesis. BMC Pharmacology and Toxicology.2020;21(1):46.
23. Tajaldini, M., Samadi, F., Khosravi,A., Ghasemnejad, A., Asadi, J. Inhibition of Growth and Migration of Esophageal Squamous Cell Carcinoma Cells by Orange Peel Extract and Naringin. Medical Laboratory Journal. 2020; 14 (2):31-35.