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Investigation of Anti-cancer potential of Karanjin in MCF-7 and MDA-MB-231 breast carcinoma cells

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Abstract

The utilization of natural flavonoids against cancer is the latest research hotspot. The present study evaluated anticancer potential of Karanjin in two breast cancer cell lines. The anticancer effects of Karanjin on proliferation were assessed by MTT assay in both breast cancer cells. The Docking assay was performed to explore the binding affinities of Karanjin for receptor and enzymes involved in breast cancer. The antimitotic activity of Karanjin was evaluated by onion root method and the cytotoxicity of Karanjin was checked by Brine Shrimp lethality assay. The docking studies proved significant interactions of Karanjin with progesterone as well as estrogen receptor. However, interaction with estrogen was significantly higher in comparison to progesterone. Also, Karanjin showed positive interactions with enzyme Aromatase and HER2 gene as confirmed by docking studies. Moreover, the MTT assay confirmed anticancer abilities of Karanjin in both cell lines. However, IC 50 concentration of Karanjin was significantly less in MCF-7 cells when compared with its IC 50 concentration in MDA-MB-231 cells. The microscopic analyses of the cell lines also revealed similar result proving higher efficacy of Karanjin in MCF-7 cells in comparison to MDA-MB-231 cells. The BSL [Brine Shrimp lethality] assay for 3 doses of Karanjin showed 500µg/mL does as the most toxic dose amongst all doses tested. Similar trend was noticed in the antimitotic assay, wherein significantly higher effects on onion root number and length were recorded at 500µg/mL concentration of Karanjin. The study confirmed that Karanjin has potential anti-cancer abilities against breast cancer. Moreover, Karanjin showed significantly higher anti-cancer potential in MCF-7 cells

Keywords: *Karanjin, cancer, breast cancer, MCF-7, MDA-MB-231*

1. Introduction

Breast carcinogenesis is the most frequent factors responsible for women mortality [1]. The deeper understating of the disease resulted in better diagnostics and effective therapeutics [2]. All above collectively contributed towards timely detection and improved outcomes. Further, multiple studies in recent past have concluded lifestyle and environmental factors as key factors responsible for the progression of the breast cancer [3-5]. Also, utilization of flavonoids as anticancer agents is an upcoming area in cancer research as flavoinds have proved their potential in multiple cancer studies [6-9]. Flavonoids act against cancer mainly by virtue of their anticancer, antioxidant, antibacterial and anti-inflammatory activities [10-11]. Karanjin is the flavonoid of interest in the present study.

Karanjin is an active furanoflavonol constituent of Fordia cauliflora Hemsl. Earlier reports have confirmed voluminous mechanisms of action behind its anticancer potential such as, its ability to stimulate ATPase activities of ATP-binding cassette [ABC] transporters ABCB1, ABCC1, and ABCG2 [12]. Studies have also proved ability of Karanjin to cause cell cycle arrest via induction of apoptosis [13]. However, scantiness of information exists with regard to its role in context of breast cancer.

The prime aim of the present study is to explore anticancer properties of Karanjin against breast cancer. We primarily performed screening studies that included docking experiments for studying binding of Karanjin with enzymes and receptors involved in breast cancer. The cytotoxicity was screened by BSL assay. The control of mitotic activity of cells is an essential anticancer property. Therefore, we have checked antimitotic activity of Karanjin by utilizing onion root model. Finally, MTT assay was performed to further establish anticancer effects of Karanjin. So, the present study is first of its kind to explore anticancer properties of Karanjin in breast cancer cells.

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MATERIALS AND METHODS

Chemicals, cell lines and Instruments

Karanjin was procured from Yucca Enterprises Wadala, Mumbai. MCF-7 and MDA-MB-231 cells were obtained from NCCS, Pune. RPMI medium was procured from Hi Media. 1% anti-biotic-anti-mycotic solution was procured from Sigma-Aldrich. 96 well plates were purchased from Thermo Fisher Scientific. Instruments used in the study included Biosafety cabinet level II, Multiskan GO microplate spectrophotometer.

Docking studies

Structure of Karanjin was downloaded from website https://pubchem.ncbi.nlm.nih.gov/ AutoDock Tool 1.5.6 [ATD] was utilized for this study [14-16]. The AutoDock Vina 1.0 software automatically computed Gasteiger charges, merged non-polar hydrogens and autodock type to each atom. Then torsions were defined, which showed rotatable and non-rotatable bonds in ligand. Finally, results were saved in pdbqt file format. AutoDock Vina software [14] was run using windows command prompt. All the programme files, ligand [.pdbqt], protein [.pdbqt] and configuration files [.conf] were saved in same folder. The computation was performed in the same folder as log .txt and ligand_out.pdbqt. Log .txt file showed binding energy of ligand to the protein and ligand_out.pdbqt file revealed sites on the proteins with binding energy. The output [.pdbqt] files obtained from the docking study were used to evaluate the hydrophobic interaction of ligand with protein. The results were then processed with chimera software version 1.8 for creation of copies of protein as well as ligand. This was followed by assessment of interactions between protein and ligand by using ligplot+ version 1.4.5.

Cytotoxicity study by BSL [Brine Shrimp lethality] assay

Artemia salina leach [Brine Shrimps eggs] were procured from Amazon.in [e-market] in the form of capsules filled with thousands of dried cysts. The experimental method involved exposures to variable concentrations of the test compound [17]. The toxicity was calculated after 1 h, 2 h, 5 h and 08 h of exposure. For confirmation of results, larvae counted as dead. Moreover, dead larvae in each treatment were compared to respective controls. The following calculation formula used:
% M = percentage [control survivors – treatment survivors].

Morphometric Antimitotic activity using Alium cepa model:

The antimitotic activity of Karanjin was evaluated by using the Allium cepa roots. Onion bulbus [Allium cepa L] were obtained from local market and were place in beaker for sprouting in tap water at room temperature. The bulbs were positioned on beakers containing Karanjin at 3 different concentrations [100, 250 & 500 µg/ml] in a way that the roots were slightly immersed [18]. The duration for each treatment was 72 hrs. The root length and number were counted post 72 hrs. We counted 10 longest roots from each group for the root length.

MTT Assay

Optimum numbers [approximately 5000 cells per well] of both MCF-7 & MDA-MB-231 cell lines were seeded in 100µl media in 96 well plates [19]. This was followed by incubation of plates for 18-24 hours in 5% CO2 incubator set at 37 °C. After 24 h cells were treated with desired Karanjin concentrations in triplicates when they reached at about 40-50% confluence. Photographs were taken of each treatment group, Thereafter 10 µl of MTT [5 mg/mL] were added in each well in the media. Plates were then incubated for 2-3 hours in 5% CO2 incubator set at 37°C until the formazan crystals are formed that could be seen under the microscope. Media was discarded gently without disturbing formazan crystals. 100-200 µl of DMSO was then added per well to solubilize the crystals. Plates were incubated on shaker in dark for 10 minutes. Absorbance at 570 nm was read and IC50 values were calculated for each sample. IC50 values were calculated using Probit analysis.

Statistical analysis

The statistical significance of the data has been determined using one way analysis of variance [ANOVA] followed by Turkey's multiple post-hoc test using SPSS Software The results are represented as Means ± S.D.

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RESULTS AND DISCUSSION

The exploitation of flavinoids against cancer is an emerging trend. Flavonoids are natural polyphenols that are omnipresent in plant-based food comprising of fruits, vegetables and teas [20]. The present study tested Karanjin against breast carcinoma by exploiting two cell lines. We have attempted assessment of every aspect of Karanjin, from molecular docking studies to toxicity profiles, from antimitotic activity to anti-cancer abilities. The study has undoubtedly presented Karanjin as an effective anticancer flavonoid against breast carcinoma.

Docking Study

Molecular docking study of Karanjin was performed with multiple key proteins [Hormones, Enzymes and Protein] responsible for breast cancer pathogenesis and pathophysiology. Predictions of binding energies of Karanjin with different molecules were estimated using AutoDock Vina and results are shown in Table-1 along with its interactions with protein residue.

Table 1: Protein ligand interactions

Complex	Energy (Kcal/mol)	Interaction bonds	
		Hydrogen bonding	Hydrophobic bonding
Progesterone - *Karanjin*	-8.4	Ser898	Phe895, Ile896, Phe895, Ile896,
Estrogen - *Karanjin*	-7.9	His524	Met427, Glu423, He424, Lys520, Glu523, Arg548, His547
Aromatase - *Karanjin*	-9.3	Gln428, Lys440	Phe432, Tyr424, Pro429, Tyr361, Phe427
Her2 - *Karanjin*	-9.0	--	Gln298, Ala317, Cys316, Val319, Tyr321, Phe349, Asn297, Glu299

The Ligplot showed hydrophobic interactions as well as hydrogen bonding with progesterone, estrogen, enzyme Aromatase and HER2 gene. Docking of Karanjin with progesterone, estrogen, aromatase and HER2 gene revealed affinity energy of -8.4 Kcal/mol, -7.9 Kcal/mol, -9.3 Kcal/mol and -9.0 Kcal/mol respectively. Protein ligand interaction by Ligplot confirmed formation of hydrogen bond with Ser898 residue of estrogen with significant hydrophobic interactions [figure-1A]. Karanjin formed one hydrogen bond with His524 amino acid of progesterone [Figure-1B] and with aromatase it forms hydrogen bonding form with two residue i.e. Gln428 and Lys440 [Figure-1C]. On the other hand, docking with HER2 gene exhibited no any hydrogen bonding [Figure-1D] while forming significant hydrophobic interaction with abundant residues. Moreover, hydrophobic interactions of Karanjin with estrogen were higher in comparison to progesterone. Also, this could be correlated with low IC50 value obtained via MTT assay with MCF-7 cell line (an estrogen positive cell line). The key significance of the observed affinities by docking studies was demonstrated by positive hydrophobic interactions of Karanjin with hormones estrogen and progesterone. This could be owed to ability of Karanjin to interfere with breast cancer resistance protein 1, BCRP1 [21]. Moreover, Karanjin also showed favorable interaction with HER2 gene and enzyme aromatase. So, the above hydrophobic interactions contributed towards better drug absorption leading to efficient anti-cancer effects.

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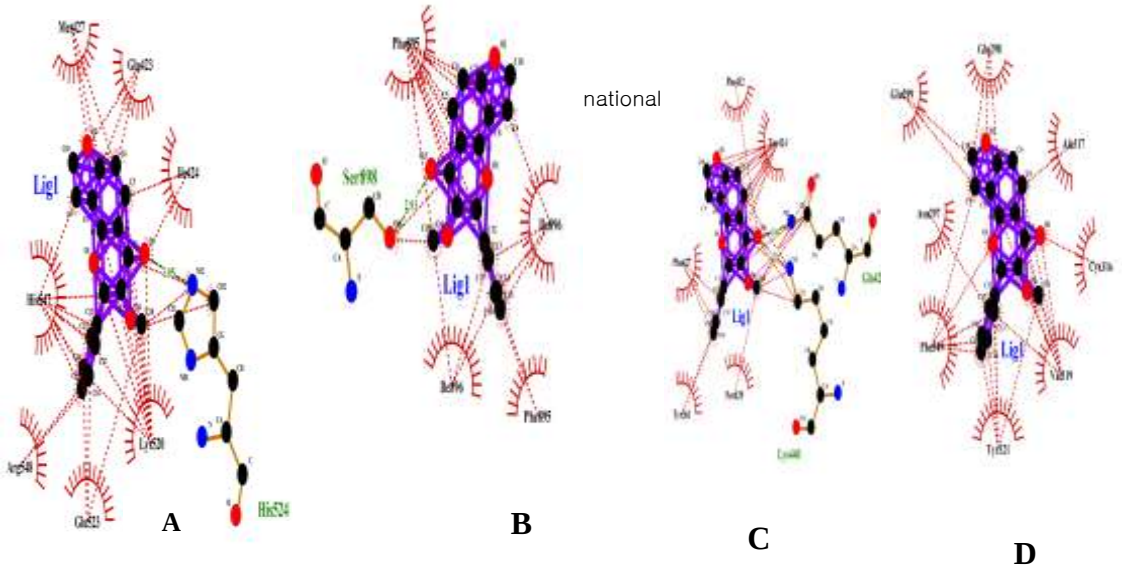

Figure 1: Protein ligand interaction by Ligplot software.

Brine Shrimp lethality Assay

The cytotoxicity of Karanjin was recognized by BSL assay and the highest 500µg/mL dose showed maximum cytotoxicity. The BSL [Brine Shrimp lethality] assay [table 2] showed cytotoxicity effects at for 3 different doses of Karanjin.

Table 2: Brine shrimp lethality assay for cytotoxicity screening

Time Point	Control	100µg/mL *Karanjin*	250µg/mL *Karanjin*	500µg/mL *Karanjin*
0.00 hr.	10	10.00	10.00	10.00
1.00 hr.	10	7.67	6.33	6.00
2.00 hr.	9.5	5.33	4.67	4.67
5.00 hr.	8.67	3.67	3.33	1.67
8.00 hr.	8	2.33	1.67	0.33

The results showed 500µg/mL does as the most toxic dose amongst all doses tested, as it resulted in lowest percentage of cells after 8 hrs. treatment. On the other hand, other two doses viz. 100µg/mL and 250µg/mL, cytotoxic effects were there in comparison to control. This assay clearly identified 500µg/mL dose as the most cytotoxic Moreover, antimitotic of Karanjin also complemented the above results as highest antimitotic activity was recorded at 500µg/mL dose levels.

Antimitotic activity

The results of antimitotic activity assay showed similar trend as followed by BSL assay [figure 2 and table 3].

Table 3: Effect of *Karanjin* on root length and root number of *Allium Cepa* root

Sr. No.	Test	Control	100 µg/ml *Karanjin*	250 µg /ml *Karanjin*	500 µg/ml *Karanjin*
1	**Number of roots in onion**	34	25	20	10
2	**Length in Cm (N=10) ± SD**	4.8 ± 0.78	2.7 ± 0.62	1.5 ± 0.88	1.0 ± 0.57

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(A) Water control (B) 100 µg/ml (C) 250 µg/ml (D) 500 µg/ml

Figure 2: Treatment of Alium Cepa root with different concentration of Karanjin.

The water control shows normal growth with greater root length and number. Onion root number as well as length was recorded shortest at 500µg/mL in comparison to other two doses. Furthermore, other doses also showed good cytotoxic effects but 500µg/mL dose of Karanjin appeared most effective of all. Other concentration displays decreased the growth gradually in dose dependent manner. The above results could be justified by the fact that Karanjin has been confirmed earlier to induce inhibitory effects on human cancer cells [22]. Another possible reason for the above observation could be the ability of Karanjin to cause cell cycle arrest at G2/M phase leading decline in the growth of cancer cells [23]. Also, Karanjin has been observed earlier to provoke apoptosis in carcinoma cells [24].

MTT Assay:

The anticancer effects were recorded in the form of MTT assay results, wherein effective virulence of Karanjin was noticed in both cell lines. The dose-response relationship of the karanjin is shown in Tables 4 for MCF-7 and MDA-MB-21 cell lines.

Table 3: Effect Of Karanjin On Cell Viability And Cytotoxicity Of Mcf-7 And Mda-Mb-231 Cell Line

Concentrations (µM)	Average Absorbance		Percent Cell Viability (%)	
	MCF-7	MDA-MB-231	MCF-7	MDA-MB-231
0 µM	0.756 ± 0.057	0.785 ± 0.033	100	100
2.5 µM	0.734 ±0.060	0.683 ± 0.016	97.13	87.04
5 µM	0.563 ± 0.068**	0.564 ± 0.062***	74.47	71.88
10 µM	0.450 ± 0.026***	0.473 ± 0.021 ***	59.52	60.32
20 µM	0.209 ± 0.000***	0.388 ± 0.007***	27.64	49.49
40 µM	0.079 ± 0.004***	0.079 ± 0.003***	10.41	10.03
IC_{50}	**11.66 µM**		**18.56 µM**	

Values are expressed as mean ± SD (n=3). Statistical analysis: One-way ANOVA followed by Tukey's post hoc test. **p < 0.01, ***p < 0.001 as compared to control.

The significant results were obtained at all doses except 2.5µM dose in both cell lines in comparison to control. Further, morphological changes (figure 6A & 6B) established significantly higher efficacy in MCF-7 cells in comparison to MDA-MB-231 cells. Similar trend was recorded in IC 50 values of Karanjin that confirmed higher anticancer effects in MCF-7 cells. IC50 concentration of Karanjin was observed to be 11.66 µM in MCF-7 cells. On the other hand, IC 50 concentration of Karanjin was noticed to be 18.56 µM in MDA-MB-231 cells. Morphological analyses [figure 3A & 3B] of both cells complimented the above results.

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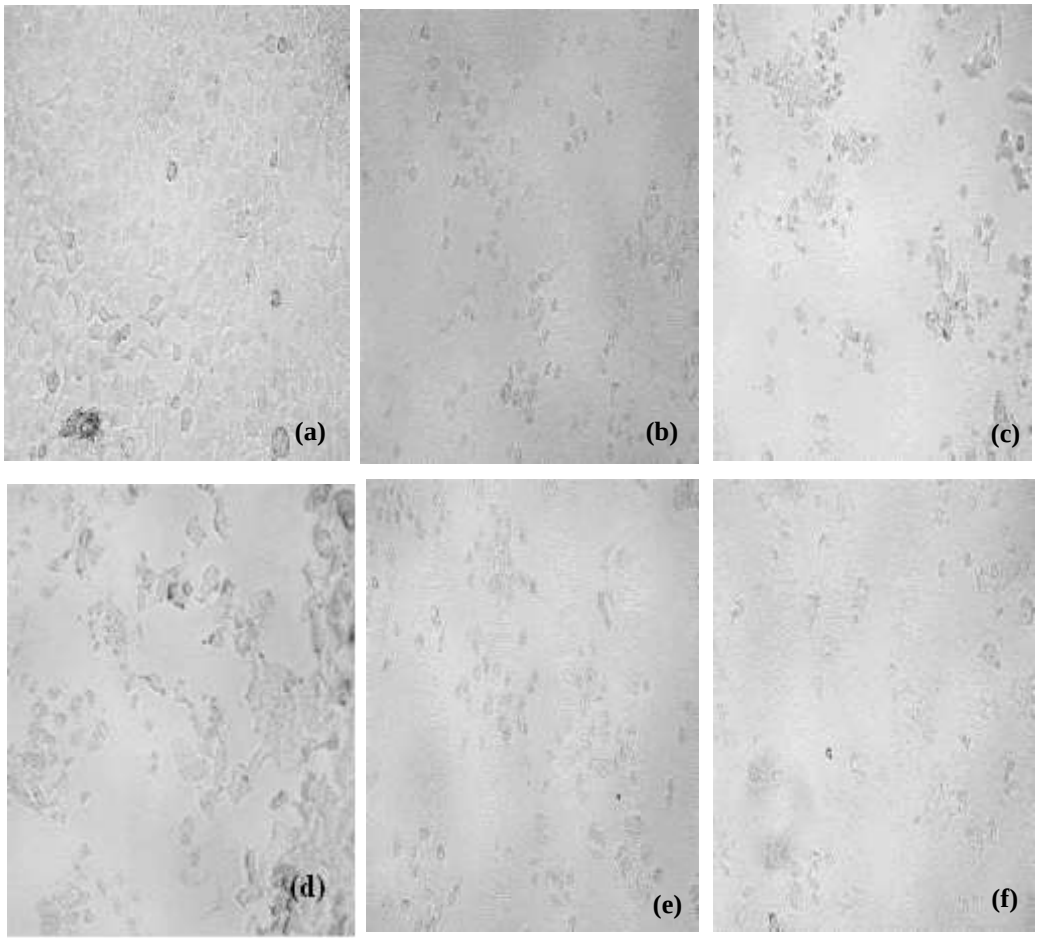

Figure 3A: effect of Karanjin on the presence of viable cells of mcf-7 through formation of formazan crystals after mtt treatment effect of varying concentrations of Karanjin on the presence of viable cells through formation of formazan crystals after mtt treatment (a) control, (b) 2.5 µm, (c) 5 µm, (d) 10µm, (e) 20µm and (f) 40µm. viability of untreated cells was considered 100 %.

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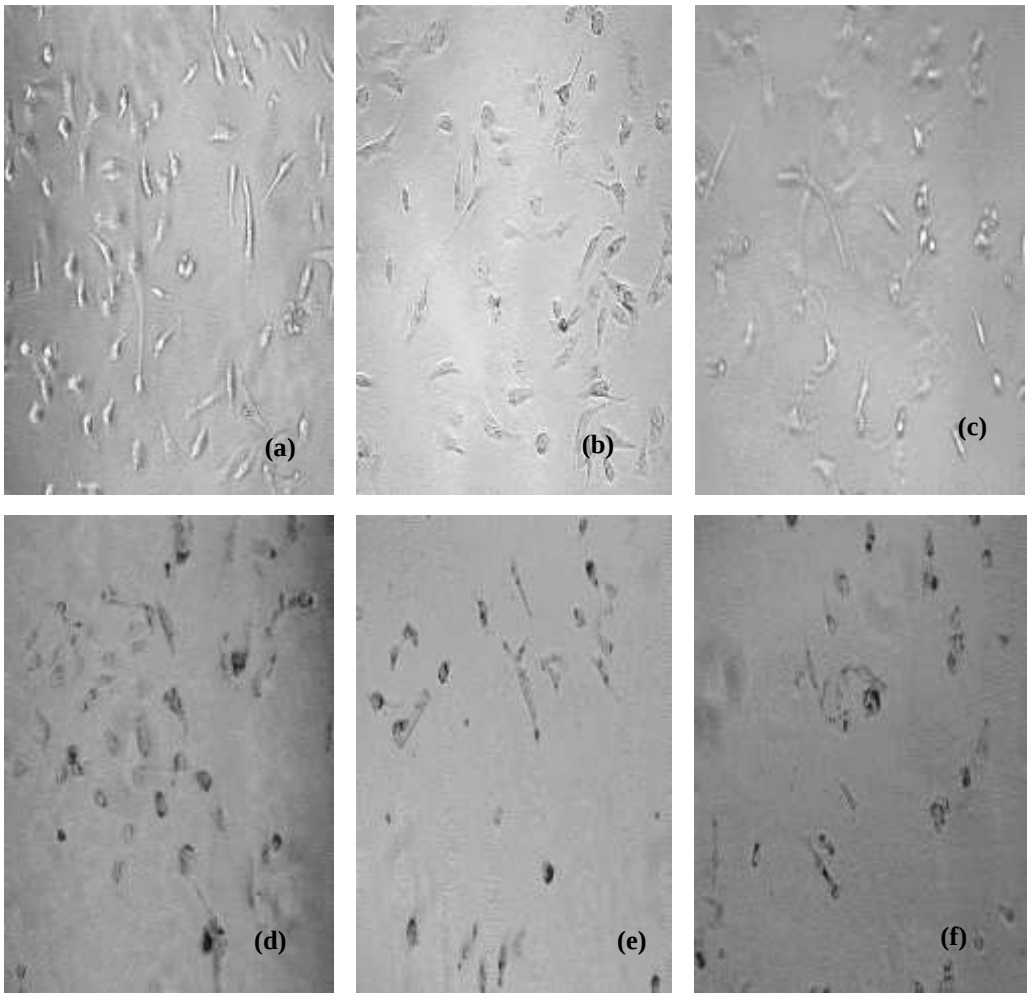

FIG 3B: Effect of *karanjin* on the presence of viable cells of mda-mb-231 through formation of formazan crystals after mtt treatment effect of varying concentrations of *karanjin* on the presence of viable cells through formation of formazan crystals after mtt treatment (a) control, (b) 2.5 µm, (c) 5 µm, (d) 10 µm, (e) 20 µm and (f) 40 µm. Viability of untreated cells was considered 100 %.

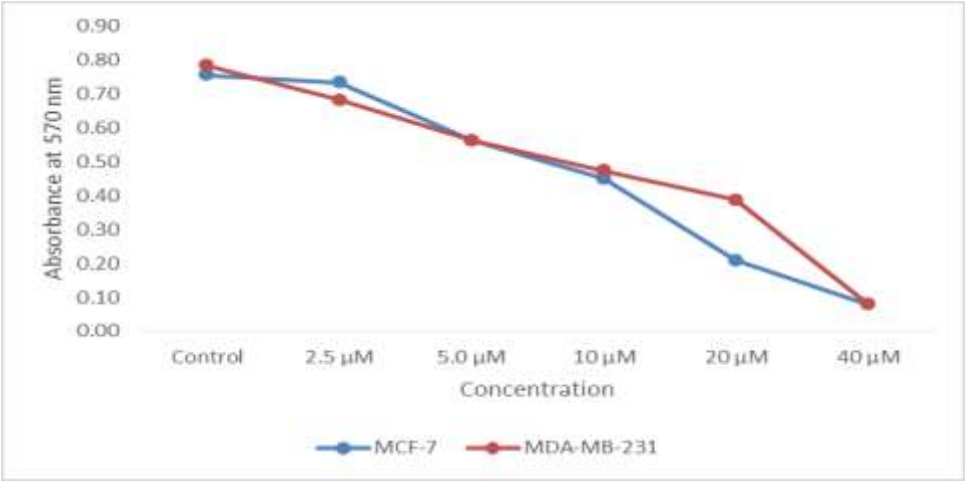

Figure 3C: Dose response of *karanjin* on MCF-7 and MDA-MB-21 cells at variable concentrations.

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These results clearly established higher efficacy of Karanjin in MCF-7 cells over MDA-MB-231 cells. The above results could be justified on the fact that MCF-7 is an estrogen positive cell line, while MDA-MB-231 cells are estrogen negative. Furthermore, our primary docking studies already predicted positive interaction of Karanjin with estrogen hormone. Similar results were reported earlier by utilizing hormone therapy on MCF-7 cells [25]. So, MCF-7 cells showed higher sensitivity as Karanjin is acting mainly by interacting with hormones [figure 3C]. The only limitation of the MTT assay is its inability to determine exact reason of cell death, as it could be cell necrosis too.

CONCLUSION

This study added alternative element to the prevailing comprehension of flavonoid Karanjin against breast carcinoma. The study concludes that Karanjin is potential anticancer flavonoid against breast carcinoma. Specifically, MCF-7 cells are more sensitive to Karanjin treatment as it acts primarily by positive interactions with hormones.

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