ANTICANCER ACTIVITY OF VITAMIN D3–TAMOXIFEN COMBINATION MICROEMULSION ON MCF-7 BREAST CELL LINE AND ITS SYNERGISTIC EFFECT

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ABSTRACT

This study investigates the anticancer activity of a combination microemulsion of vitamin D3 and tamoxifen (TMX-VD3 ME) for a synergistic effect on the MCF-7 breast cancer cell line. The combination microemulsion was prepared by dissolving the drugs in oil, mixing the oil with a surfactant, cosurfactant, and water. ME (VD3 1.5 mg mL^{-1}, TMX 10 mg mL^{-1}) was optimized for stability, globule size, and PDI. MTT assay was used to evaluate the cytotoxicity of the microemulsion. The results demonstrated a concentration-dependent increase in cell uptake with a decrease in cell viability. Flow cytometry revealed enhanced apoptosis and cell cycle arrest in G0/G1 phase and 3.45-fold enhanced efficacy in the migration assay. Additionally, the combination TMX-VD3 ME microemulsion exhibited enhanced anticancer efficacy compared to individual treatments of vitamin D3 ME or tamoxifen ME alone, indicating a synergistic effect. The zebrafish model revealed synergistic antiangiogenic activity of the vitamin D3 ME formulations.

Keywords: Vitamin D3, tamoxifen, breast cancer, cell proliferation, apoptosis anti-tumor, anti-cancer, microemulsion

INTRODUCTION

A leading cause of cancer-related death in females is breast cancer (BC),[1] as it represents 11.7 % of all cancer cases[2]. Further, breast cancer caused 10.6 % (90408) of all fatalities and 13.5 % (178361) of all cancer cases in India[3]. Early-stage and advanced estrogen-positive breast cancer can be treated with tamoxifen (TMX), a selective estrogen receptor modulator[4]. However, when given orally, it exhibits significant intra- and interpatient variation in bioavailability.

Among various approaches evaluated to improve tamoxifen bioavailability, nano-based approaches have shown great promise. TMX SNEEDS – self-nano emulsifying drug delivery systems - showed a considerable increase in release rate compared to drug suspension[4]. Oral nanosponges of TMX-loaded cyclodextrin showed 1.4-fold increased bioavailability compared to plain tamoxifen[5]. Similarly, TMX loaded with the polymer Poly Lactic-co-Glycolic Acid (PLGA) nanoparticles revealed increased oral bioavailability by more than 4 fold compared to base and salt form (TMX citrate).

A nanoemulsion of TMX (globule size 47 nm) displayed 20-fold greater inhibition in cell proliferation and increased 4-fold higher cell apoptosis in the breast cancer cell line (HTB-20)[6]. TMX-NLC was more cytotoxic to mouse (4T1) and human (MCF-7) mammary breast cancer cell lines[7]. Also, chitosan (CH) nanoparticle-based smart pH-responsive drug delivery system (DDS) enables more intelligent controlled release and improves the chemotherapeutic effectiveness of TMX. Additionally, lowering pH from 7.4 to 4.0 significantly improved the anticancer activity in the human breast cancer cell line, i.e., MCF-7, which is beneficial for cancer therapy.

To increase activity against tumor cells, TMX has been combined with other drug molecules[8]. Nanosponge particles (NPs) co-loaded with TMX, and quercetin (QT) showed improved bioavailability and enhanced anti-cancer effects, demonstrating the suitability of nanosponges as a dual-release drug delivery system[9]. A combination of TMX and tranilast led to a marked decrease in growth and

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proliferation compared to either agent alone[10]. Similarly, combination therapy with methylsulfonylmethane (MSM) and tamoxifen inhibited tumor growth and metastasis[11].

The anticancer properties of vitamin D3 have been published in the literature. Its activity on breast, prostate, thyroid, hematopoietic tumor cells, and retinoblastomas is exerted through Vitamin D receptor (VDR)[13]. Vitamin D3 can stop the cell cycle in the G0/G1 phase, induce apoptosis, and reduce cell growth. Due to these qualities, vitamin D3 and its metabolites have been tested in both pre-clinical and clinical settings as anti-cancer agents[12]. Wijingaarden et al. examined the antiproliferative effects of four novel vitamin D3 analogs on breast cancer cells, alone or in combination with tamoxifen. These analogs exhibited increased growth inhibition and reduced calcemic activity[13]. Furthermore, vitamin D3 also exhibits poor oral bioavailability, like TMX (REF). Hence, we report a combination microemulsion of TMX with vitamin D3 in this study to enable bioenhancement and high anticancer efficacy.

MATERIALS AND METHODS

Materials

Vitamin D3 was received as a gift sample from Fermenta Biotech Limited, Mumbai, India. Capmul® MCM (Glycerol Monocaprylocaprate) Abitec Corporation, India was obtained as a gift sample. Transcutol® HP (highest purity) was a gift sample from Gattefosse Private Limited, India. BioXera Pharma Pvt. Ltd., Mumbai gifted tamoxifen citrate. D-alpha tocopheryl polyethylene glycol 1000 succinate (TPGS) was obtained as a gift sample from BASF, Mumbai. Propylene glycol, ethanol, polyethylene glycol 400 (PEG 400), methanol and acetonitrile were procured from Merck India Private Limited. Analytical reagent grade or HPLC grade chemicals were procured. Fetal bovine serum (FBS) and DMEM-Dulbecco's modified eagle medium were obtained from the Thermo Fisher Scientific Pvt. Ltd. JC-1, and DRAQ5 dyes and hydroxypropyl methylcellulose [HPMC (E5)] were purchased from Invitrogen and Colorcon, respectively. Amphotericin antibiotic solution, FITC (fluorescein isothiocyanate), and MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium salt were all obtained from the Sigma Aldrich St. Louis and propidium iodide (PI) from Sigma Aldrich Private Limited, India.

Solubility study of vitamin D3 and tamoxifen

The solubility of vitamin D3 and tamoxifen was determined in oils, surfactants, and cosurfactants by adding an additional drug into 1 mL of each selected surfactant and oils in centrifugation tubes (2 mL) and kept at 37 °C (± 0.5) in a shaker water bath to achieve equilibrium for 48 h. These samples were centrifuged for 15 minutes at 10,000 rpm, and the supernatant was quantified using a UV spectrophotometer at 265 nm for vitamin D3 and 275 nm for tamoxifen[14].

Surfactant- Cosurfactant emulsification study

Different surfactants (Tween® 80, Span® 80, Kolliphor® TPGS, Cremophor® RH 40, Labrasol®) and Cosurfactants (Polyethylene glycol 400, Transcutol® HP, propylene glycol) were examined for the oil phase's capacity to emulsify. The choice of surfactant was made based on % transparency and ease of emulsification. Later, 100 mg of the oil phase and 300 mg of the surfactant were combined. The mixtures were gradually heated at 40-50 °C to homogenize the constituent parts. 50 mg of each mixture was then diluted to a volume of 50 mL in a stoppered conical flask using distilled water. The count of flask inversions necessary to produce a homogenous emulsion was used for the assessment of ease of emulsification. The emulsions were left to stand for 2 h. A double-beam UV spectrophotometer was used to measure the emulsions percent transparency at a maximum wavelength of 640 nm, while the blank used was distilled water. The emulsions were also visually evaluated for symptoms of turbidity or phase separation. The experiment was repeated three times[15].

Table I: Surfactant emulsification study

Surfactant	No of flask inversion	% Transparency ± SD	No of flask inversion	% Transparency ± SD
	Vitamin D3		Tamoxifen	
TPGS	08 ± 1.12	94.22 ± 0.12	12 ± 2.16	84.52 ± 0.26
Tween® 80	10 ± 3.01	90.45 ± 0.18	14± 4.22	94.57 ± 0.21
Solutol® HS 15	12 ± 2.10	90.22 ± 0.28	18 ± 3.18	94.66 ± 0.54
Labrasol®	12 ± 1.08	87.24 ± 0.30	22 ± 4.48	96.74 ± 0.36
Myrj®- 52	18± 1.14	74.66 ± 0.24	23± 1.14	89.22 ± 0.18

Pseudo-ternary phase diagram

Pseudo ternary phase diagrams were drawn using the water titration method to investigate the concentration and range of components at the existing boundary of MEs to determine the region of microemulsion existence. As per the result of the emulsification study, the surfactant, and cosurfactants selected were TPGS and Transcutol® HP, respectively. Based on the solubility study, the oil employed in the present study was Capmul® MCM. The Smix was prepared by mixing surfactant with cosurfactant in fixed weight ratios such as 1:1, 2:1, and 1:2. Further, oil and Smix were added at RT (25 ˚C) in the ratios of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9 (w/w). These mixtures were diluted dropwise with water under moderate agitation. When they appeared as clear liquids, the samples were classified as microemulsions[16].

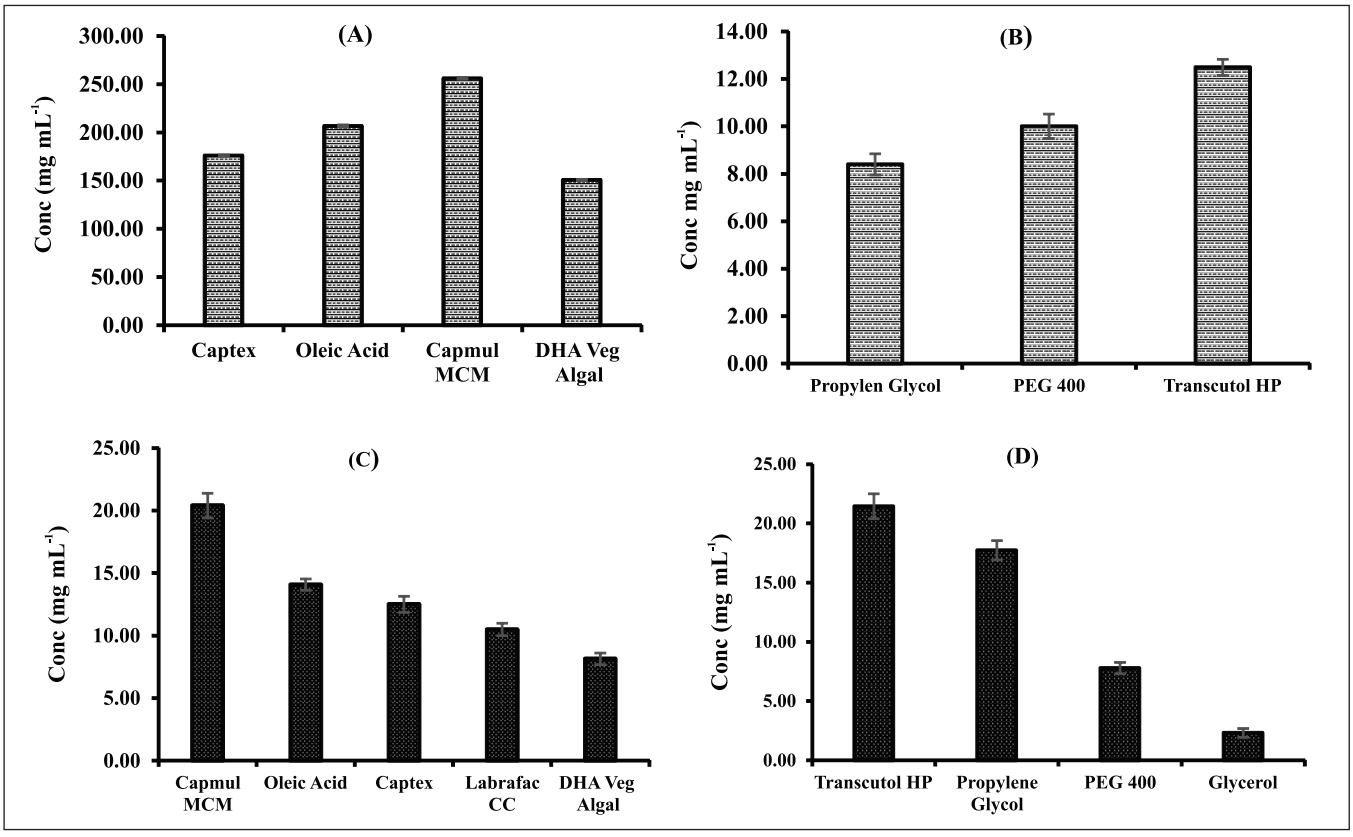

Fig. 1: Vitamin D3 solubility in (A) oils (mg mL^{-1}) and B) cosurfactant (mg mL^{-1}), tamoxifen solubility in (C) oils (mg mL^{-1}) and (D) cosurfactant (mg mL^{-1})

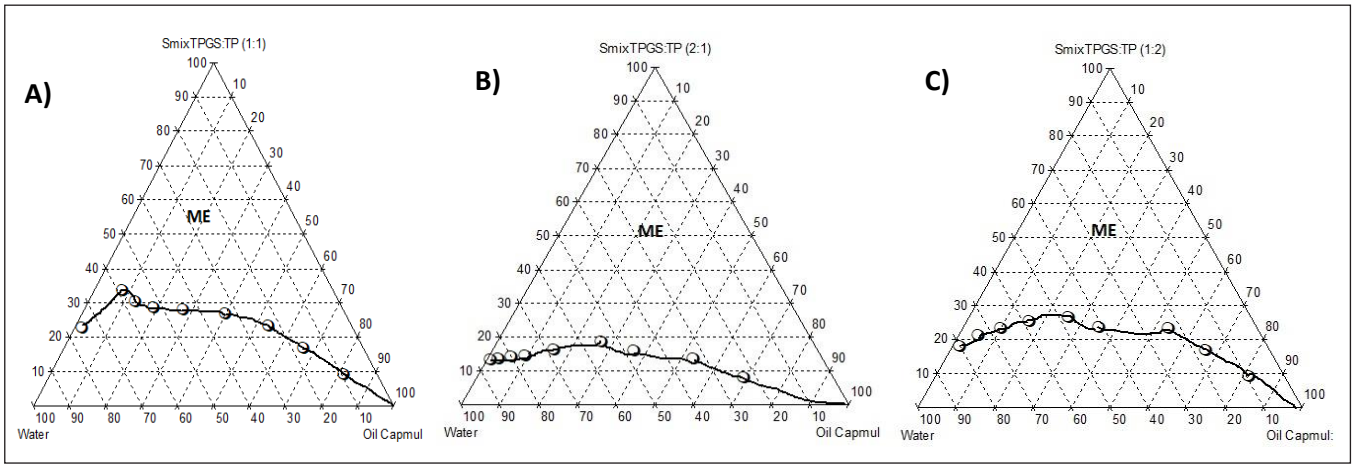

Fig. 2: Diagram depicting the pseudo-ternary phase of the selected components (A) 1:1 ratio (B) 2:1 ratio (C) 1:2 ratio

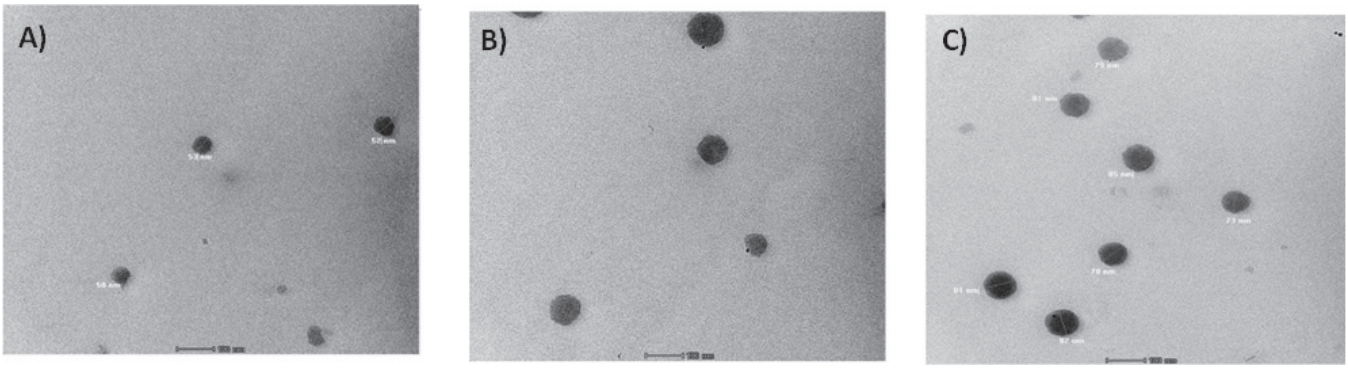

Fig. 3: TEM images of (A) vitamin D3 ME and (B) tamoxifen ME (C) TMX-VD3 ME

Preparation of vitamin D3 and tamoxifen microemulsion

The optimized microemulsion (ME) comprised 10 % V/V Oil, 60 % V/V Smix [TPGS: Transcutol® HP, (2:1)], and 30 % V/V of water. Combination ME (1 mL) was prepared by dissolving TMX (10 mg) and vitamin D3 ME (1.5 mg) in 0.1 mL oil and 0.6 mL Smix and vortexed on a cyclomixer. This was mixed with 0.3 mL of water to generate the TMX-VD3 ME. Tamoxifen ME (10 mg mL^{-1}) was prepared similarly, excluding vitamin D3, and VD3ME was prepared excluding TMX.

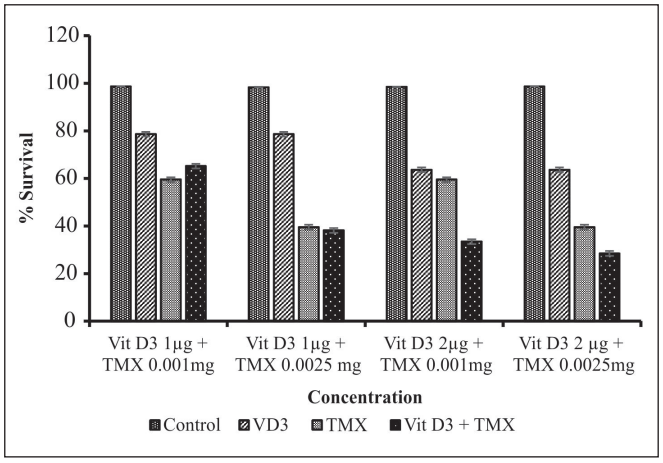

Fig. 4: Cell viability assay of VDE ME, TMX ME and TMX-VD3 ME in combination

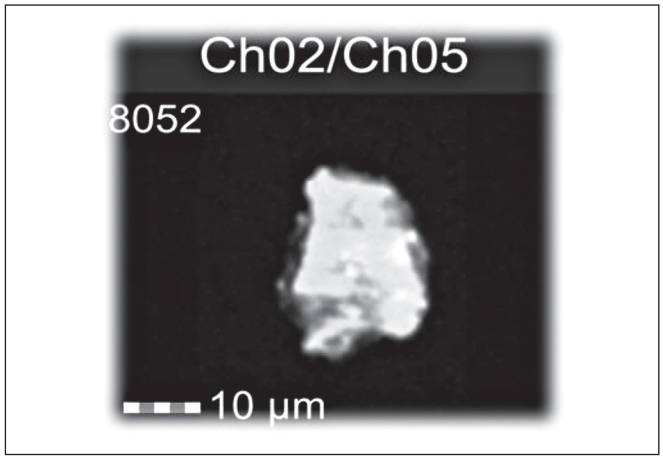

Fig. 5: Cell apoptosis

Physicochemical characterization

Globule size, PDI (Polydispersity index), and zeta potential

The average globule size, PDI (polydispersity index), and zeta potential of MEs were measured using a zeta sizer [ZS 90, Malvern, UK] at RT 25 °C after diluting with double distilled water.

Drug content

Drug-loaded MEs were diluted with HPLC grade methanol and checked for its drug content by HPLC for vitamin D3 at λmax 265 nm using mobile phase acetonitrile: methanol (70:30 V/V), and for tamoxifen, at λmax 275 nm using a mobile phase of pH 3, potassium dihydrogen phosphate, adjusted by using orthophosphoric acid and acetonitrile (ACN) (30:70 V/V), column (C-18), the flow rate was maintained at 1 mL min^{-1}, and diode array detector was used. Forced degradation studies were performed, and stability indicating HPLC method was developed.

Transmission electron microscopy (TEM)

An FEI Tecnai 12 BT instrument running at 120 kV was used to conduct TEM (transmission electron microscopy) analyses. VD3ME was mixed by vortexing on a cyclcomixer after diluting with water (Milli-Q ®) in the ratio of 1:50. On a transmission electron microscopy grid with holey carbon film covering it, a drop of the ME was applied. The grid was then stained for 10 minutes with 2 % uranyl acetate, and a thin liquid film was created after blotting with filter paper.

Stability

For stability evaluation TMX-VD3 ME, VD3ME, and TMX ME was maintained for one year in sealed amber glass bottles at 30 ± 2 ^{0}C/ RH 65 ± 5% and 40 ± 2 ^{0}C/ RH 75, 5% for 6 months. Vitamin D3 and tamoxifen content in the preconcentrate were determined at certain intervals in accordance with ICH (International Conference on Harmonisation) guidelines by HPLC. As per the ICH guidelines, vitamin D3 and tamoxifen concentration in the preconcentrate were analyzed using HPLC at recommended time intervals. The formulations were also evaluated for globule size, PDI, and zeta potential.

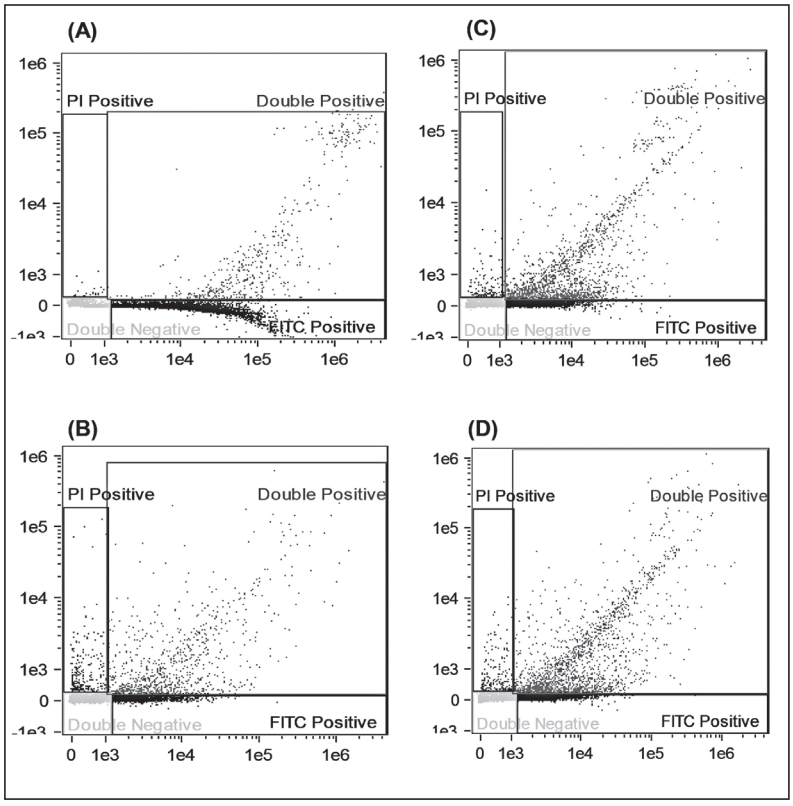

Fig. 6: Cell apoptosis assay of (A) control (B) VD3 ME (C) TMX ME (D) TMX-VD3 ME

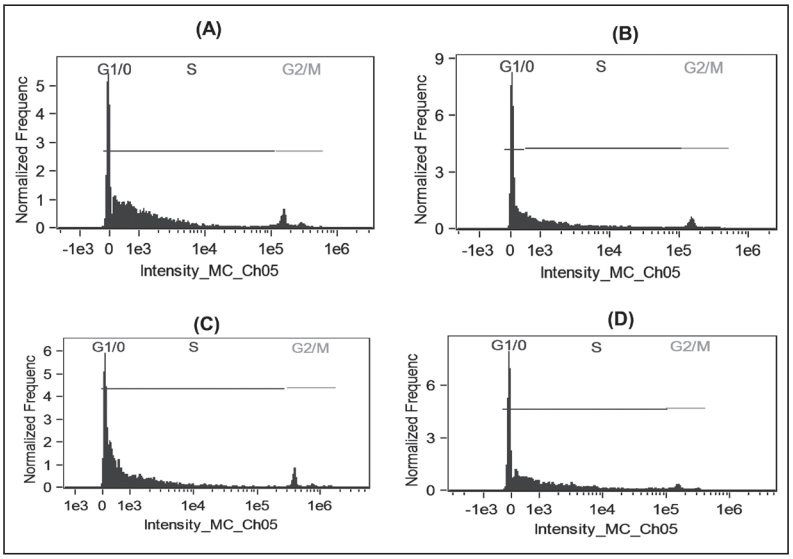

Fig. 7: Cell cycle analysis: (A) Control (B) VD3 ME (C) TMX ME (D) Vit D3 – TMX ME

Anti-cancer efficacy in MCF-7 cell line

Human breast cancer cells

MCF-7 (Human breast cancer cells) cell lines were obtained from the National Centre for Cell Science (NCCS), Pune. They were incubated at 37 ^{0}C in a carbon dioxide incubator containing CO_2, 5 %, in Dulbecco's Modified Eagle Medium, complete medium (which contained heat inactivated FBS (10 %) and 0.4 % amphotericin) and maintained until they became confluent.

Cytotoxicity assay

MTT assay was used to assess cytotoxicity. In a nutshell, DMEM complete medium was used to seed 5000 cells per well in 96-well plates having a flat bottom, followed by incubation for 24 h to allow cells to adhere. VD3ME, TMXME, and TMX-VD3 ME were sterilized in a laminar airflow cabinet by filtration (using 0.22 µm membrane) and used for further treatment. Aliquots of VD3 ME (0.5, 1, 2, 4, 8, and 16 µg mL^{-1}) and tamoxifen ME (0.001, 0.0025, 0.005, 0.0075, and 0.015 mg mL^{-1}) and its combination vitamin D3+TMX ME (1µg + 0.001mg, 1 µg +0.0025 mg, 2 µg + 0.001 mg and 2 µg +0.0025mg mL^{-1}) were added and then plates were placed in a CO_2 incubator. After 24 and 48 h, 100 µL of MTT reagent (5 mg mL^{-1}) was introduced, and the plates allowed to stand for further 4 h. After removal of the medium, 200 µL of DMSO (dimethyl sulfoxide) was introduced to solubilize the formed formazan crystals. Cell viability was assessed, by recording absorbance at 570 nm using a microplate reader (Bio-Rad, USA). Suspensions of vitamin D3 and tamoxifen 0.2 % HPMC E5 in PBS served as controls. The cell viability was determined using the formula below:

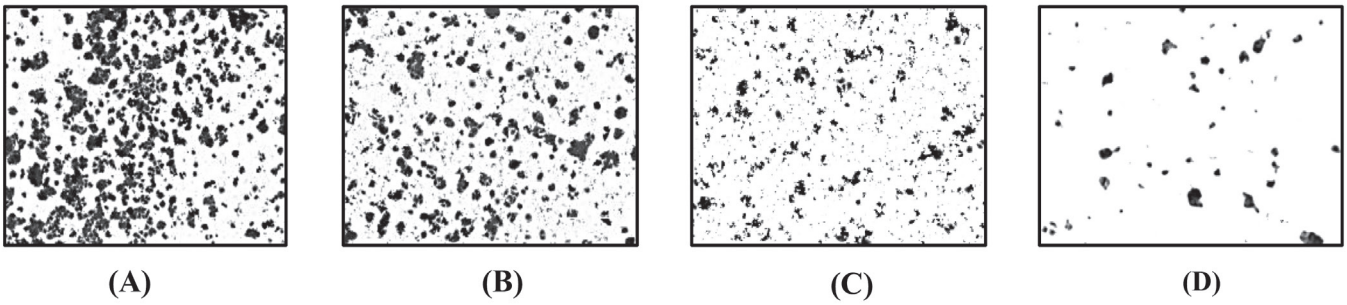

Fig. 8: Cell migration assay (A) Cell control (B) VD3 ME 2 µg mL^{-1} (C) TMX ME- 0.0025 mg mL^{-1} (D) VD3 2 µg mL^{-1}+ TMX- 0.0025 mg mL^{-1} ME

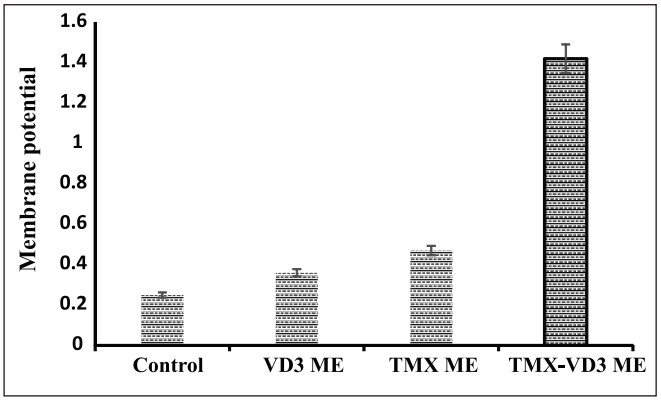

Fig. 9: CBMN Assay- quantitative analysis of mitochondrial membrane potential disruption (n = 3, Mean ± SE)

$$\text{Cell viability (\%)} = \frac{\text{Absorbance samples}}{\text{Absorbance of control}} \text{ X } 100$$

------ [Equation…1]

Imaging flow cytometry (IFC) – *In vitro* assays

Cell uptake assay

MCF-7 cells at a density of 10,000 cells well^{-1} were added to a 6-well plate containing coverslips and allowed to stand for a duration of 24 h. They were then exposed to fluorescein isothiocyanate (FITC - 10 µg mL^{-1}) ME for 24 h. The cells were washed thrice with ice-cold phosphate-buffered saline (PBS), resuspended in PBS, and centrifuged at 1,000 rpm for 5 minutes. Cells were then dispersed in PBS (350 µL) and analyzed on flow cytometer to evaluate the intracellular uptake of ME. Luminex ImageStream® X Mark II Imaging Flow Cytometer, USA, was used to perform IFC assays[17].

Cell apoptosis assay

The stage of apoptosis was measured using the Annexin V-FITC (5 µL) and PI (propidium iodide) (4 µL) double staining assay. MCF-7 cells were subjected to vitamin D3 (2 µg mL^{-1}), tamoxifen ME (0.0025 mg mL^{-1}), and a combination of vitamin D3- tamoxifen ME (2 µg mL^{-1} and 0.0025 mg mL^{-1}) for 24 h. Then cells were dyed with Annexin V-FITC and PI after washing twice with PBS buffer. Samples were analysed on Amnis Image Stream®X Mark II flow cytometer. Fluorescence was monitored at excitation and emission wavelengths of 488nm and 505-560 nm, respectively, in channel 2 for Annexin and for PI in channel 4. Direct cell counting was used to distinguish between the number of cells intact, early, and late apoptotic[18].

Cell cycle analysis

In 12-well plates, MCF-7 cells (10,00,000 cells well^{-1}) were introduced and incubated for 24 h to allow adherence to the surface of the culture plate. vitamin D3 (2 µg mL^{-1}), tamoxifen ME (0.0025 mg mL^{-1}), and a combination of vitamin D3- tamoxifen ME (2 µg mL^{-1} and 0.0025 mg mL^{-1}) were added to the wells, and then incubated for 48 h. Cells were trypsinized following incubation, centrifuged (1000 rpm, 5 minutes, 4 °C) and dispersed in 100 µL PBS. Cells were dyed with 5 µL of PI solution (50 µg mL^{-1}) for a duration of 30 minutes while being kept in the dark following treatment with 10 µL of enzyme ribonuclease A (100 µg mL^{-1}) for 15 min at 37 °C. Fluorescence was recorded on IFC at excitation 488 nm and emission 596–643 nm. Image recognition characteristics were employed to differentiate mitotic events and the G0/G1, S and G2 phases based on nuclear morphological changes in the MCF-7 cells[19].

Migration assay

MCF-7 cells were grown until 80 % confluent, recovered from the plate with 0.5 mol L^{-1} EDTA (pH 6.8) and washed twice in Dulbecco's PBS. The cells were then suspended in a starvation medium. In this study, 24-well transwell chambers with the membrane of polycarbonate having a pore-size of 8 µm (Corning® BioCoat™ Control Inserts, BD Bioscience) were used. The top well, which

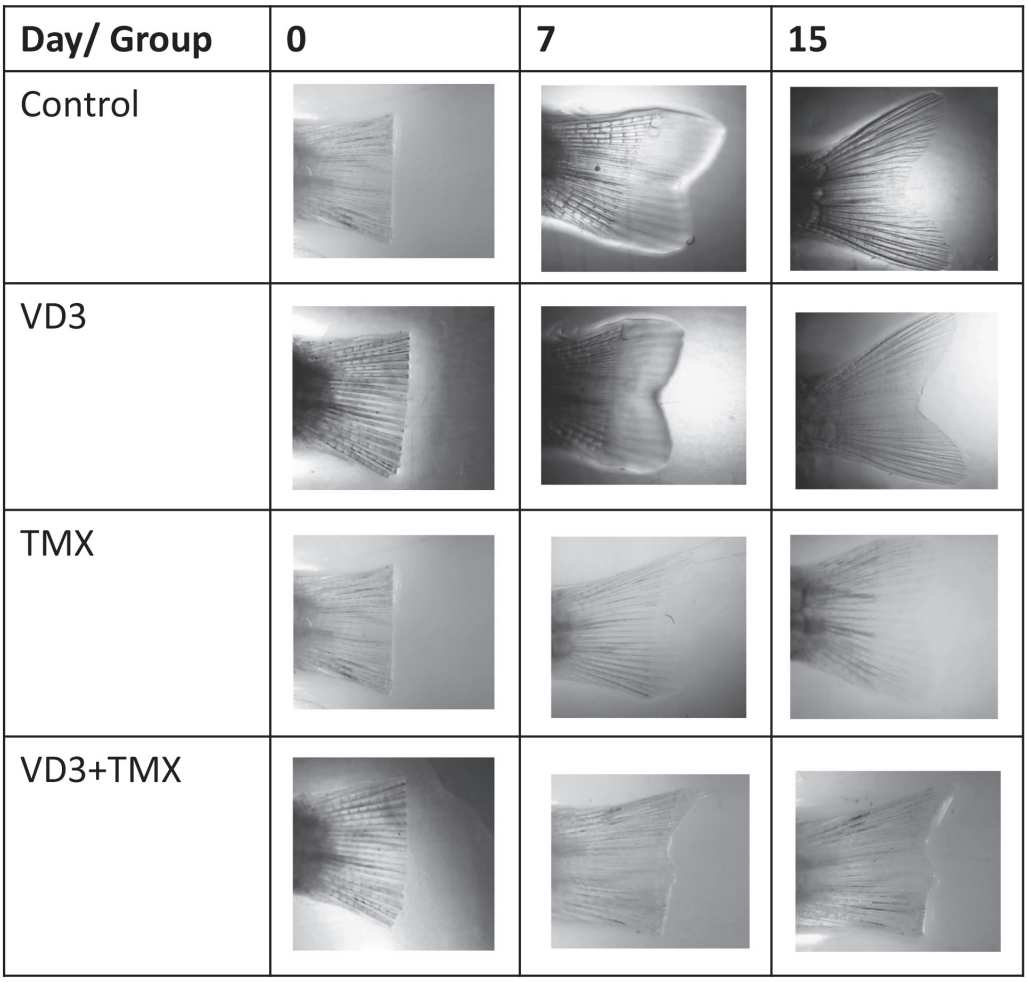

Fig. 10: Tail fin regeneration (angiogenesis) assay in zebrafish with control, VD3 ME, TMX ME and TMX-VD3 ME formulation

contained cells at a density of 50,000 well^{-1} was placed in a bottom well containing 0.5 mL of either complete medium or 5 µg mL^{-1} TGF or 10 % FBS, vitamin D3 (2 µg mL^{-1}) tamoxifen ME (0.0025 mg mL^{-1}), and a combination of vitamin D3- tamoxifen ME (2 µg mL^{-1} & 0.0025 mg mL^{-1}) was added to the top well. After 24 h at 37 °C in a 5% CO_2 incubator, the non-migrated cells were scraped off with a cotton swab and fixed and coloured using Diff-Quik kit. Migrated cells were measured by counting 12 fields at 400X magnifications. The experiment was carried out in triplicate, and the results were averaged[20].

Invasion assay

Using 24-well Matrigel invasion chambers (Corning® BioCoat™ Matrigel®), cell invasion was evaluated. The inserts had 50 µL of Matrigel® pre-coated (1:4 dilution; BD Biosciences). Serum-free DMEM was used to suspend the cells. Subsequently, 1×10^4 cells were added to the upper chambers. DMEM containing 10 % FBS (foetal bovine serum) was injected into lower chambers. Vitamin D3 (2 µg mL^{-1}), tamoxifen ME (0.0025 mg mL^{-1}) and a combination of vitamin D3- tamoxifen ME (2 µg mL^{-1} & 0.0025 mg mL^{-1}) was added in the upper chambers. The chambers were incubated for 24 h at 37 °C, fixed with 3.7 % paraformaldehyde for 10 min, and stained for 30 min at room temperature (RT) with 2 % crystal violet. The number of cells that penetrated the membrane in 10 microscopic fields/filter (about 100) was used to assess cell penetration through the membrane[21].

Scratch assay

Human breast cancer cell line MCF-7 was grown in 12-well plates. The cells were provided either full media (10% FBS) or 1 g L^{-1} anti-TGF (R&D Systems, clone: 1D11). Cells were scratched using a sharp 10 µL pipette tip when they reached 100% confluence. Vitamin D3 (2 µg mL^{-1}) tamoxifen ME (0.0025 mg mL^{-1}) and a combination of vitamin D3- tamoxifen ME (2 µg mL^{-1} & 0.0025 mg mL^{-1})

was added in the wells. Using a Nikon camera mounted on a microscope, photographs were taken. The size of the gap was calculated and converted into a percentage of wound closure. The findings are based on an average of four experiments.

CBMN (Cytokinesis-block micronucleus) assay

The cytokinesis-block micronucleus (CBMN) experiment was performed on the IFC platform in line with the Fenech et al. protocol. MCF-7 cells were taken at a concentration of 1 x 10^6 cells per well in plate (12-wells). After allowing samples to adhere over 24 h, vitamin D3 (2 µg mL^{-1}) tamoxifen ME (0.0025 mg mL^{-1}), and a combination of vitamin D3- tamoxifen ME (2 µg mL^{-1} & 0.0025 mg mL^{-1}) were added and samples incubated for 48 h. Cells were collected after trypsinization, spun for 5 minutes at 1000 rpm, 4 °C, then dispersed in 1 mL of PBS. The cell suspensions were collected in 1.5 mL PP (polypropylene) tubes and centrifuged for 8 min at 1200 rpm. The samples were incubated at room temperature for 10 minutes. Following the aspiration of the supernatant, 30 mL of BD FACS lysing solution (Conc: 1x) was added. Then samples were resuspended in 100 µL of PBS after being centrifuged twice at 3400 rpm with PBS (10 mL) solution. DRAQ5 was added to 100 µL of cell suspension until a final concentration of 50 µM was reached. Bright-field pictures were recorded in channel 1, while samples were processed on the IFC platform in channel 5 with a 642 nm laser set to 20 mW. The mononucleated to binucleated cells ratio was used as the predictor of cell nuclear division[22].

Antiangiogenesis assay on zebrafish

Angiogenesis assay is quick and affordable, making it a cutting-edge tool for studying tumour angiogenesis and for the development of antiangiogenic drugs. Adult fish danio orange zebra (400–450 mg) were kept in a glass container in water at a temperature of 25 ±0.5 ^{0}C, with an aerator system and maintained in a 14 h light and 10 h dark cycle. The fish were placed on glass slides after being immersed in trignocaine solution at a concentration of 0.2% for 20-25 seconds until the movement of the gills stopped. A stereomicroscope (Olympus IX71) fitted with a microscopic camera was used to capture the image prior to amputation. The mid-fin level of the tail fin was severed using a surgical blade. After the post-amputation photograph was taken, the fish was put back in the beaker and allowed to heal. The fish that had been severed were divided into four groups of six fish each. Group I: PBS control; Group II: vitamin D3 ME (2 µg mL^{-1}); Group III: tamoxifen ME (0.0025 mg mL^{-1}); and Group IV: vitamin D3-TMX- ME (2 µg mL^{-1} & 0.0025 mg mL^{-1}). The fish were put into 250 mL of water (aerated) and kept at 25 ± 0.5 ^{0}C for the next 3 days (72 h) to permit the tail fins to regenerate. The fins of the fishtail were captured with a stereomicroscope on days 3, 6, 9, 12, and 15 and measured using ImageJ software.

RESULTS AND DISCUSSION

Combination therapy is a viable option for improved efficacy of chemotherapeutic drugs with fewer adverse effects[23]. Combination therapy is advantageous as it attempts to combine already-existing medication molecules to produce a synergistic impact. This study presents a simple yet promising design strategy in the form of a readily scalable combination microemulsion of vitamin D3 and tamoxifen for improved anticancer efficacy.

Solubility of vitamin D3, tamoxifen and surfactant emulsification study

The solubility of vitamin D3 and tamoxifen was studied in different types of oils, surfactants and co-surfactants, and is reported in Fig. 1. Both vitamin D3 and tamoxifen exhibited maximum solubility in Capmul® MCM and Transcutol®, which were therefore selected as the oil and co-surfactant phase of the ME, respectively. The surfactant emulsification study results are provided in Table I, wherein the emulsification capacity was found in the order TPGS > Tween® 80 > Solutol H 15 > Cremophor® RH 40. Hence, TGPS was selected as the surfactant for ME development.

Construction of pseudo-ternary phase diagram

The pseudo ternary phase diagrams were plotted using Capmul® MCM and TPGS and Transcutol® HP as the Smix (Fig. 2) and O/W microemulsion regions were identified. The 1:1 Smix areas showed lower microemulsion region than other 2: 1, 1:2 ratios. Further 1:1 and 1:2 ratio exhibited globule size > 100 nm. Therefore, 2:1 ratio which showed globule size <100 nm was selected for further study.

The optimized ME comprised 10 % Capmul® MCM as the oil, 65 % V/V Smix (TPGS: Transcutol® HP, 2:1) and 25 % V/V of water. Vitamin D3 (VD3 ME), tamoxifen ME (TMX ME), and tamoxifen-vitamin D3 combination ME (TMX-VD3 ME) exhibited a globule size of 52.82 ± 3.16 nm, 65.26 ± 2.46 nm, and 76 ± 2.68 nm respectively, PDI was found to be 0.239, 0.258 and 0.218 for VD3ME, TMXME and TMX-VD3 respectively. MEs demonstrated good colloidal stability with zeta potential in the -20 to -25 mV range. Vitamin D3 ME displayed 99.5 % of drug

content, tamoxifen ME showed 99.67 % drug content and the combination ME showed 98.92 % drug content. All the MEs exhibited stability up to one year at 30 ^{0}C/ 60% RH (Assay > 95 %).

TEM (Transmission electron microscopy) study

The TEM (transmission electron microscopy) images of vitamin D3 microemulsion, tamoxifen microemulsion and combined formulation, i.e., TMX-VD3 ME formulations, are displayed in Fig. 3. The TEM images revealed spherical morphology and size below 100 nm corroborating with DLS size data.

Anti-cancer study on cell line (MCF-7)

Worldwide, breast cancer affects more women than any other type of cancer, yet few effective treatments are available[24]. Applications of chemotherapy that have been approved and received positive clinical reviews have minimal efficacy and undesirable side effects. Hence, there is a need for newer effective therapies with fewer side effects. This paper presents one such effort through a combination ME of Vitamin D3 and tamoxifen.

Cytotoxicity assay

Cytotoxicity assay evaluates a MEs capacity to result in cell death or damage. As shown in Fig. 4, MCF-7 cell viability decreased when VD3 ME and TMX ME levels increased. VD3 ME, TMX ME, and its combination showed superior anticancer efficacy against MCF-7 cells. The IC_{50} value of vitamin D3 ME was 2 µg mL^{-1}, and for TMX ME it was 0.0025 mg mL^{-1}. The nano-sized globule's ability to promote high intracellular absorption through endocytosis or phagocytosis contributes to increased cytotoxicity[25]. The poor aqueous solubility of vitamin D3 and tamoxifen, a BCS class II drug, restricts cell entry through diffusion. As a result, using microemulsion formulation could help these medications work more effectively in cell lines and produce the desired effects.

Cell uptake assay

The flow cytometry analysis revealed significant uptake of TMX-VD3 ME by MCF-7 cells in a time-dependent manner. However, TMX-VD3 ME revealed a 1.6-fold greater uptake at 1 h and 6.7-fold at 4 h compared to plain drug suspension, confirming the microemulsion's role in achieving high intracellular drug concentration. These findings support the potential role of TMX-VD3 ME as a therapeutic agent in combination with breast cancer management, highlighting its ability to be internalized by cancer cells.

Cell apoptosis assay

Annexin V - FITC and propodium iodide (PI) were used in a double staining procedure to track early and late apoptosis. Fig. 5 displays representative pictures of cells going through apoptosis. Fig. 6's dot plot shows the proportion of cells that underwent early and late apoptosis after being incubated with VD3 ME, TMX ME, and TMX-VD3 ME, Annexin V, Annexin-FITC positive, PI positive, and PI positive (Double Positive), and Double Negative, which happens when no Annexin and PI staining is present, were separated into the four categories. Phospholipid phosphatidylserine, present on the membrane's inner side, is where the protein annexin V binds. Thus, Annexin V can detect initial apoptotic cells that develop apoptotic blebs, and also lose their original spherical shape as a result of phosphatidylserine exposure. Cellular membrane damage during late apoptosis enables PI to pass through and bind directly to the DNA. These cells were identified as double positive because they would exhibit Annexin V and PI staining. Cells identified in the bright field channel but not activated and stained by either dye are known as double-negative cells. Necroptotic cells are PI-positive cells because they permit deep PI penetration and show diffused and strong PI staining. IFC analyses the morphology of apoptotic cells in a single measurement by combining flow cytometry and microscopy while enabling concurrent quantification and objective analysis. Fig. 6 shows the percentage of cells going through various stages of apoptosis. The early and late apoptotic cell percentages at 48 h were 22.42 %, 18.62 %, 14.12 %, and 7.08 %, and 5.78%, 4.4 % for TMX-VD3 ME, VD3 ME, and TMX ME, respectively. This indicates a 3.2-fold increase in the apoptotic potential of TMX-VD3 ME compared to VD3 ME and TMX ME. The cytotoxicity and cell cycle study results are consistent with the significantly high apoptotic effect observed with TMX-VD3 ME, reiterating the benefit of TMX-VD3 ME in enabling high efficacy.

Cell cycle analysis

Depending on how the cell cycle progresses from the G0/G1 phase to the S and G2/M phases, cell cycle arrest may enhance the generation of apoptosis and hinder cell growth[26]. Fig. 7 displays the results of a cell cycle analysis together with associated IFC pictures. With the combination formulation. TMX-VD3 ME, as opposed to VD3 ME and TMX ME. A significant rise in the number of cells in the G0/G1 phase with a decrease in S phase cells, indicated a stronger arrest of the cell cycle in the G0/G1 phase. This was consistent with studies showing that vitamin D3 can cause apoptosis by stimulating the mitochondrial pathway, which causes cancer cells to be arrested in the G0/G1 phase. Importantly, TMX-VD3 ME

exhibited significantly better inhibition than individual formulation i. e. VD3 ME and TMX ME. The importance of nanonization of vitamin D3 and tamoxifen combination was validated as it showed greater inhibition than the control.

Migration and invasion assay

The cell migration concentration was determined after 48 h of exposure of MEs. Vitamin D3 ME induced increased levels in MCF-7 cell migration and invasion compared with those observed in the untreated cells, while the same activity was observed for TMX. The mechanism of action of anticancer agent on the MCF-7 cell line involves binding the agent to its receptor within the cells. This receptor-ligand contact sets off a sequence of intracellular signalling events that influence gene expression and cellular behaviour, which in turn affects the MCF-7 cells' capacity for migration and invasion. The transwell cell migration assay quantifies a cell's capacity to move towards a chemoattractant chemotactically. In this case, we tested three formulations, namely VD3 ME, TMX ME, and TMX-VD3 ME. We measured the migration and invasion of untreated and treated MCF-7 cells to evaluate the effects of these on the dynamics of cell migration and invasion. A 1.76-fold time increase in anticancer activity was observed, whereas in the case of VD3 ME formulation, as compared to a 2.26-fold increase using TMX ME and a whopping 3.45-fold increase using TMX-VD3 ME formulation. Fig. 8 displays the cell migration in the MCF7 cell line.

Scratch assay

The percentage of wound closure was measured at two intervals, i.e., 12 h and 24 h. At the end of 12 h, the wound closure was 66.44 % found in the control group, 44.93 % in the VD3 ME group, 34.9 % in the TMX ME group, and 26.92 % in the VD3+TMX group. In 24 h, we found wound closure 74.15 % in the control, 56.21% in the VD3 ME group, 40.06 % in the TMX group, and 31.36 % in the TMX-VD3 ME group. There were substantial differences in the percentage of wound closure between the VD3, TMX, and TMX-VD3 ME groups compared to the control group.

CBMN (Cytokinesis-block micronucleus) assay

This is a novel and inclusive method for assessing genotoxicity, cytostasis, DNA damage, and cytotoxicity in several lymphocytes, tissue types, etc. There were substantial differences in the proportion of participants compared to the control group of cells. A rise in the proportion of mononucleated to binucleated cells indicates a reduction in a nuclear division that would validate the cytostatic effect. The lobe count feature was used for the DRAQ 5 channel wound closure identified between the VD3 ME, TMX ME, and TMX-VD3 ME groups to differentiate between mononucleated and binucleated cells based on DRAQ 5 staining.

We observed images of mononucleated, binucleated, trinucleated and polynucleated cells using IFC. Calculations were made using a similar quantitative study of mitochondrial membrane potential disturbance, as shown in Fig 9. MCF-7 cell line had characteristic binucleated cells that indicate cell division throughout the various cell cycle stages. After exposure to vitamin D3, tamoxifen and combination, the MFI ratio was found to be 4.68 ± 0.34. Contrary to the control, where the MFI ratio was 2.24 ± 0.14, this showed a considerable rise in mononucleated cells and a commensurate decline in binucleated cells.

Antiangiogenesis assay (zebrafish model)

The growth and metastasis of tumors depend on angiogenesis[27]. Widespread acceptance exists of the regenerated zebrafish tail fin as an *in vivo* angiogenesis research model[28]. The flow of vital nutrients to the tumor mass can be stopped by inhibiting blood vessel growth, thereby helping to halt metastasis. Zebrafish tail fins were severed at the mid-fin level for the study on tail fin regeneration, and they were then given time to heal. By one day after the amputation (dpa-day post amputation), the ends of amputated blood vessels have healed, and by two dpa, anastomosis has allowed arteries and veins to rejoin. Endothelial cell networks in the regenerated tissue form a vascular plexus that reaches the tip of the fin by 3 dpa (days post-amputation) and continues to develop for an additional 15 days. After treatment, the control group showed nearly full fin regeneration (93.14 %), while VD3ME group showed 86.5 % regeneration. At the end of 15 days, the TMX ME group also showed 74.22 % regeneration of the tail fin that had been severed. The TMX-VD3 ME group's much lower regeneration rate of 62.28 % demonstrated the superior effectiveness of TMX-VD3 versus TMX. VD-TMX in its nanonized form has a strong anti-angiogenic action, as evidenced by the TMX-VD3 ME's significant reduction of tail fin regeneration shown in Fig. 10.

CONCLUSION

According to the current investigation, a combination microemulsion of vitamin D3 and tamoxifen has superior anticancer efficacy. The study also demonstrates that

the effectiveness of BCS II medications, such as vitamin D3 and TMX can be significantly enhanced through formulation as microemulsion, as seen from the results of the *in vitro* anticancer assays in the MCF-7 cell line and the zebrafish anti-angiogenesis assay. Importantly, the study also offers a straightforward, scalable combination microemulsion of TMX and vitamin D3 as a promising approach for breast cancer treatment.

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