Study of anti-cancer effects of *Withania somnifera* and *Asparagus racemosus* in 3D breast cancer cultures

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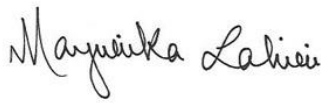
Certificate

This is to certify that this dissertation entitled '**Study of anti-cancer effects of *Withania somnifera* and *Asparagus racemosus* in 3D breast cancer cultures**' towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents the work carried out by **Shrunal Mane** at IISER Pune under the supervision of **Dr. Mayurika Lahiri**, Associate Professor, Biology Division, IISER Pune during the academic year 2018-2019.



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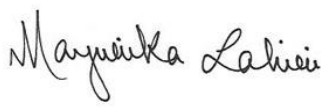
Declaration

I hereby declare that the matter embodied in the thesis entitled "**Study of anti-cancer effects of *Withania somnifera* and *Asparagus racemosus* in 3D breast cancer cultures**" are the results of the work carried out by me at the Department of Biology, IISER Pune, under the supervision of **Dr. Mayurika Lahiri**, Associate Professor, IISER Pune and the same has not been submitted elsewhere for any other degree.



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Abstract

Breast cancer is one of the primary causes of death in women worldwide. Ayurveda is an ancient branch of medicine in India that uses plants and their extracts for the treatment of numerous diseases including cancer. With an increase in drug resistance and cytotoxic effect of traditional chemotherapeutics, emphasis is given on finding alternate, less harmful treatment options for cancer. *Withania somnifera* and *Asparagus racemosus* are two plants used in Ayurveda for the treatment of inflammation, heart diseases, and cancer. This project aims to evaluate the anticancer effects of these two plant extracts in 3D breast cancer model. 2D cultures fail to mimic the *in vivo* microenvironment of a tumor. Cancer cells, when grown on an extracellular matrix, such as Matrigel, form three-dimensional, multicellular, spheroidal structures that resemble the *in vivo* tumor. Hence, they are an ideal platform to test the effects of *Withania somnifera* ethanolic and water (WSE and WSW, respectively) extract and *Asparagus racemosus* (ARE and ARW, respectively) in breast cancers. Doxorubicin, which is a commonly used chemotherapeutic, is used as a positive control for the study. MTT assay was performed to determine the IC_{50} concentration for each extract and doxorubicin in ER^{+} MCF7 cells and a TNBC cell line, MDA-MB-231. Morphometric analysis of 3D cultures shows a significant reduction in spheroid size upon treatment with WSE, WSW, ARE and ARW. The data suggests that these extracts possess anti-cancer activity and may be developed as potential chemotherapeutic drugs.

Keywords: Breast cancer, cancer chemotherapy, *Withania somnifera*, *Asparagus racemosus*, 3D culture

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I am grateful to the Biology department at IISER Pune for providing me excellent facilities to carry out my project. I also would like to thank the IISER Pune Microscopy facility for helping me complete major part of my thesis work. I want to thank Pharmazan Pvt. Ltd and AYUSH CoE, Pune for providing the extracts used in the study.Finally, I would also like to thank Ministry of AYUSH and IISER Pune for providing financial support.

Introduction

Breast Cancer in India

Cancer in the breast tissue occurs when the epithelial cells proliferate uncontrollably (Hanahan and Weinberg, 2011). According to the recent Globocan data, breast cancer has been ranked first for incidence, mortality as well as prevalence rates in women across the globe. Almost as many as 2.1 million new cases were diagnosed in 2018 worldwide. In India, of all the new cases registered in 2018, more than 27% are of breast cancer (Bray, Ferlay and Soerjomataram, 2018). The trend of breast cancer cases in India is different from what one sees in western populations. There has been a significant increase in diagnosed cases in the past 20 years. The age of detection in the Indian cohort is much lower than that in the west, and cancer at the time of detection tends to be more aggressive (Dhillon *et al.*, 2018). Lack of proper awareness, lack of early detection, unaffordable treatment options are few of the contributing factors to poor prognosis of breast cancer patients in India (Malvia *et al.*, 2017). There are four molecular subtypes of breast cancer. Based on the estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor 2 receptor (HER2) statuses, breast cancer is classified as $ER^+PR^+Her2^-$ (Luminal A), $ER^+PR^+Her2^+$ (Luminal B), $ER^-PR^-Her2^+$ (Her2 enriched) and $ER^-PR^-Her2^-$ (Triple Negative Breast Cancer TNBC/ Basal-like). In India, the most common subtypes seen are Her2 enriched and Triple Negative breast cancer (TNBC) (Desai *et al.*, 2000; Thakur, Bordoloi and Kunnumakkara, 2018).

Prognosis of a patient is identified by stage (i.e., tumor size, location, whether the disease has spread to lymph nodes and other parts of the body), grade, recurrence of the disease, and the age and health of the patient. Patients that have a good prognosis are usually offered less invasive treatments, such as lumpectomy and radiation or hormone therapy, while patients with poor prognosis are usually offered more aggressive treatment, such as mastectomy and one or more chemotherapy drugs (Maughan, Lutterbie and Ham, 2010). There are two types of therapy treatments given to patients, Neoadjuvant therapy (NAT) and Adjuvant therapy (AT). NAT includes chemotherapy (CT), radiation therapy (RT) or hormone therapy (HT) given before

surgical removal of the lump/breast. Adjuvant therapy (AT) includes therapy treatment post-surgery to avoid relapse (Breast Cancer Treatment (PDQ®), 2019). The traditional chemotherapy drugs include Taxanes like paclitaxel (Taxol), which inhibits microtubule depolymerization and arrests the cells in cell cycle (Weaver, 2014). Doxorubicin, a topoisomerase II inhibitor, which stalls the cells during replication and signals the cell to undergo apoptosis (Thorn *et al.*, 2011). The mechanism of action of these drugs target highly proliferating and dividing cells. Due to which they are also used for the treatment of various other cancers such as lung cancer, bladder cancer, ovarian cancer, etc. (Hande, 2008; Alqahtani *et al.*, 2019). Tissue-specific or targeted therapies for breast cancer are also available which are given depending on the tumor subtype. For ER^+ early-stage tumors, Tamoxifen is used as NAT. Tamoxifen selectively binds to the estrogen receptor and hence, does not allow estrogen signaling (Abe *et al.*,1998). Anastrozole is an aromatase inhibitor; it blocks the conversion of androgens to estrogen. It is also used for the treatment of ER+ tumors as adjuvant therapy (AT) (Cuzick *et al.*, 2010). Some of the tumor subtypes differentially overexpress a protein or a receptor. Targeted therapies can be developed for such patients. For example, Trastuzumab (Herceptin) is a monoclonal antibody that targets Her2 enriched tumors (Lewis Phillips *et al.*, 2008). As TNBC is negative for all receptor statuses, there is no targeted therapy for it. Currently, treatment of TNBC includes use of PARP inhibitors (Olaparib), tyrosine kinase inhibitors (Lapatinib) and mTOR inhibitors (Everolimus) (Khan *et al.*, 2019).

These drugs, even though effective in killing cancer cells, also have a cytotoxic effect on healthy cells, which lead to various side effects like nausea, loss of hair, weakness, weight loss and may also compromise host immunity. Some of these drugs also have long-term effects such as anemia, thrombocytopenia (bleeding and bruising), inflammation of other organs like lungs, heart diseases, fertility issues, etc. (American Cancer Society, 2015). Moreover, multiple studies show that patients are becoming resistant to traditional medicines in use (Gottesman, Fojo and Bates, 2002). Hence, it has become a challenge today to sensitize the tumors to these treatments to effectively target cancer. It is also essential to avoid undesirable side effects of these drugs without compromising their anti-tumor activity. Due to these challenges, much emphasis has

been given to complementary, alternative and less harmful treatment options for breast cancer.

Ayurveda and Cancer: *Withania somnifera* and *Asparagus racemosus*

Ayurveda is an ancient branch of Indian medicine that uses natural botanical products for the treatment of various diseases. Ayurvedic literature has shown the use of plant products for the management of cancers from as early as the 7th century BC (Balachandran and Govindarajan, 2005). Medicinal plants have various advantages over synthetic drugs. There is an increasing demand for treatment based on natural products as they are inexpensive and considered relatively safe as they are better compatible with the human body (Nema, Khare, Jain, Pradhan, *et al.*, 2013). The use of Ayurvedic medicine is known to improve life span and general health. They have no short or long term side effects. Plants and their secondary metabolites are potential anti-cancer therapeutics because of their anti-proliferative activity (Baliga, 2010). Many chemotherapeutics in use are plant-derived. Paclitaxel (Taxol) is a purified alkaloid found in the bark of the Pacific yew tree (*Taxus brevifolia*). Vincristine and Vinblastine are anti-cancer agents derived from *Vinca rosea* (Levitsky and Dembitsky, 2015). With this in mind, many herbs and plants like bitter leaf (*Vernonia amygdalina*), Amazon shrub Suma (*Pfaffia paniculata*), grapevine (*Vitis vinifera*), *Taxus buccata*, *Tinospora cordifolia* and numerous more are under evaluation for their anti-cancer properties *in vitro* and *in vivo* (Rastogi, 2010).

Withania somnifera (WS), commonly known as Ashwagandha, is a plant belonging to the Solanaceae family. Ashwagandha is rich in secondary metabolites like steroidal lactones (withanolides and withaferin), alkaloids, and saponins (Singh and Dagenais, 2000). Both leaf and root extracts of ashwagandha are shown to have tumor preventive activity. Many researchers have shown that Withaferin A, a withanolide purified from the extracts, has antitumor activity in lung, breast, ovarian, osteosarcoma and colon cancer cell lines (Nema, Khare, Jain and Pradhan, 2013; Jayaprakasam *et al.*, 2003; Wadhwa *et al.*, 2013). Administration of *Withania somnifera* root extracts reduced MNU-induced mammary carcinogenesis in female Sprague-Dawley rats (Khazal *et al.*, 2013). Whole plant (ethanolic) extract shows reduced tumor incidence in urethane-induced lung

adenoma in Swiss albino rats (Singh *et al.*, 1986). *W. somnifera* extracts have been reported to have an immunoprotective role by reducing pro-inflammatory cytokines and increased oxygen scavenging activity (Muralikrishnan, Dinda and Shakeel, 2010). WS has also shown to have immunomodulatory activity by inducing cellular immune response in immunosuppressed mice (Agarwal *et al.*, 1999).
Asparagus racemosus (AR), commonly known as Shatavari, is a plant belonging to Liliaceae family. It is rich in secondary metabolites like steroidal saponins (shatavarins), alkaloids and isoflavones (Alok *et al.*, 2013). Studies show root extracts of *A.racemosus* have anti-cancer properties in vitro using colon, kidney, and breast cancer cell lines (Verma, Tripathi and Das, 2014; Bhutani *et al.*, 2010; Bousserouel *et al.*, 2013). *A.racemosus* reduces DMBA-induced mammary carcinogenesis in mice models (Rao, 1981). Wistar rats pre-treated with AR extracts do not show hepatocarcinogenesis induced by diethylnitrosamine (DEN) (Agrawal *et al.*, 2008). Root extracts of AR show cell toxicity and morphological change in non-small cell lung cancer cell line (Biswas *et al.*, 2018). Treatment with AR extract reduces tumor volume in Ehrlich ascites carcinoma (EAC) tumor-bearing mice (Prakash *et al.*, 2012). Studies show that there is a presence of phytoestrogens in AR extracts. Hence AR can be developed as a potential anti-cancer drug for treatment of ER+ breast cancers (Sharma and Jaitak, 2018).

Withania somnifera and *Asparagus racemosus* are two important botanical plants used in Ayurveda. Their ability to induce apoptosis in cancer cells has attracted researchers to study their anti-cancer properties *in vitro* to develop drugs for the treatment of several cancers (Diwanay, Chitre and Patwardhan, 2004). These plant extracts also possess cancer-preventive properties and immunomodulatory activity (Malik *et al.*, 2009; Diwanay, Chitre and Patwardhan, 2004). As mentioned previously, researchers have evaluated the effect of the bioactive compound or the purified compound of these extracts in various cancer cell lines and have been successful in showing their anti-tumor activity. However, we are still unaware of the cytotoxic effect they might have on healthy cells. Conventional preparation methods in Ayurveda practices usually involve extensive drying the root or the leaves, followed by grinding into a fine powder (Baliga, 2010). Extraction procedures are performed using aqueous or organic solvents.

Ayurveda literature mentions that the secondary metabolites of the plant matrix enhance the activity of the primary metabolite (bioactive compound). Crude extracts also enhance the immune response and function of the body in a diseased state (Roy, Ahuja and Bharadvaja, 2017; Nema, Khare, Jain, Pradhan, *et al.*, 2013). Therefore, for this study, it would be interesting to evaluate the effects of crude aqueous (water) and organic (ethanol) extracts of both, *Withania somnifera* and *Asparagus racemosus* in MCF7 (ER^+) and MDA-MB-231 (TNBC) cell lines using three-dimensional cultures as a model system.

3D Cultures as a Model for Drug evaluation

The mammary gland is made up of multiple lobules which are responsible for producing milk during the lactation period. These lobules are connected via ducts, which carry the milk from the lobules to the nipple. Acinus is a single subunit of a lobule. It is made up of a bilayer of inner luminal epithelial cells and outer myoepithelial cells, which are in contact with the basement membrane. The myoepithelial cells help in contraction of the lobules for transporting milk via ducts (Vidi, Bissell and Lelièvre, 2013). The breast tissue is continually going under changes during the lifetime of a woman. There are changes in morphology and signaling during puberty, pregnancy, lactation, and menopausal events. Cancer in the breast may occur when one of these signaling pathways is perturbed (Campbell and Watson, 2009).

When MCF10A, non-malignant breast epithelial cells, are grown on a matrix resembling the extracellular matrix (ECM), they formed a three-dimensional structure with a hollow lumen. The cells showed apicobasal polarity and tight cell-cell junction. When stimulated with casein, these structures also produced milk. Similarly, when breast cancer cell lines are grown on a matrix, they form multicellular, spheroidal structures with a filled lumen (Debnath and Brugge, 2005).

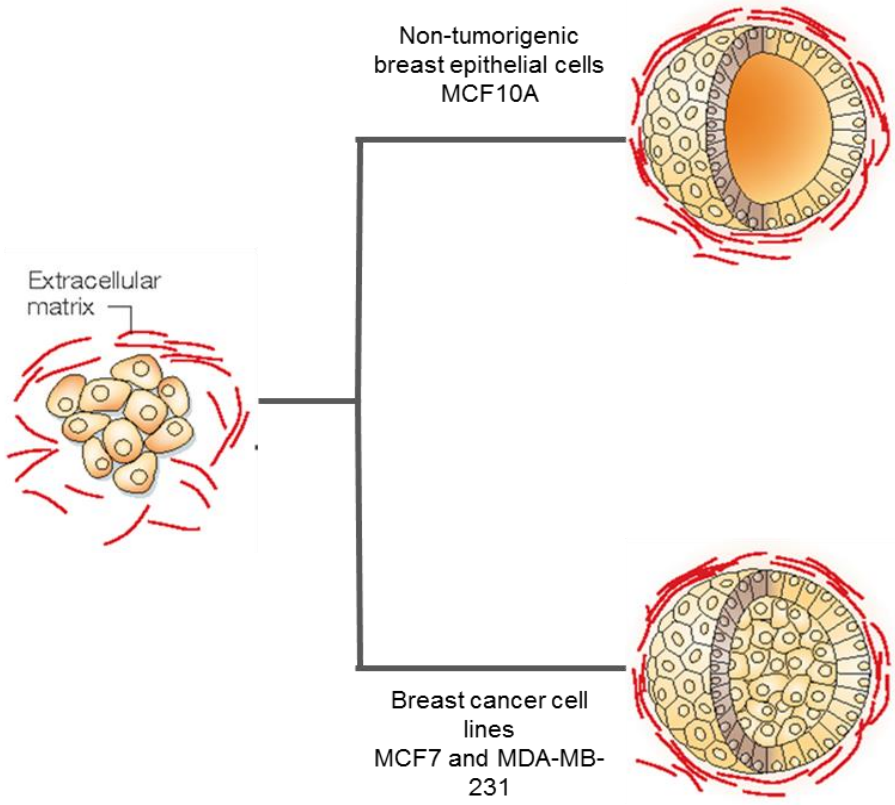


Figure1: Schematic showing Non-tumorigenic breast epithelial cells, MCF10A, grown in Matrigel (ECM) form 3D acini with hollow lumen, whereas breast cancer cells grown Matrigel form multicellular spheroid. Adapted from J. Debnath *et al*. (2005) *Nat Rev Cancer.*

One of the foremost issues faced by medical oncologists is failure of drugs in Phase II or III trials of drug testing. One of the reasons for the failure is that the conventional method of drug development includes testing the drugs in two dimensional, monolayer cultures of cancer cells (Lovitt, Shelper and Avery, 2018). We now know that there is a cross-talk between the cancer cells and its microenvironment. The extracellular matrix (ECM) plays a significant role in cancer cell behavior and signaling (Bissell *et al.*, 1999). It provides cell structural support and affects the behavior in a tissue-specific manner. ECM signals the cell to grow and proliferate to form 3D structures in the body (Vidi, Bissell and Lelièvre, 2013). 2D cultures fail to imitate this microenvironment and the signaling that happens in 3-dimensional tumors. To bridge this gap between 2D cultures and mice studies and to better recapitulate the *in vivo* environment of tumors, many researchers are leaning towards the use of three-dimensional cultures (3D cultures) of

cancer cells as a platform for drug testing (Lovitt, Shelper and Avery, 2013; Langhans, 2018). By studies done on cancer cell 3D models, we know that the ECM may alter or affect the drug response. The response of cells in direct contact with the ECM is different from the cells in the luminal space. The import and export of the drug are also dependent on the ECM. Hence, contact with the ECM might play a role in drug resistance (Imamura *et al.*, 2015; Lovitt, Shelper and Avery, 2018). Hence, a 3D culture of cancer cells is an ideal platform for preliminary studies in drug development.
For this study, I am using Matrigel as the matrix to grow MCF7 and MDA-MB-231 breast cancer cell lines as 3D spheroids. Matrigel is derived from extracts of EHS tumors, which were found to have most of the basement membrane components. It is a laminin-111 rich basement membrane (lr-BM) containing other BM components such as collage-IV, entactin, and heparan sulfate proteoglycan. Matrigel is a form of natural hydrogel scaffold which is biocompatible and has naturally adhesive properties.(Kleinman and Martin, 2005). These components form a matrix that provides structural integrity to the cells to grow in 3D. Matrigel facilitates cell attachment through integrin which leads to activation of cell signaling pathways like cell proliferation, establishment of apicobasal polarity, and differentiation. This integrin receptor interaction also modulates drug response of the cells (Tibbitt and Anseth, 2009; Langhans, 2018).

Studies have shown the anticancer properties of AR and WS in 2D models but not in 3D. Hence this study aims to evaluate the effect of ethanolic and water extracts of *Withania somnifera* (WSE and WSW respectively) and ethanolic and water extracts of *Asparagus racemosus* (ARE and ARW respectively) in 3D breast culture of MCF7 and MDA-MB-231.

Materials and Methods

Cell lines

MCF7 was purchased from the European Collection of Cell Cultures (ECACC). MDA-MB-231 was borrowed from the NCCS cell repository.

Cell culture

MCF7 and MDA-MB-231 cells were grown on 100mm 2D tissue culture-treated dishes (Corning) with Dulbecco's Modified Eagle Medium (DMEM; Lonza) containing high glucose, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) and 100 units/ml of penicillin-streptomycin (Invitrogen).

Chemicals

Doxorubicin Hydrochloride (Relic Biotechnology Pvt. Ltd.) was purchased and dissolved in DMSO (Sigma-Aldrich). 100gm packets of powdered *Withania somnifera* hydro-ethanolic and water extract (WSE and WSW respectively) and *Asparagus racemosus* hydro-ethanolic and water extract (ARE and ARW respectively) were provided by Pharmanza Herbal Pvt. Ltd. in collaboration with Prof. Bhushan Patwardhan, Savitribai Phule Pune University.

MTT assay

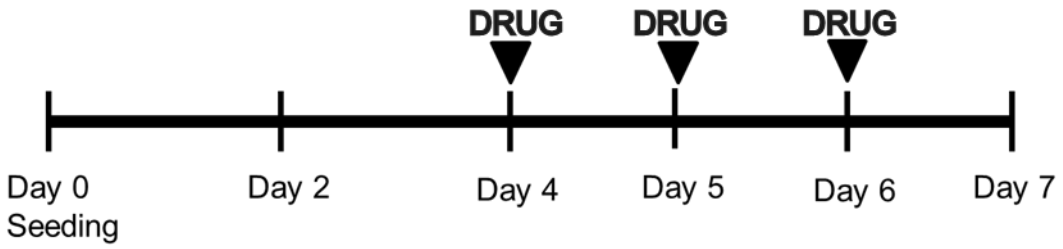
Five thousand cells of MCF7 and MDA-MB-231 suspended in DMEM were seeded in each well of a 96-well plate. 24hrs post-seeding, cells were treated with different concentrations of Doxorubicin, ARE, WSE, ARW and WSW and incubated for 48hrs at 37℃. After 48hrs, the media containing drug/extract was aspirated. 0.5mg/ml thiazolyl blue tetrazolium (MTT; Sigma-Aldrich) dissolved in DMEM was added on top of the cells and incubated for 4hrs at 37℃. After 4hrs, the MTT solution was aspirated, and 100µl of dimethyl sulfoxide (DMSO; Thermofisher) was added to each well to dissolve the formazan crystals. The absorbance was measured at 570nm wavelength using Varioskan Flash (Thermo Scientific).

MCF7 and MDA-MB-231 3D culture

The cells were cultured using the “on-top” method of 3D culturing (Lee *et al.*, 2007). MCF7 (3500 cells/well) and MDA-MB-231 (2000 cells/well) were suspended in DMEM supplemented with 10% FBS, 1x penicillin-streptomycin containing 5% Matrigel® (Corning). This cell suspension was seeded in an 8-well chambered coverglass (Nunc Lab tek; Thermo Scientific) pre-coated with 55µl Matrigel®. The culture was maintained at 37℃ with 5% CO_2 incubators for seven days. Media change was given every two days.

Treatment Regime for 3D culture

Cells are seeded on day 0 and allowed to grow for 7 days. A triple dose regime was standardized. The drug/extracts are added on days 4, 5 and 6 incubated for 24hrs each. The culture is fixed on day 7.



Immunofluorescence for 3D cultures

On day 7, 3D cultures were fixed using 4% paraformaldehyde in PBS for 20 mins at RT. Cells were washed with 1XPBS twice for 10 mins after fixation. Cells were permeabilized with cold 0.5% Triton-X 100 in PBS at 4℃ for 10 mins (extreme care is to be taken during this step). After permeabilization, cells were washed with PBS-glycine twice for 15 mins each, and one only-PBS wash for 10 mins. Primary blocking was done with 10% goat serum (Abcam) for 1hour at RT followed by secondary blocking with 1% F(ab’)2 fragment goat anti-mouse IgG (Jackson ImmunoResearch) in goat serum for another 1 hour. Alexa Fluor® Phalloidin 488 prepared 1:100 in goat serum was incubated for 1hr at RT. After incubation, cells were washed with 1X IF buffer for 20

mins followed by two washes with 1X PBS for 10 mins each. After the washes, cells were incubated with 0.5µg/ml of Hoechst 33258 in PBS for exactly 5 mins. Cells were then washed thrice with 1XPBS for 10 mins each. After the last wash, excess PBS is removed, and Slow Fade® Gold antifade reagent was added to mount the cells. The cells were kept at RT for 1hr before keeping them at 4˚C overnight. The chamber is kept at RT the next day for 30 mins before imaging. The cells are then imaged using Leica Sp8 confocal microscope using a 63x oil immersion objective.

Morphometry of 3D spheroids

Morphometric parameters like Volume and Surface Area of spheroids were obtained using the Phalloidin staining in the Huygens Essential software (Scientific Volume Imaging). The number of cells per spheroid was quantified by manually counting the number of nuclei per spheroid using ImageJ software (NIH).

Statistical Analysis

Results were analyzed and plotted using GraphPad Prism (GraphPad Software, CA, USA). Mann-Whitney U test was used as a statistical test of significance.

Aim and Objectives

Aim: To study the anti-cancer effects of *Withania somnifera* and *Asparagus racemosus* extracts in breast cancer

Objectives

1. Determine the dose range and regime of the extracts in 2D cultures of breast cancer cell lines.
    a) Determine the IC_{50} dose range of Doxorubicin (positive control) in MCF7 and MDA-MB-231 cell lines.
    b) Determine the IC_{50} dose of WSE, WSW, ARE and ARW in MCF7 and MDA-MB-231 cell lines.
2. Standardize the dose range and regime in 3D cultures of MCF7 and MDA-MB-231.
3. Morphometric analysis of the 3D spheroids
    a) Volume and surface area of spheroids.
    b) The number of cells per spheroid.
4. Assays to determine DNA damage and apoptotic activity of the extracts in 3D cultures.

Results

Determining IC_{50} dose of Doxorubicin DOX in MCF7 and MDA-MB-231 cell lines

Doxorubicin Hydrochloride in powder form was dissolved in Dimethyl Sulfoxide (DMSO) to prepare 1mM main stock. A further working stalk of 100μM was prepared by diluting in DMSO. 5000 cells of MCF7 and MDA-MB-231 were seeded in each well of a 96-well plate. 24hrs post-seeding, drug addition was done. The concentration range of 0-1.5 μM was used for MCF7 cells and 0-20μM for MDA-MB-231 cells. The drug was incubated for 48hrs. After incubation, the media was aspirated, and the cells were incubated with 0.5mg/ml MTT+DMEM solution for 4hrs. MTT is a yellow-colored tetrazolium dye. When it is taken up by live cells, the mitochondrial dehydrogenase reduces it to form purple-colored formazan crystals. After incubation with MTT, the solution is aspirated and DMSO is added to each well to dissolve the formazan crystals. The absorbance of each well is recorded using Varioskan Plate reader at a wavelength of 570nm. % viability is calculated as follows,

$$\% \text{ viable cells} = \frac{(abs_{sample} - abs_{blank})}{(abs_{control} - abs_{blank})} \times 100$$

The viability of cells as a function of the concentration of doxorubicin was plotted. The Concentration at 50% viability is considered as IC_{50} for doxorubicin.

The IC_{50} for DOX determined in MCF7 is 0.75 μM or 0.435 μg/ml and in MDA-MB-231 is 9.77 μM or 5.66 μg/ml (Fig. 2).

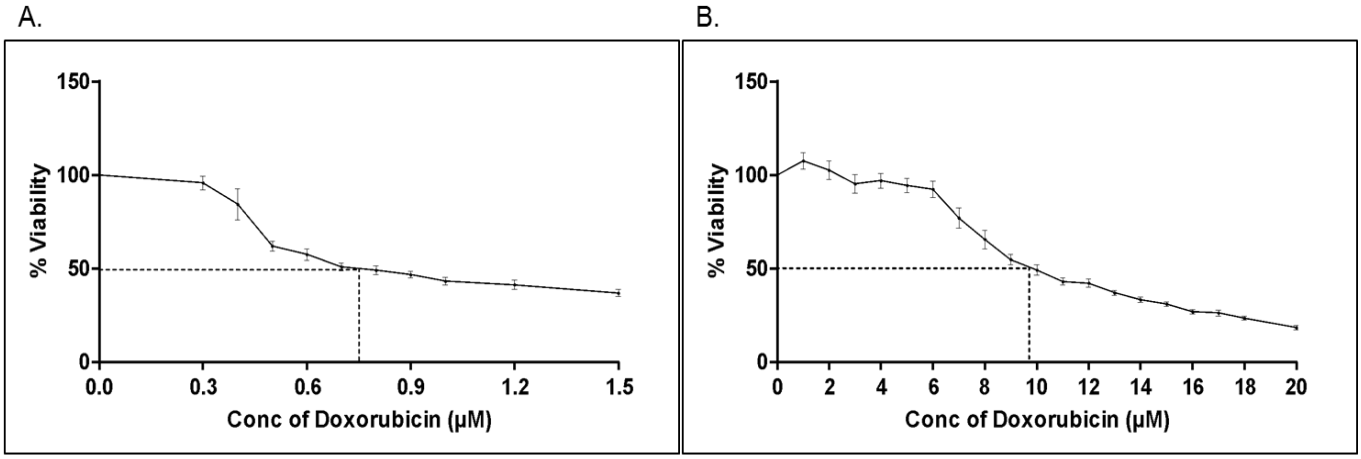


Figure 2: A) MCF7 and B) MDA-MB-231 cells were treated with Doxorubicin of wide range concentrations,incubated for 48hrs. Cells were then subjected to MTT assay. The percentage of cell survival as a function of drug concentration was plotted and results are expressed as mean ± SEM (N=3). The IC_{50} is determined to be 0.75 µM or 0.435 µg/ml for MCF7 cells and 9.77 µM or 5.66 µg/ml for MDA-MB-231 cells

Determining IC_{50} dose of *Withania somnifera* ethanolic (WSE) in MCF7 and MDA-MB-231 cell lines

50mg powder of crude *Withania somnifera* roots extracted in ethanol (organic solvent) was was weighed and dissolved in 1ml DMSO to make a 50mg/ml main stock. MTT assay was performed. The IC_{50} for WSE determined in MCF7 is 52 µg/ml and in MDA-MB-231 is 55 µg/ml (Fig. 3).

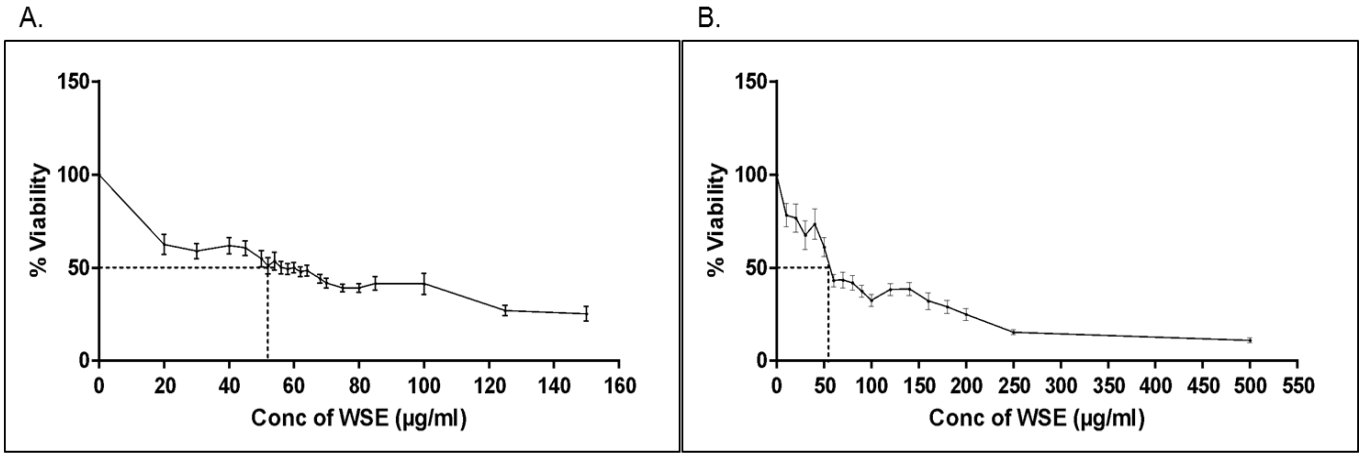


Figure 3: A) MCF7 and B) MDA-MB-231 cells were treated with a wide range concentrations of *Withania somnifera* ethanolic extract, incubated for 48hrs. Cells were then subjected to MTT assay. The percentage of cell survival as a function of drug concentration was plotted and results are expressed as mean ± SEM (N=3). The IC_{50} is determined to be 52 µg/ml for MCF7 cells and 55 µg/ml for MDA-MB-231 cells

Determining IC_{50} dose of *Withania somnifera* water (WSW) in MCF7 and MDA-MB-231 cell lines

100mg powder of crude *Withania somnifera* roots extracted in water (aqueous solvent) was weighed and dissolved in 1ml ultrapure distilled water to make a 100mg/ml main stock. MTT assay was performed. The IC_{50} for WSW determined in MCF7 is 1000 µg/ml and in MDA-MB-231 is 2000 µg/ml (Fig. 4).

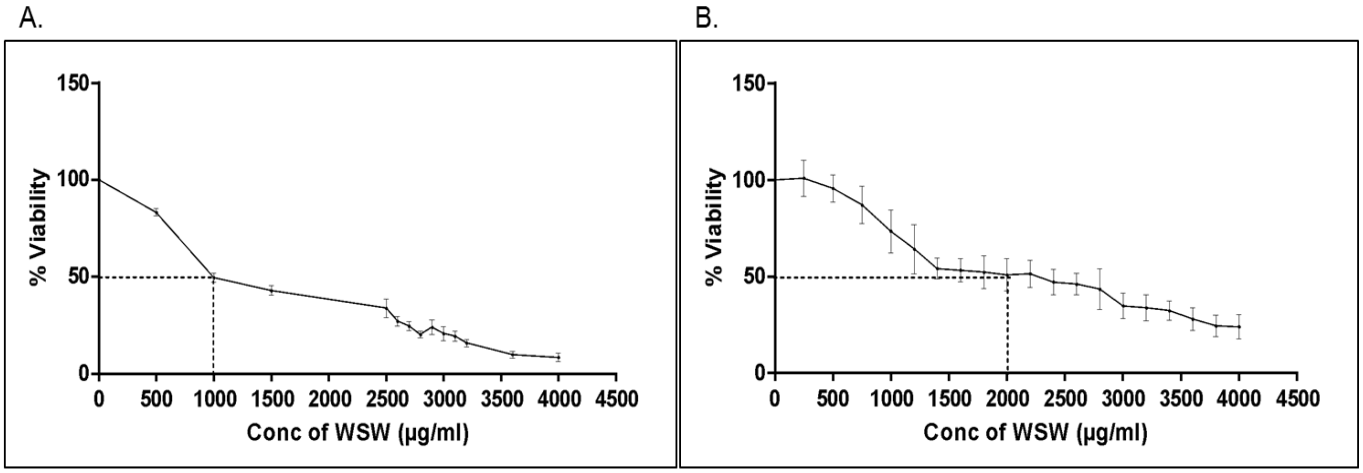


Figure 4: A) MCF7 and B) MDA-MB-231 cells were treated with a wide range concentrations of *Withania somnifera* water extract, incubated for 48hrs. Cells were then subjected to MTT assay. The percentage of cell survival as a function of drug concentration was plotted and results are expressed as mean ± SEM (N=3). The IC_{50} is determined to be 1000 µg/ml for MCF7 cells and 2000 µg/ml for MDA-MB-231 cells

Determining IC_{50} dose of *Asparagus racemosus* ethanolic (ARE) in MCF7 and MDA-MB-231 cell lines

50mg powder of crude *Asparagus racemosus* roots extracted in ethanol (organic solvent) was weighed and dissolved in 1ml DMSO to make a 50mg/ml main stock. MTT assay was performed. The IC_{50} for ARE determined in MCF7 is 315 µg/ml and in MDA-MB-231 is 510 µg/ml (Fig. 5).

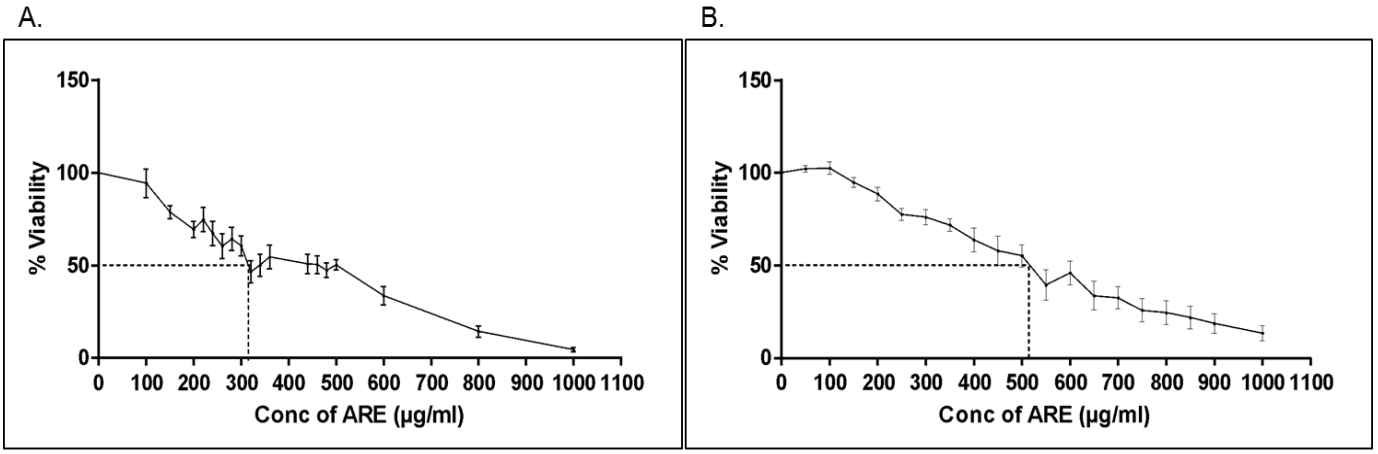


Figure 5: A) MCF7 and B) MDA-MB-231 cells were treated with a wide range concentrations of *Asparagus racemosus* ethanolic (ARE) extract, incubated for 48hrs. Cells were then subjected to MTT assay. The percentage of cell survival as a function of drug concentration was plotted and results are expressed as mean ± SEM (N=3). The IC_{50} is determined to be 315 µg/ml for MCF7 cells and 510 µg/ml for MDA-MB-231 cells

Determining IC_{50} dose of *Asparagus racemosus* water (ARW) in MCF7 and MDA-MB-231 cell lines

100mg powder of crude *Asparagus racemosus* roots extracted in water (organic solvent) was weighed and dissolved in 1ml ultrapure distilled water to make a 100mg/ml main stock. MTT assay was performed. The IC_{50} for ARW determined in MCF7 is 510µg/ml and in MDA-MB-231 is 550µg/ml (Fig. 6).

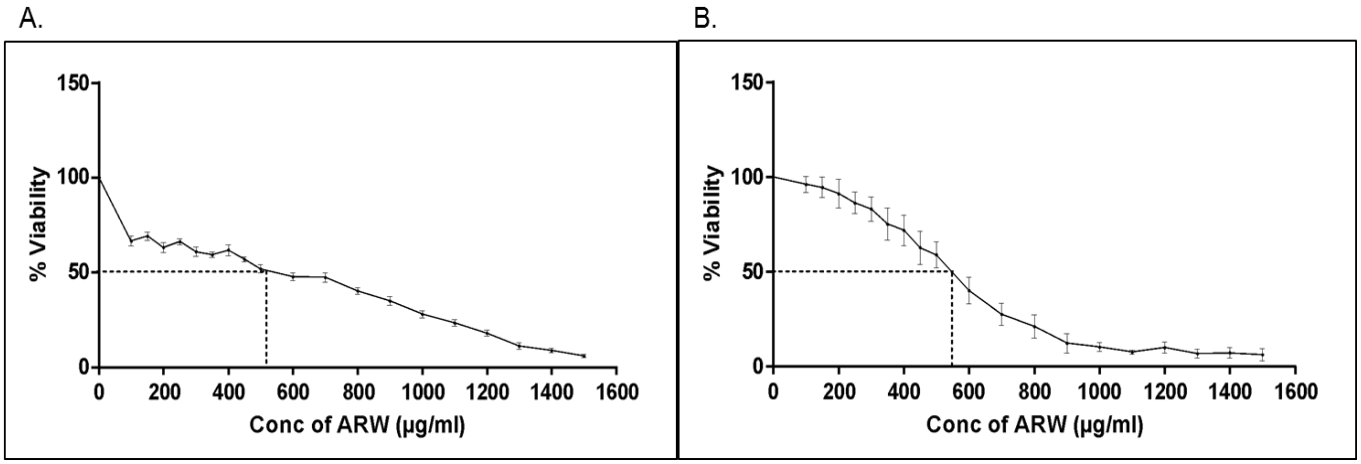


Figure 6: A) MCF7 and B) MDA-MB-231 cells were treated with a wide range concentrations of Asparagus racemosus water (ARW) extract, incubated for 48hrs. Cells were then subjected to MTT assay. The percentage of cell survival as a function of drug concentration was plotted and results are expressed as mean ± SEM (N=3). The IC_{50} is determined to be 510 µg/ml for MCF7 cells and 550 µg/ml for MDA-MB-231 cells

Table 1: **Summary of IC_{50} determined in both cell lines for each extract and doxorubicin**

Extract/Drug	MCF7	MDA-MB-231
Doxorubicin (DOX)	0.435 µg/ml	5.66 µg/ml
W.Somnifera ethanolic extract (WSE)	52 µg/ml	55 µg/ml
W.Somnifera water extract (WSW)	1000 µg/ml	2000 µg/ml
A.Racemosus ethanolic extract (ARE)	315 µg/ml	510 µg/ml
A.Racemosus water extract (ARW)	510 µg/ml	550 µg/ml

Spheroid formation of MCF7 and MDA-MB-231 cells grown on Matrigel

As the IC_{50} values of all the extracts were determined, the next part of the study was to grow MCF7 and MDA-MB-231 cells as 3D cultures. For standardizing the culture protocol, MCF7 (5000 cells/well) and MDA-MB-231(4000 cells/well) were seeded in a 48-well dish. The cells were maintained as 2D monolayer cultures. On the day of seeding, the cells were trypsinized and resuspended in DMEM. Matrigel bed was prepared. Media with 5% matrigel and the cell suspension was prepared and seeded on top of the Matrigel bed. The culture was maintained for eight days. The growth of spheroids was monitored by taking phase-contrast images using Eclipse TS100 Nikon microscope (Fig. 7 for MCF7 and Fig. 8 for MDA-MB-231)

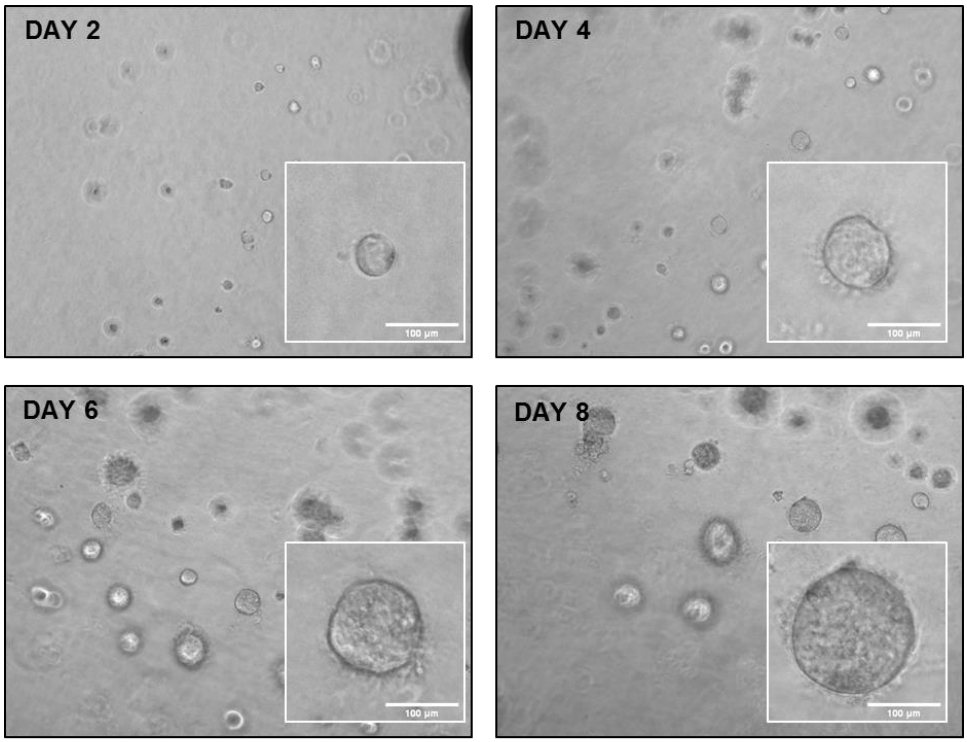


Figure 7: **Growth of MCF7 3D spheroids for 8 days.**
Phase contrast images of MCF7 3D culture taken on day 2, 4, 6 and 8. The images are taken using Eclipse TS100 Nikon microscope at 10x magnification

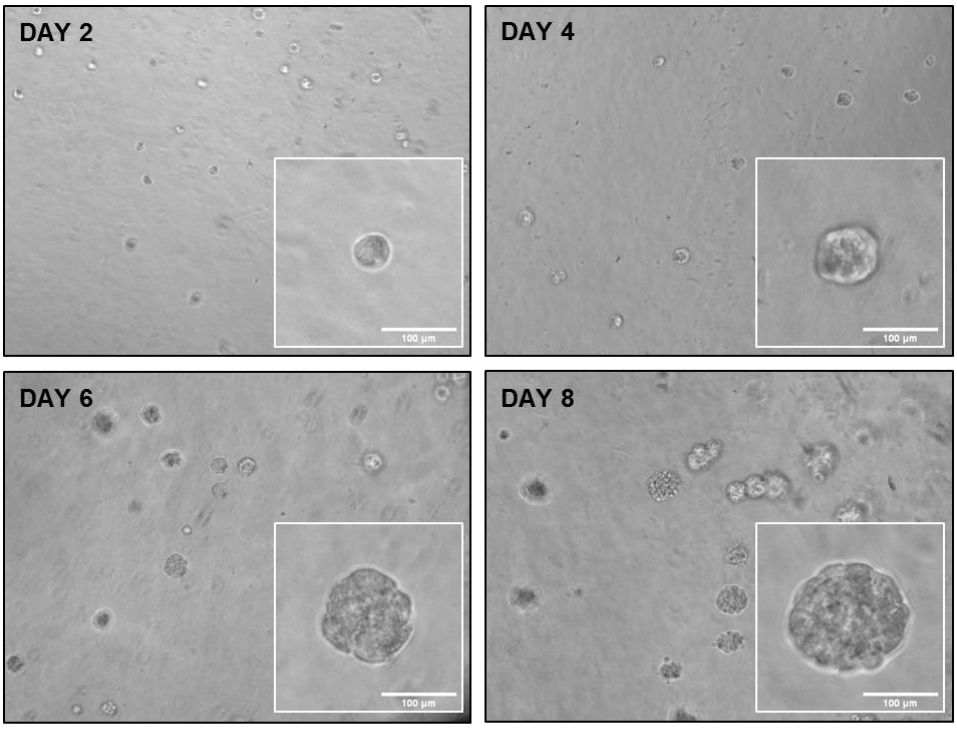


Figure 8: **Growth of MDA-MB-231 3D spheroids for 8 days.**
Phase contrast images of MDA-MB-231 3D culture taken on day 2, 4, 6 and 8. The images are taken using Eclipse TS100 Nikon microscope at 10x magnification.

Along with the standardization of 3D culture, the treatment regime was standardized using doxorubicin in both the cell lines. The treatment regime was adapted from Imamura *et al.*, 2015. The cells are seeded on day 0, and the drug is added on days 4, 5 and 6, incubated for 24hrs each. Media is changed every two days and the growth of 3D cultures was monitored using Eclipse TS100 Nikon microscope (Fig 9 for MCF7 and Fig.10 for MDA-MB-231).

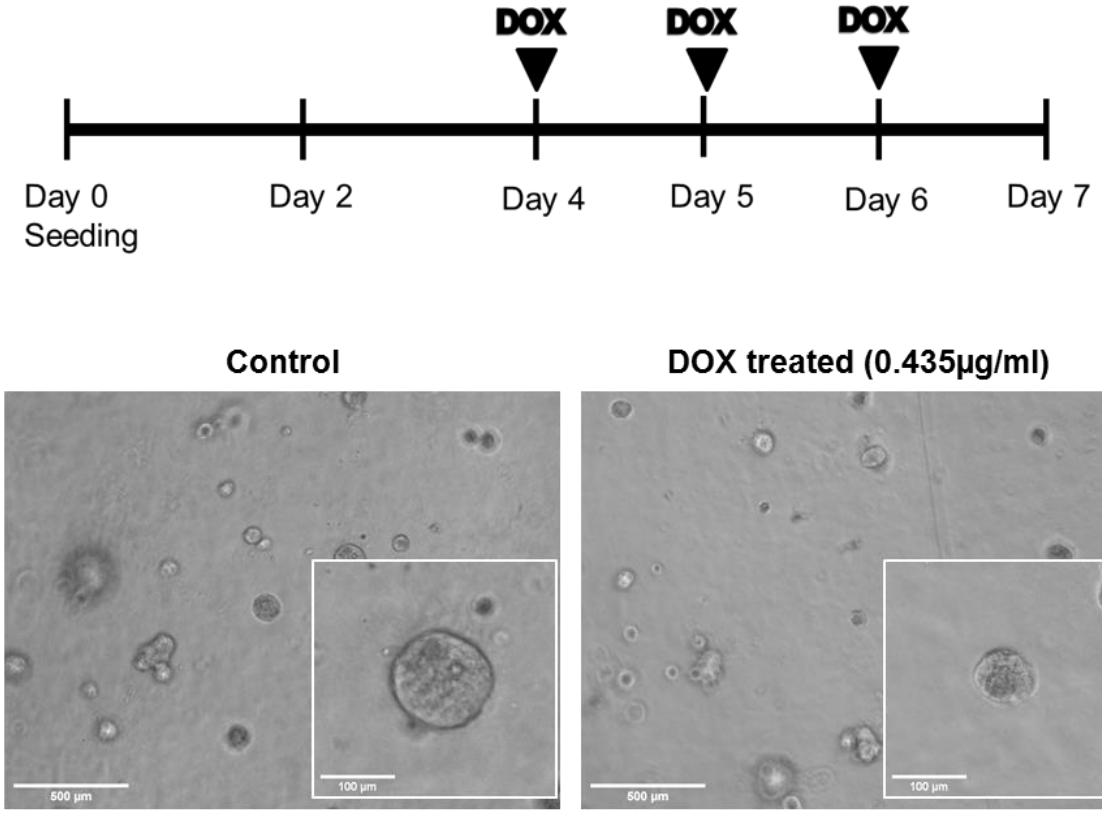
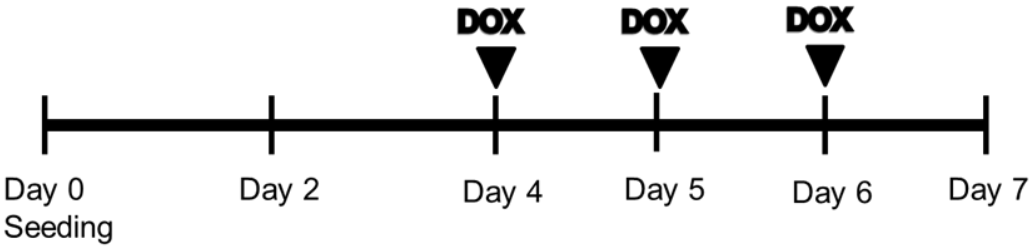


Figure 9: **MCF7 spheroid show reduction in size upon triple dose treatment with DOX**
Phase contrast images of MCF7 3D culture taken on day 7 after treatment with triple dose of IC_{50} concentration of doxorubicin. The images are taken using Eclipse TS100 Nikon microscope at 10x magnification.



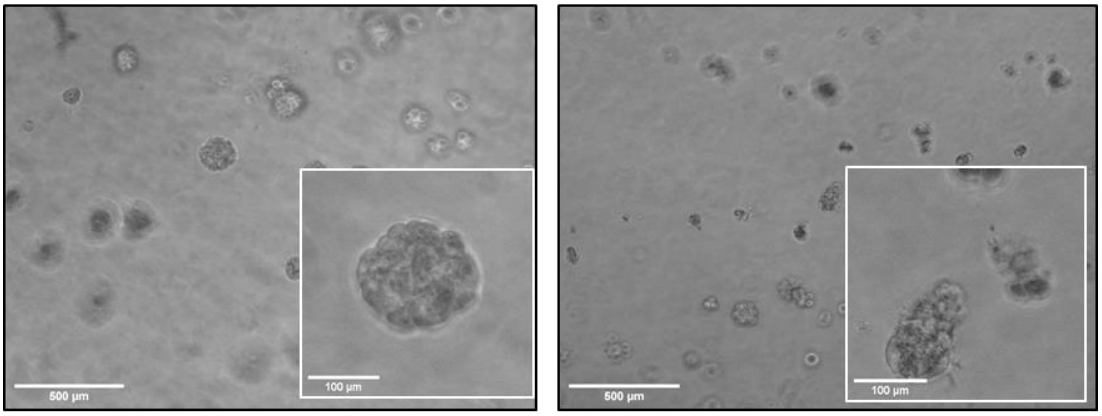


Figure 10: **MDA-MB-231 spheroid show reduction in size upon triple dose treatment with DOX**
Phase contrast images of MDA-MB-231 3D culture taken on day 7 after treatment with triple dose of IC_{50} concentration of doxorubicin. The images are taken using Eclipse TS100 Nikon microscope at 10x magnification.

Morphometric analysis of MCF7 3D cultures using Immunofluorescence (IF) staining

To evaluate and quantify the effect of DOX, WSE, WSW, ARE and ARW in 3D cultures of MCF7, and the cultures were treated and fixed on day 7. Cells were then subjected to IF assay as mentioned in Materials and Methods. The staining was performed using Phalloidin Alexa Fluor 488. Fluorescently-labeled phalloidin is a peptide that has a high affinity to bind to F-actin in the cells. Nuclei were counterstained with Hoechst 33258.

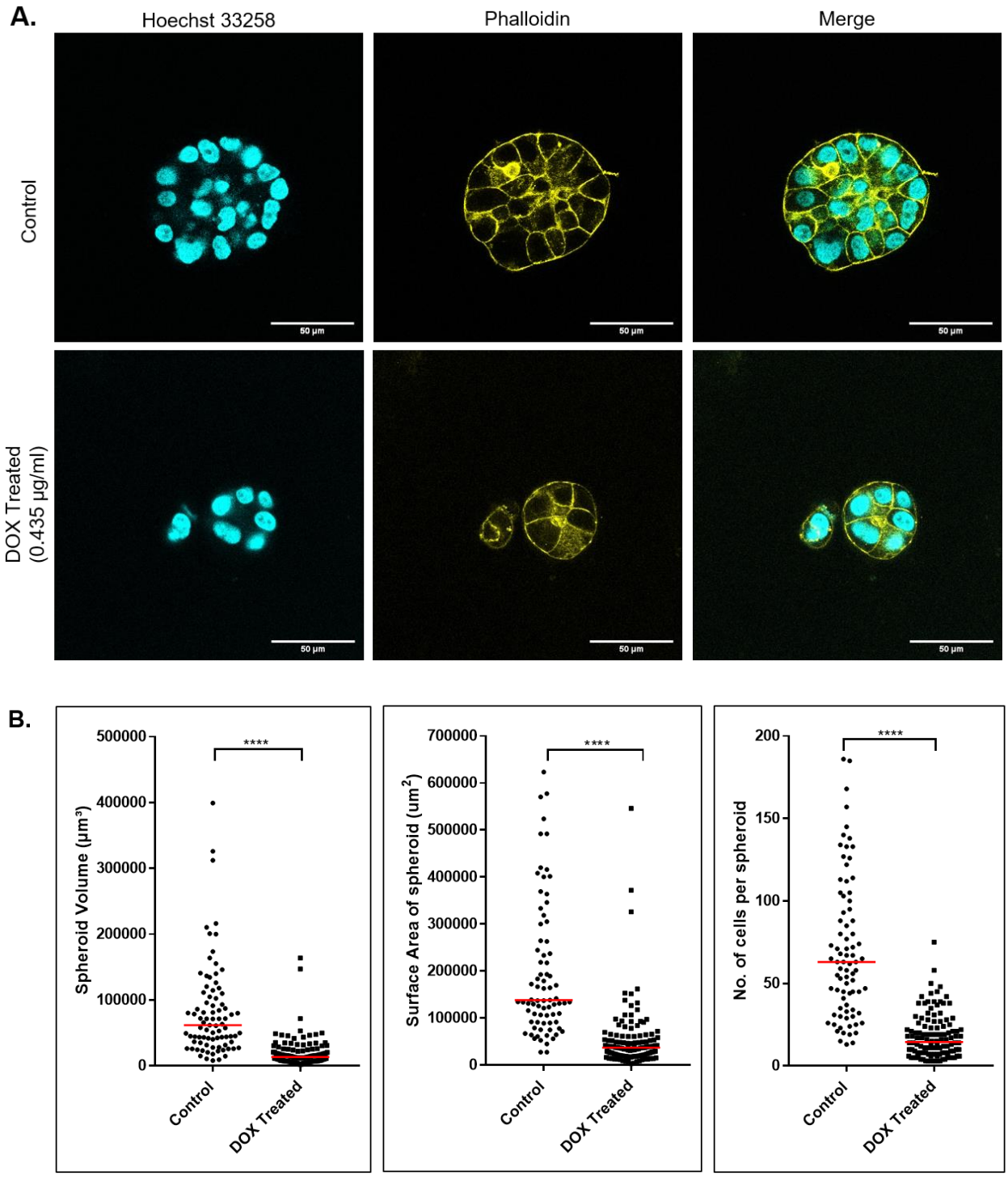


Figure 11: **Doxorubicin treatment leads to reduction of MCF7 spheroids.**
A) Representative image of MCF7 3D spheroid showing reduction in size. B) Morphometric analysis showing reduction in parameters like spheroid volume, spheroid surface area and number of cells per spheroid. 85 spheroids for control and 123 spheroids for DOX treated from 2 biological experiments were analysed. Statistical analysis was done using Mann Whiney test (**** p<0.0001)

Doxorubicin is a commonly used chemotherapy drug for the treatment of breast cancer. Hence, it is used as a positive control. MCF7 spheroids reduce in size upon treatment with DOX (Fig. 11). The volume and surface area of spheroids are analyzed using Huygens Essential software. The number of cells per spheroid is analyzed by counting the number of nuclei through different stacks using the Image J software.

WSE, WSW, ARE and ARW treated MCF7 spheroids were processed similar to the positive control (DOX). WSE treated spheroids show disrupted structure in addition to reduction in size (Fig. 12). WSW treated spheroids also show a significant reduction in size as compared to control (Fig. 13).
ARE and ARW also effectively reduce the size of MCF7 spheroids upon treatment (Fig. 14 and Fig.15 respectively).

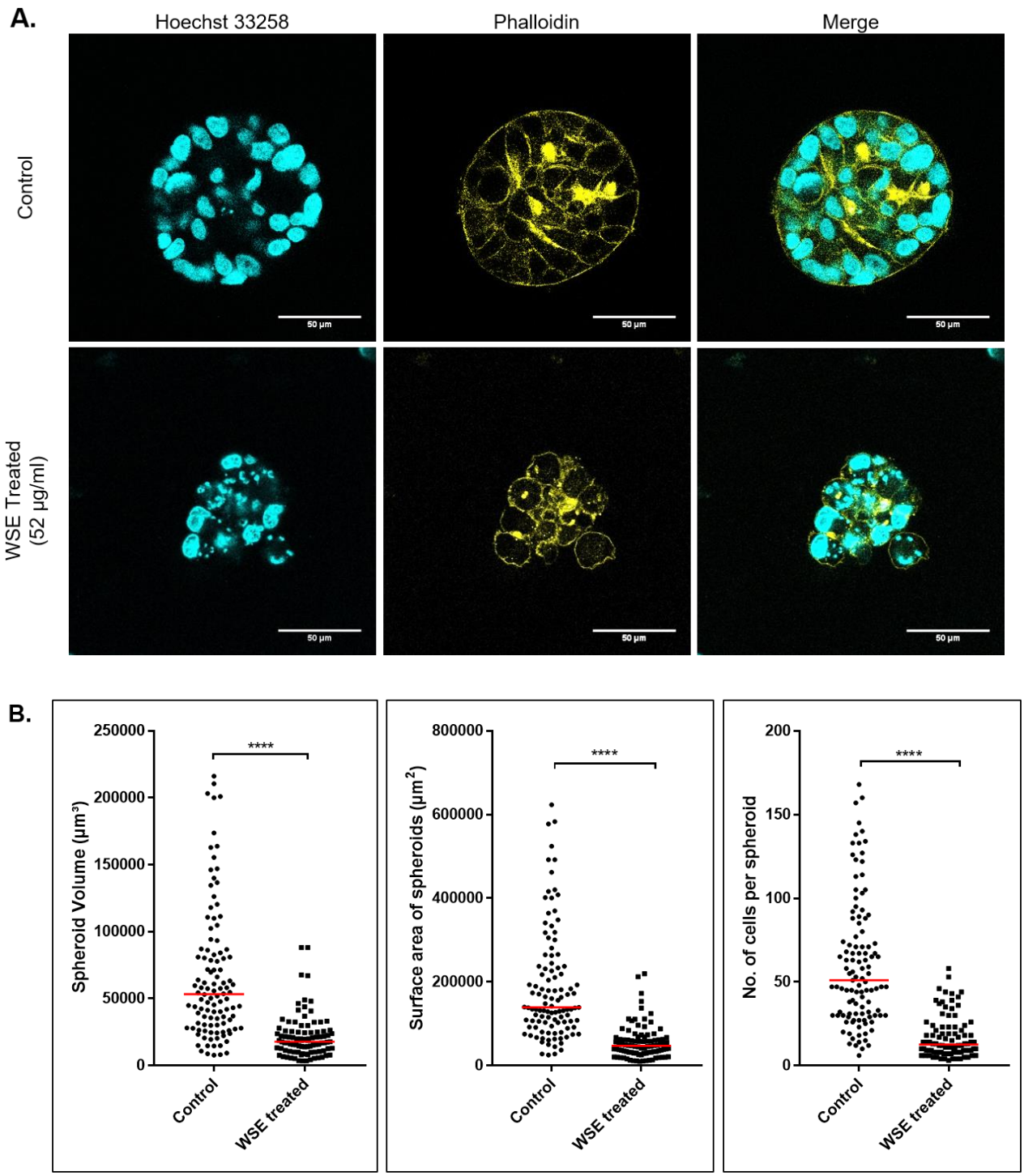


Figure 12: **MCF7 spheroid are disrupted and reduce in size upon treatment with *Withania somnifera* ethanolic (WSE) extract.** A) Representative image of MCF7 3D spheroid showing reduction in size. B) Morphometric analysis showing reduction in parameters like spheroid volume, spheroid surface area and number of cells per spheroid. 113 spheroids of control and 94 spheroids of WSE treated from 2 biological experiments were analysed. Statistical analysis was done using Mann Whiney test (**** p<0.0001)

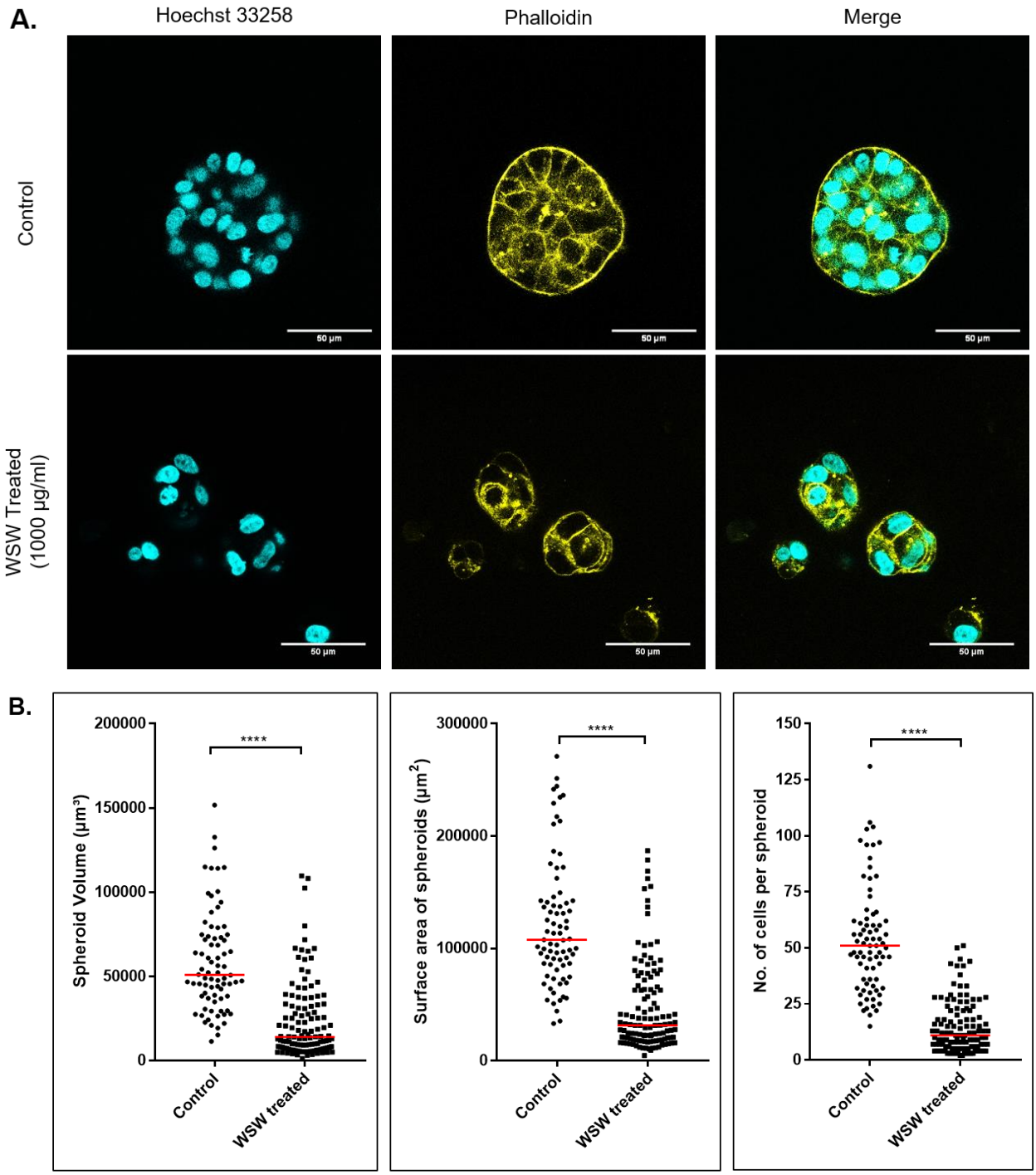


Figure 13: **MCF7 spheroids reduce in size upon treatment with *Withania somnifera* water (WSW) extract.**
A) Representative image of MCF7 3D spheroid showing reduction in size. B) Morphometric analysis showing reduction in parameters like spheroid volume, spheroid surface area and number of cells per spheroid. 78 spheroids of control and 124 spheroids of WSW treated from 2 biological experiments were analysed. Statistical analysis was done using Mann Whiney test (**** $p<0.0001$)

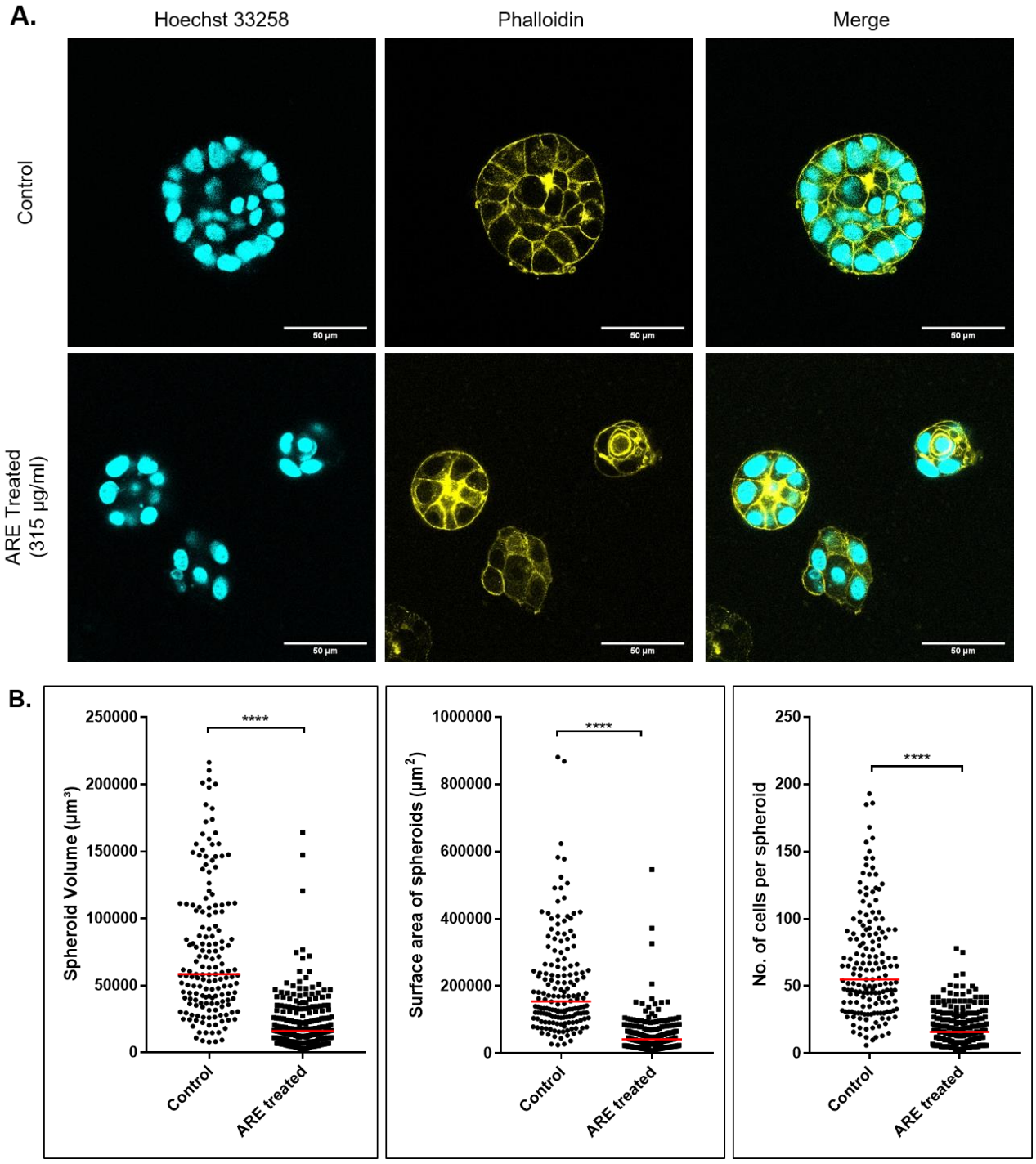


Figure 14: **MCF7 spheroids reduce in size upon treatment with *Asparagus racemosus* ethanolic (ARE) extract.**
A) Representative image of MCF7 3D spheroid showing reduction in size. B) Morphometric analysis showing reduction in parameters like spheroid volume, spheroid surface area and number of cells per spheroid. 168 spheroids of control and 238 spheroids of ARE treated from 3 biological experiments were analysed. Statistical analysis was done using Mann Whiney test (**** $p<0.0001$)

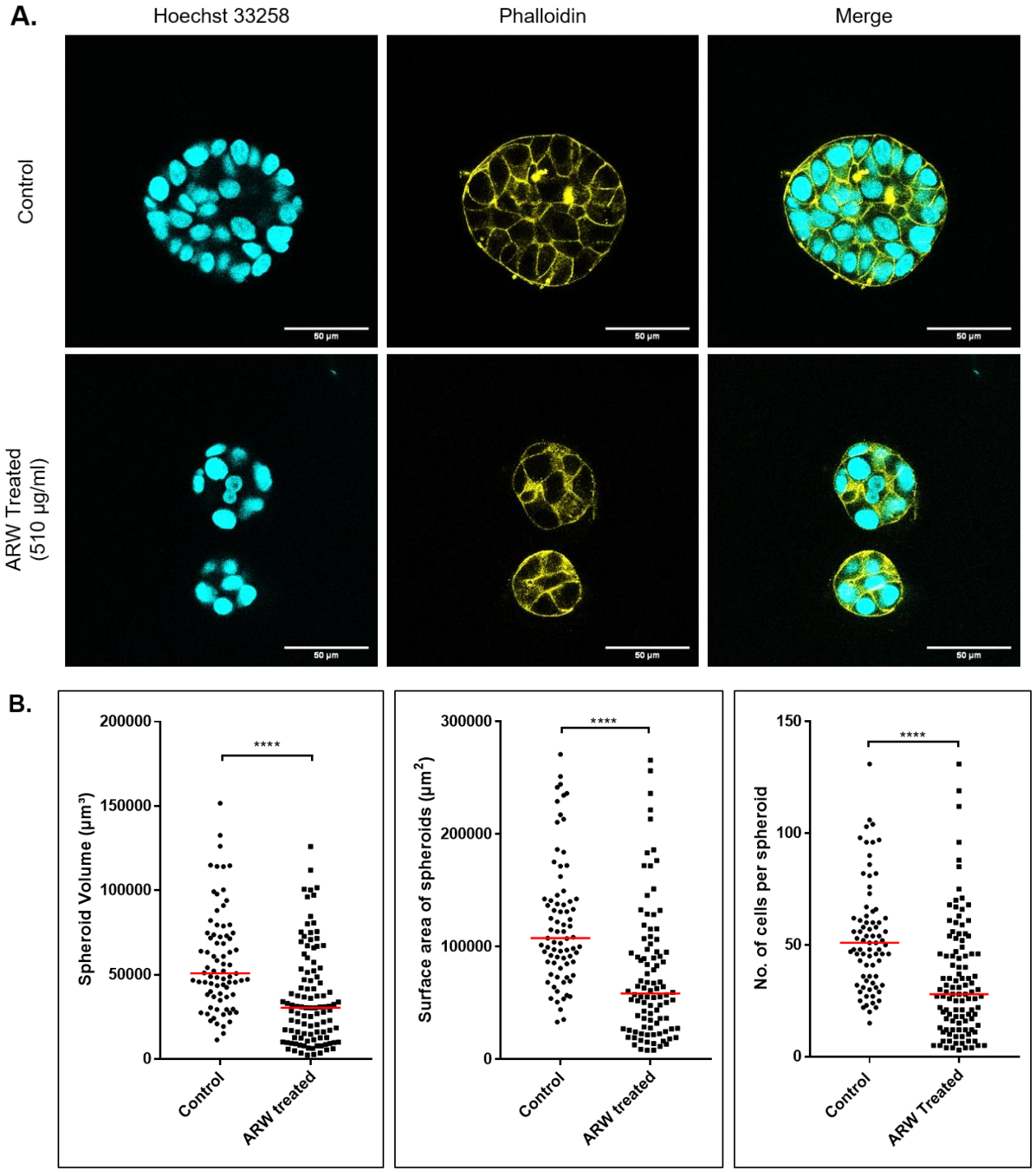


Figure 15: **MCF7 spheroids reduce in size upon treatment with *Asparagus racemosus* water (ARW) extract.**
A) Representative image of MCF7 3D spheroid showing reduction in size. B) Morphometric analysis showing reduction in parameters like spheroid volume, spheroid surface area and number of cells per spheroid. 78 spheroids of control and 108 spheroids of ARW treated from 2 biological experiments were analysed. Statistical analysis was done using Mann Whiney test (**** p<0.0001)

Morphometric analysis of MDA-MB-231 3D cultures using Immunofluorescence (IF) staining

To evaluate and quantify the effect of DOX, WSE, WSW, ARE and ARW in 3D cultures of MDA-MB-231, the cultures were fixed on day 7 and subjected to IF assay, as mentioned in Materials and Methods. The staining was performed using Phalloidin Alexa Fluor 488. Nuclei were counterstained with Hoechst 33258. Doxorubicin (positive control) showed reduction in spheroid size (Fig. 16).

Similarly, MDA-MB-231 spheroids treated with WSE showed reduction and disruption of spheroids (Fig. 17). WSW treatment showed reduced spheroids (Fig. 18).
ARE treated cultures also showed significant reduction in size of the spheroids. There were many spherical structures with one or two cells that were not quantified as spheroids (Fig. 19). ARW treatment completely disrupted the 3D structures of MDA-MD-231. Many spherical structures with one nucleus were observed (Fig. 20).

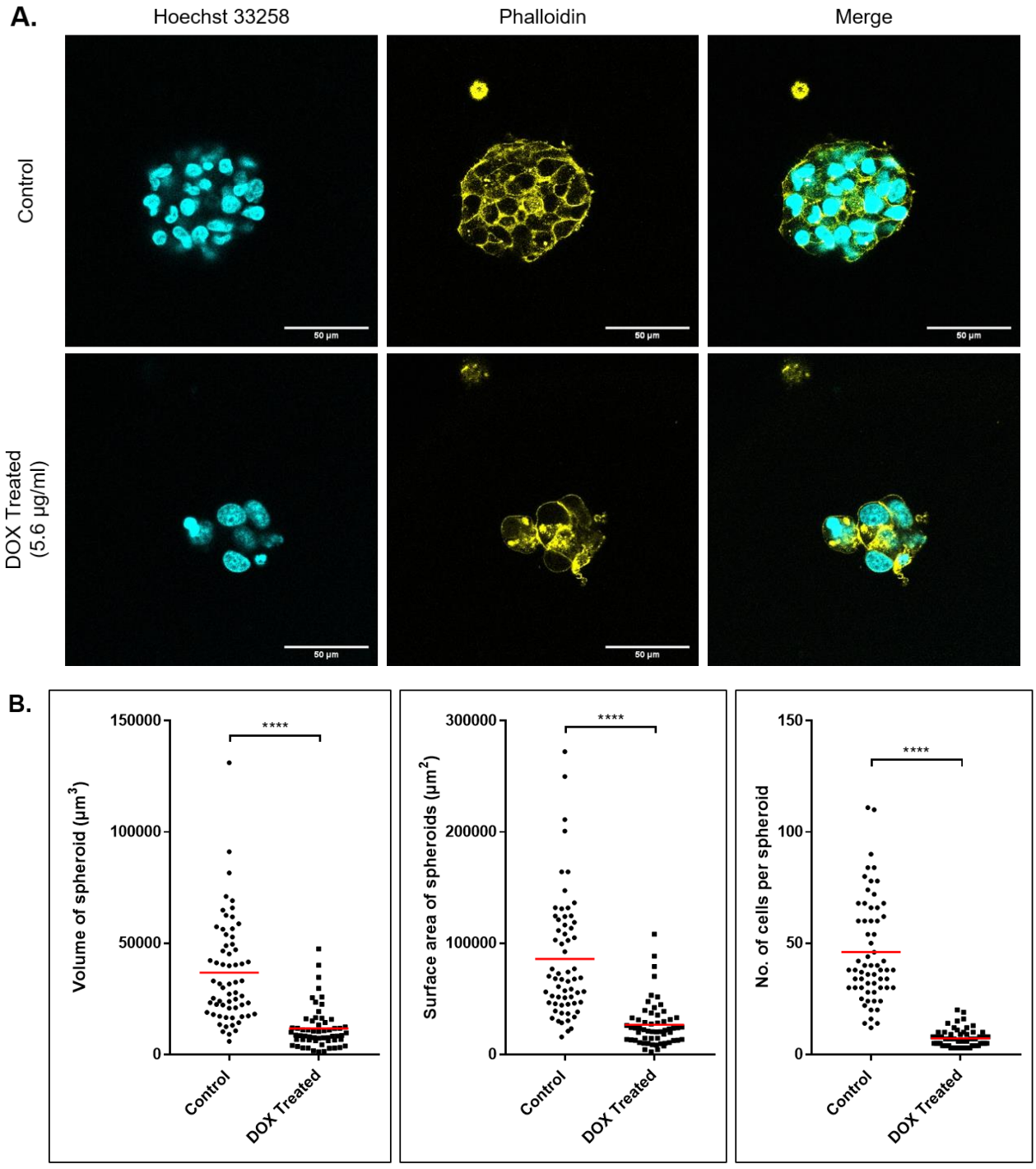


Figure 16: **MDA-MB-231 spheroids get disrupted and reduce in size upon treatment with Doxorubicin (DOX).**
A) Representative image of MDA-MB-231 3D spheroid showing reduction in size. B) Morphometric analysis showing reduction in parameters like spheroid volume, spheroid surface area and number of cells per spheroid. 63 spheroids of control and 59 spheroids of DOX treated from 1 biological experiment were analysed. Statistical analysis was done using Mann Whiney test (**** $p<0.0001$)

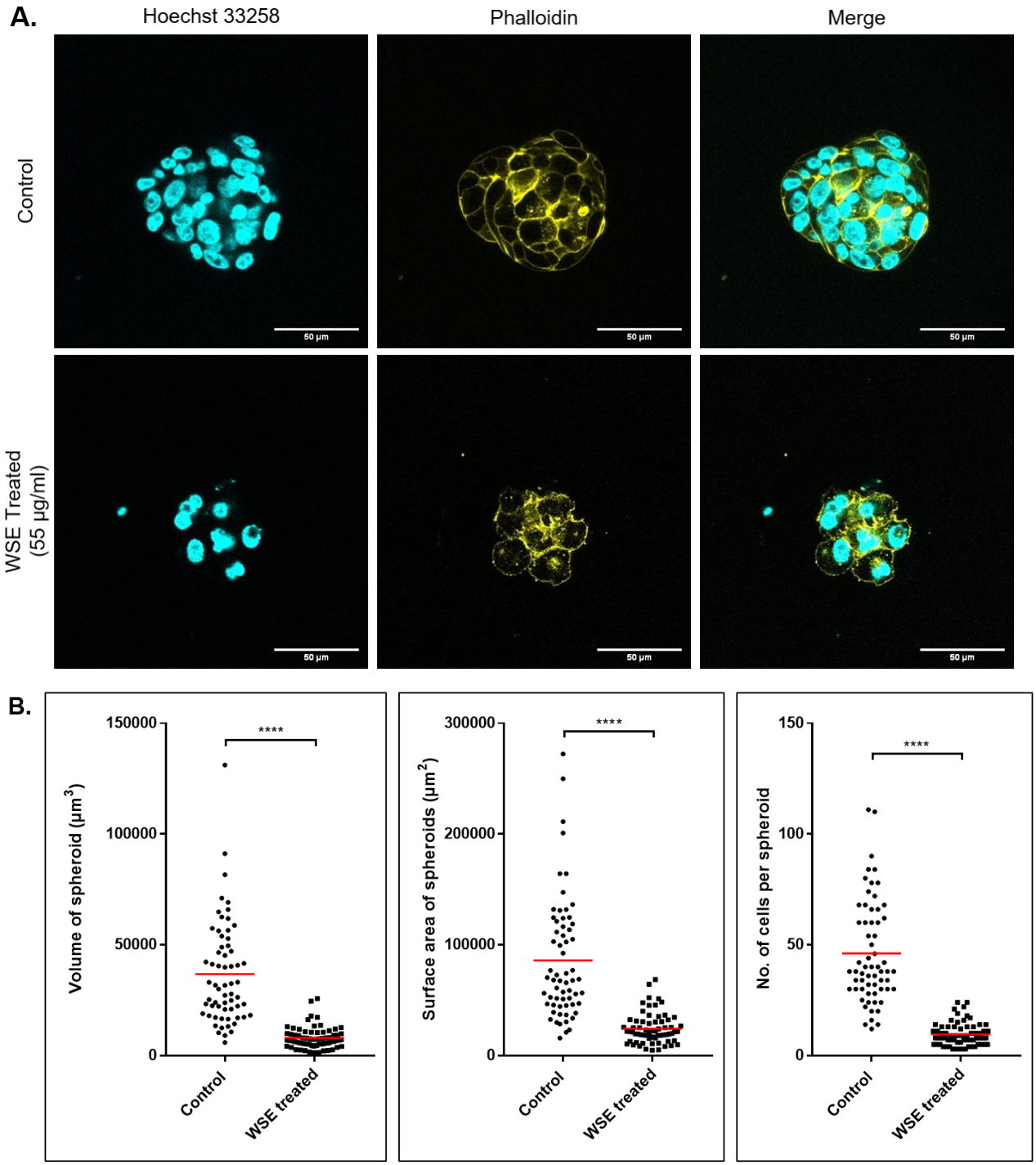


Figure 17: **MDA-MB-231 spheroids get disrupted and reduce in size upon treatment with *Withania somnifera* ethanolic (WSE) extract.**
A) Representative image of MDA-MB-231 3D spheroid showing reduction in size. B) Morphometric analysis showing reduction in parameters like spheroid volume, spheroid surface area and number of cells per spheroid. 63 spheroids of control and 68 spheroids of WSE treated from 1 biological experiment were analysed. Statistical analysis was done using Mann Whiney test (**** p<0.0001)

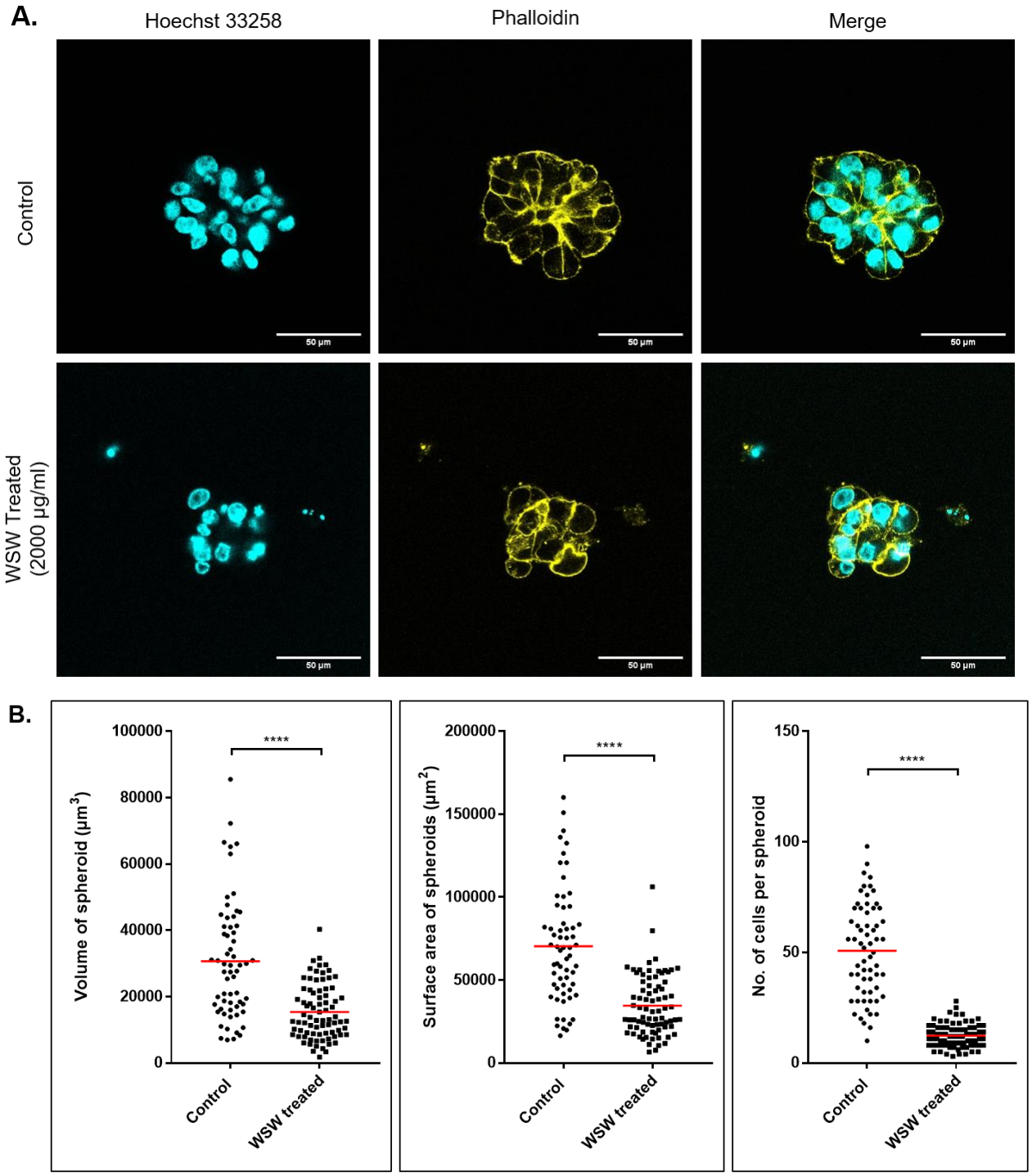


Figure 18: **MDA-MB-231 spheroids get disrupted and reduce in size upon treatment with *Withania somnifera* water (WSW) extract.**

A) Representative image of MDA-MB-231 3D spheroid showing reduction in size. B) Morphometric analysis showing reduction in parameters like spheroid volume, spheroid surface area and number of cells per spheroid. 62 spheroids of control and 79 spheroids of WSW treated from 1 biological experiment were analysed. Statistical analysis was done using Mann Whiney test (**** p<0.0001)

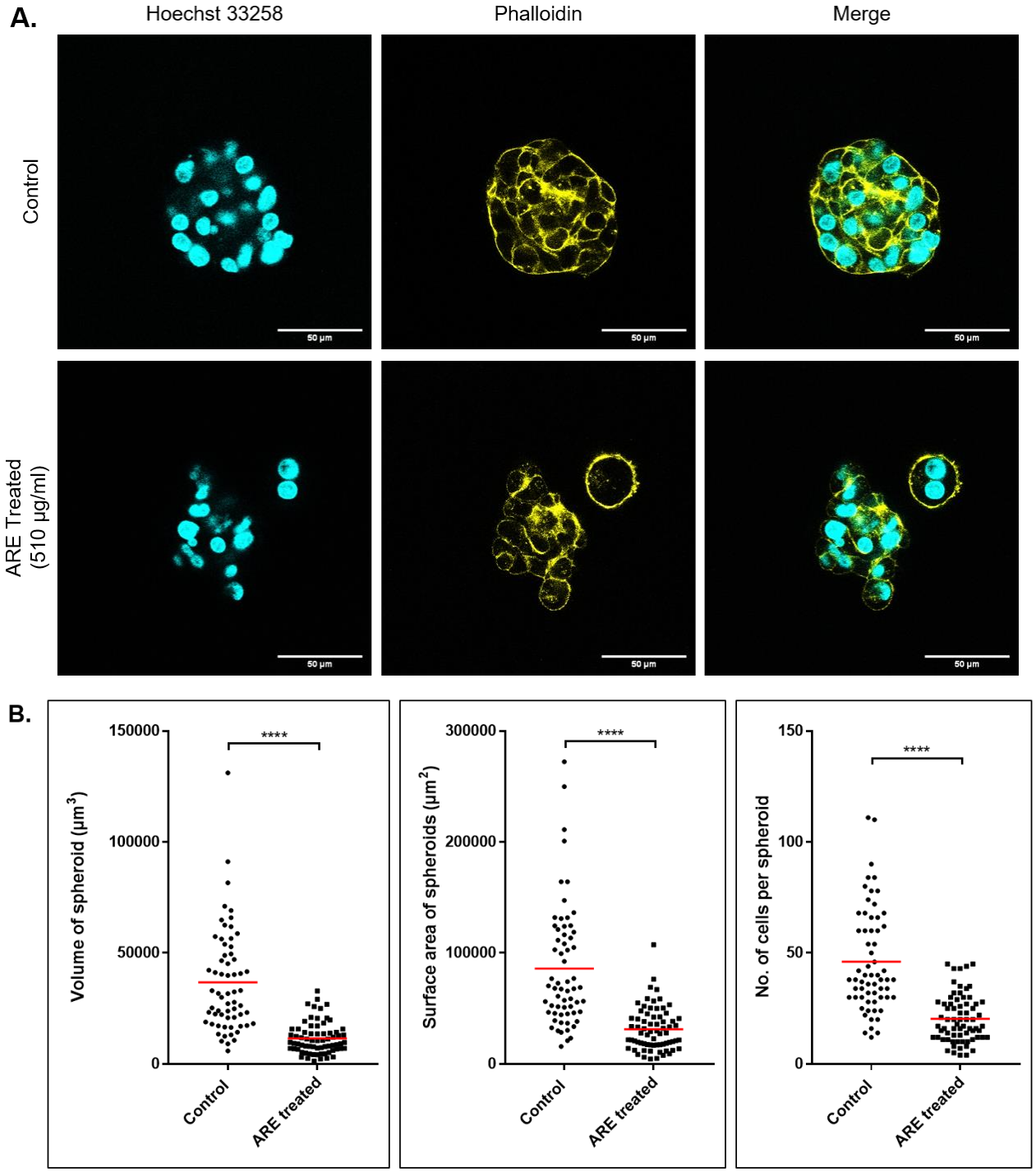


Figure 19: **MDA-MB-231 spheroids get disrupted and reduce in size upon treatment with *Asparagus racemosus* ethanolic (ARE) extract.**
A) Representative image of MDA-MB-231 3D spheroid showing reduction in size. B) Morphometric analysis showing reduction in parameters like spheroid volume, spheroid surface area and number of cells per spheroid. 63 spheroids of control and 70 spheroids of ARE treated from 1 biological experiment were analysed. Statistical analysis was done using Mann Whiney test (**** $p<0.0001$)

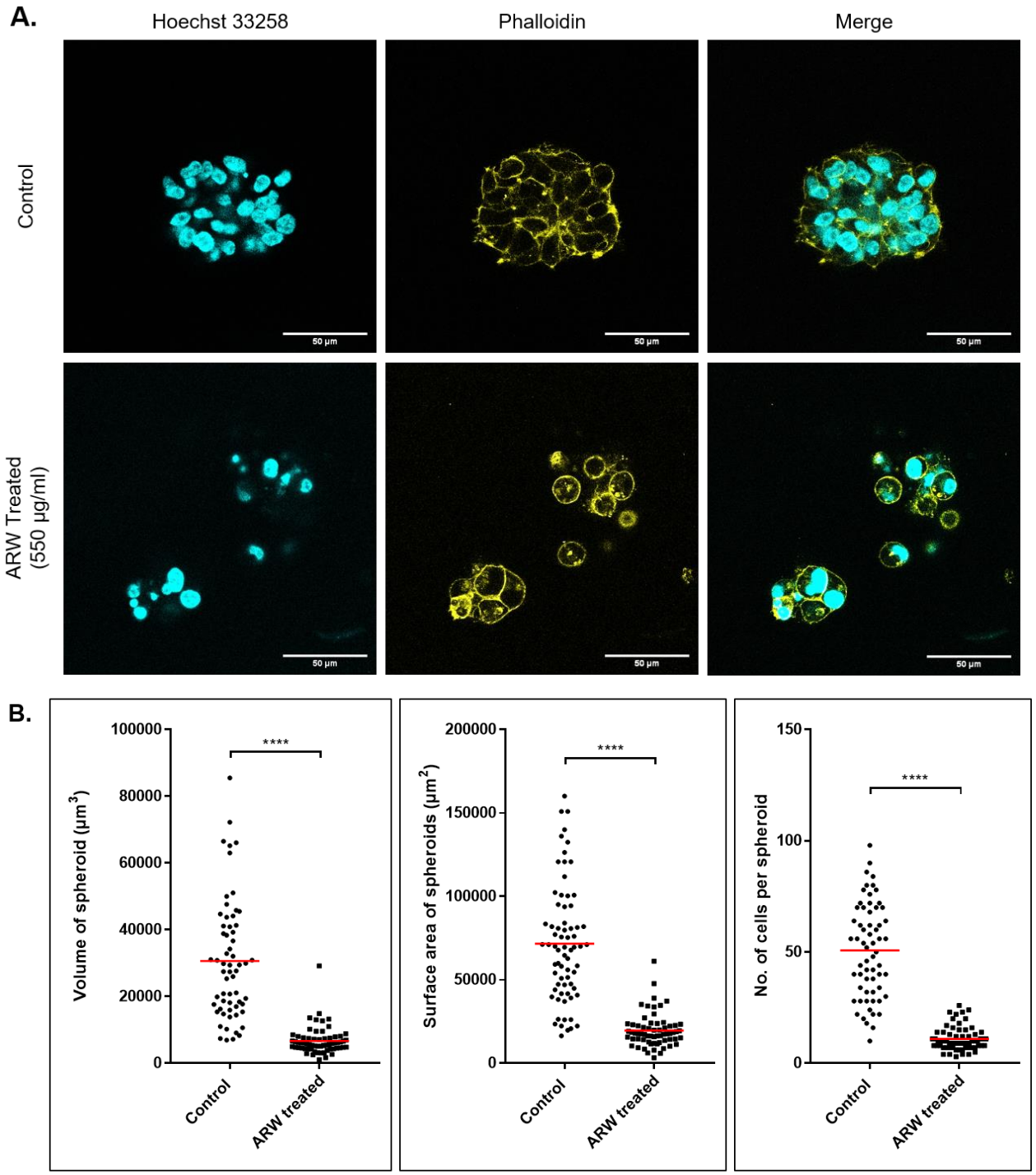


Figure 20: **MDA-MB-231 spheroids get disrupted and reduce in size upon treatment with *Asparagus racemosus* water (ARW) extract.**

A) Representative image of MDA-MB-231 3D spheroid showing reduction in size. B) Morphometric analysis showing reduction in parameters like spheroid volume, spheroid surface area and number of cells per spheroid. 63 spheroids of control and 70 spheroids of ARE treated from 1 biological experiment were analysed. Statistical analysis was done using Mann Whiney test (**** p<0.0001)

Discussion

Breast cancer is one of the foremost causes of mortality in women. A tumor mass is a heterogeneous population of cells that are capable of rapidly changing its signaling according to the cues in its microenvironment. Reduced apoptosis or resisting apoptosis is shown to play a role in carcinogenesis (Hanahan, Weinberg and Francisco, 2000). Oncologists all over the world face the problem of multi-drug resistance in their patients. Upon recurrence or relapse of cancer, the treatment becomes even more difficult as cancer cells develops resistance to drugs that have previously been used for their treatment (Lovitt, Shelper and Avery, 2018). It has become essential to develop novel therapies to target cancer and avoid relapse effectively. There are various treatments available for breast cancer. Oncologist use these drugs individually or in combination with other drugs to enhance the effect of treatment. Along with their anti-cancer activity, these drugs pose harm to healthy cells too. Hence complementary and alternative therapies for cancer treatment have become an emergent field (Garodia *et al.*, 2007).

Ayurvedic medicine use plant extracts for the treatment of various diseases. In Ayurvedic literature, there is a classification of plants based on their activity or effect on the human body. One such classification is “Rasayana,” which includes plants that strengthen the immune system of an individual. *Withania somnifera* and *Asparagus racemosus* fall under this category (Balachandran and Govindarajan, 2005; Diwanay, Chitre and Patwardhan, 2004).

Withania somnifera and *Asparagus racemosus* extracts were prepared in both aqueous and organic solvents to determine which solvent would be ideal and more effective in killing the cancer cells. Literature had already mentioned the anticancer effects of these extracts, but none of them have shown using 3D culture models. Monlolayer cultures fail to mimic the structure and signaling happening in the tissue. Culturing cancer cells on Matrigel forms 3D spheroids which resembles the *in vivo* tumor mass. To overcome this challenge and to better recapitulate the drug responce, we use 3D cultures of breast cancer cells as a platform for drug evaluation. We chose MCF7, which is an ER^+ breast cancer cell line and MDA-MB-231, which is an aggressive TNBC cell line for this study. The IC_{50} was determined in monolayer cultures, and we went on to use that dose in 3D

cultures of the two cell lines.

Upon treatment with WSE, the MCF7 and MDA-MB-231 spheroids significantly reduced in size which was quantified and analyzed using morphometry. We could observe that cells of the treated spheroids showed fragmented nuclei. This is a known morphological phenotype of apoptotic cells (Wong, 2011). Further studies need to be done to confirm the our observation. EtBr staining or Annexin stain are few assays that can be used quantify the phenotype. Ethidium Bromide is a cell-impermeable, fluorescent, DNA-binding dye. It is taken up by cells which have ruptured membrane. Fluorescently labeled Annexin V can be used to detect phosphatidylserine (PS) that is exposed on the outside of early-apoptotic or necrotic cells. Unlike the ethanolic extract of *Withania somnifera*, the aqueous (WSW) extract treatment showed different responses in both the cell lines. MCF7 cells treated with WSW showed significant reduction in spheroid size upon quantification but, one interesting observation was the presence of multiple single-cell structures upon treatment with WSW. These structures were small, spherical and contained one or two nuclei. A similar observation was seen in a study done by Lovitt, Shelper and Avery, (2018) where they showed that treatment using a very high dose of Doxorubicin in 3D cultures of MCF7 and MDA-MB-231 lead to complete deterioration of the spheroids, resulting in similar single-cell structures. In MDA-MB-231 spheroids treated with WSW, we observed two significant phenotypes, reduction in size and disruption of cell structure. The cells of the WSW-treated spheroids did not look like that of the control. The actin staining around each cell of WSW treated MDA-MB-231 showed irregular structure. In a study by Malik *et al.*, 2009, a mechanism by which *Withania* may induce apoptosis was proposed. The study showed HL-60 (Colon cancer cell line) cells treated with *Withania somnifera* extract enhanced the production of Nitric oxide species (NOS). This leads to disturbance of the mitochondrial membrane potential which leads to release of cytochrome c. Therefore *Withania* may be inducing apoptosis in breast cancer cells due to oxidative stress. The drawback of the referred study is that monolayer cultures do not recapitulate the 3D environment and signaling. Therefore, further studies need to be done to confirm this hypothesis. We need study various apoptosis pathway players to elucidate the mechanism of action of *Withania* in MCF7 and MDA-MB-231 3D cultures. In another study by Yang *et al.*, (2013), demonstrates that oral administration of *Withania* extract in MDA-MB-231 xenograft mice breast

cancer model reduces lung metastasis or lung nodule formation, which suggests *Withania* may play a role in preventing metastasis. Overall, literature and the data from the current study strongly suggest that *Withania somnifera* (both ethanolic and water) extracts possess anti-tumor or anti-cancer activity.

When MCF7 and MDA-MB-231 cells were treated with *Asparagus racemosus* ethanolic (ARE) extract, one could observe a significant decrease in the spheroid size of both the cell lines. A study done by Sharma and Jaitak, (2018) has shown similar results. They show that *Asparagus* methanolic extract has presence of compounds (phytoestrogens) that can target the ERα receptor on T47D cells in monolayer. These compounds have also been shown to have a cytotoxic effect on those cells. Similar to the ethanolic extract, water extract of *A.racemosus* (ARW) also showed a significant reduction in size of MCF7 treated spheroids. A different response was noted in the TNBC cell line MDA-MB-231. Upon treatment with ARW, the spheroids of MDA-MB-231 cells completely disintegrated. One could still see clumps of individual cells with intact cell membranes stained by phalloidin, but these clumps were not as tightly packed as that of control. Multiple studies have shown that triple-negative breast cancer cell lines are sensitive to TRAIL-mediated apoptosis (Rahman *et al.*, 2009). In a study carried out by Bousserouel *et al.*, (2013) show that the methanolic root extract of a different species of *Asparagus* induced apoptosis via the TRAIL-mediated pathway in colon cancer cell lines. This may be a plausible explanation to why we see a disintegration of structure when MDA-MB-231 spheroids are treated with ARW. This can be used as a starting point for further studies to elucidate the mechanism of action of *Asparagus racemosus* in TNBC cell line MDA-MB-231.

In conclusion, all the four extracts used in this study possess an anti-cancer effect on MCF7 and MDA-MB-231 cells grown as 3D spheroids. Many researchers have also shown their anti-tumor activity or cancer preventive nature in mice models (Rao, 1981; Khazal *et al.*, 2013). Hence they can be developed as potential drugs for treatment of breast cancer. These extracts have also been shown to possesses immunomodulatory activity. When *Withania* is administered in tumor-bearing mice, there is an upregulation in the immune response of the body (Diwanay, Chitre and Patwardhan, 2004). The use of a high dose of chemotherapy has various side effects in the body. Studies have

shown that oral administration of *Withania* for the long term does not have cytotoxic effect on the health of female Sprague-Dawley rats (Khazal *et al.*, 2013). Although, long term use of *Asparagus* has been shown to have negative impact on mammary gland and genitals of female albino rats (Pandey *et al.*, 2005). As these plant extracts have mechanisms to target cancer cells only, they can be used as adjuvant therapy in treating breast cancer. As they have anti-cancer activity, we may also be able to reduce the concentration of the cytotoxic drug/chemotherapy agent when used in combination with the extracts. For future studies, we would like to elucidate the exact mechanism by which the drugs are inducing apoptosis or how the spheroid size reduces in 3D. It would be interesting to check the combined effect of each of these extracts with doxorubicin and to elucidate the mechanism of cell death upon combination therapy.

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Response to evaluation report and comments

Supervisor's report 2: The dissertation written by Shrunal Mane is comprehensive and adequately addresses the problem that she set out to investigate. She was capable of standardising 3D culturing of cancer cells and the results that she obtained after extract treatment were very interesting. Will be great if she can check whether decrease in spheroid volume is due to apoptosis or some other phenomena.

Response: I will be checking for various cell death markers implicated in apoptosis, autophagy or necrosis cell death pathways using Western blotting.

Expert's report 2: The thesis is well written, has logical flow of information, experimental details, systematic evaluation of anti-proliferation effect of the extracts and critical analysis of data. Findings are very interesting. However, doses are very high. In my opinion, the thesis is complete in all respect. Therefore, in my opinion, the thesis is acceptable in its current form and the viva voice examination of the candidate should be held.

Response: I agree that as compared to the positive control (DOX), the IC50 of extracts are very high. It may be because these are crude plant extracts and not purified compounds. The secondary metabolites may be having effect on the main active compound rendering me to use high does of the whole extract. Also, as each extract are dissolved in organic (ethanol) and inorganic (water) solvent, may also affect the activity of the main compound. Further studies need to be done to purify the active compound and check the difference in the IC50 values.