Invitro Anticancer and Cytotoxicity Screening and Comparison of Ethanolic and Aqueous Extract From Ficus Religiosa (Peepal Tree) by Using Cancer Cell line Culture (Breast Cancer)

Vyas Grilesh Faculty of science, department of chemistry, pacific university, pacific hills, airport road, pratap nagar extension, debari, udaipur -313024, rajasthan E-mail: grileshvyas24@gmail.com

Dr. Paras Tak Faculty of science, department of chemistry, pacific university, pacific hills, airport road, pratap nagar extension, debari, udaipur -313024, rajasthan

Dr. Vishnu Suturiya – Shree dhanvantary pharmaceuticals analysis & research center, kim- 394110, gujarat

Dr. Mayank Mehta Chemistry department, p.h.g municipal arts & science college, kalol dist: gandhinagar, 382721,gujarat

Abstract

Herbs and Plants have been principle form of medicine in India since ancient times. Hundredof medicinal plants have been used to cure diseases. Medicinal plants played a dynamic role in manufacturing human health and improving the quality of human life for thousands of years. Medicinal plants have curative properties due to presence of various complex chemical substance of different composition. Medicinal plants have served humans well as valuable components of medicines, flavorings, beverages, cosmetics and dyes. Ficus religiosa is one of the medically important plants belonging to the family of Moraceae. It has been used broadly in Ayurvedic practitioner in India to treat various disease. The present work is an endeavor to compare the Invitro anticancer and cytotoxicity impact from different extracts of Ficus religiosa.

Key word: Ficus religiosa, MTT, Ethanol Extract, Water Extract, Cell Line

Introduction

Ficus religiosa is a large evergreen tree and commonly found growing in shrines and buildings. It is commonly known as peepal tree. It has a heart shape leaves. Ficus religiosa belongs to the family of Moraceae which consistent more than 700 species. Ficus religiosa steam, leaves, bark and roots are known for its medicinal properties and usuage as individual or whole plant. [12]. Ficus religiosa is used as Hypoglycemic activity, Hypolipidemic activity, Anti-Inflammatory, Anelgesic activity, Antimicrobial activity, Antiviral activity, Wound Healing Activity, Anticonvulsant activity, Antioxidant activity, Antiasthmatic activity, Immunomodulatory activity, Anti-ulcer activity, Anticancer activity, Anti acetylcholinesterase activity.[3] Ficus religiosa has a proven substance to use in Aryuveda and multiple therapeutic effect. The objective of this study is to evaluate the in-vitro cytotoxic activity (using MTT assay) of two different extracts from the leaves of Peepal (Ficus religiosa) tree on cancer cell line (using Breast Cell Line).

MATERIAL AND REAGENT

Fresh leaves of Ficus religiosa were collected during the month of May-June 2020 from Bharuch city, Gujarat India.The human breast cancer line MDA-MB-468 is an adherent cell line that was isolated in1977 from a 51-year-old female human with metastatic adenocarcinoma of breast, and is commonly used in breast cancer research. MDA-MB-468 cells were extracted from a pleural effusion of mammary gland and breast tissues, and have proven useful for the study of metastasis, migration, and breast cancer proliferation. This cell line is primarily Estrogen receptor (ER) negative but expresses Epidermal growth factor (EGF); Transforming growth factor alpha (TGF alpha) and Aryl hydrocarbon (Ah) receptors. This was obtain from the National Centre for Cell Science, Pune and stored at Ribosome Research Center Pvt. Ltd. Kim, Surat, Gujarat India.

Other Chemical like L-15 (Hi-Media Laboratories Pvt. Ltd), Fetal Bovine Serum (Hi-Media), Antibiotics Antimycotic Solution (Sigma-Aldrich), Trypsin EDTA solution (Sigma-Aldrich), DPBS

(Sigma-Aldrich), Thiazolyl Blue Tetrazolium (MTT) (Sigma-Aldrich), DMSO (Himedia), Trypan Blue (Logos) was used in the study.

METHODS

The general purpose of the MTT assay is to measure viable cells in relatively high throughput (96 well plates) without the need for elaborate cell counting. Therefore the most common use for to determine cytotoxicity of several drugs at different concentrations. The principle of the MTT assay is that for most viable cells mitochondrial activity is constant and thereby an increase or decrease in the number of viable cells is linearly related to mitochondrial activity. The mitochondrial activity of the cells is reflected by the conversion of the tetrazolium salt MTT into formazan crystals, which can be solubilised for homogenous measurement. Thus, any increase or decrease in viable cell number can be detected by measuring formazan concentration reflected in optical density (OD) using a plate reader at 570 nm. For drug sensitivity measurements the OD values of wells with cells incubated with drugs are compared with OD of wells with cells not exposed to drugs.

The MTT assay is suitable for the measurement of drug sensitivity in established cell lines as well as primary cells. For dividing cells (usually cell lines) the decrease in cell number reflects cell growth inhibition and the drug sensitivity is then usually specified as the concentration of the drug that is required to achieve 50% growth inhibition as compared to the growth of the untreated control (50% inhibitory concentration, IC50). For primary (nondividing) cells, drug sensitivity is measured as enhanced cell kill of treated cells as compared to the loss of cells already commonly seen in untreated cells. The experiment is designed based on different research paper review as per sr.no. 1 to 21.

Preparation of plant extract:

The plant leaves were air dried at room temperature and crushed in electric grinder to make powder. Powder size is less than 45 micron. The powder was successfully extracted throughSoxhlet Assembly by using Ethanol and HPLC water. Both extract was in brownish color. Both the extract was filtered through filter media by vacuum pump. After filtration, Ethanol extract and Water Extract was concentrated through Rotary Evaporator (Make-Heidolph) under reduced pressure. Extracted concentrated mass was stored at ambient temperature.

Method for MTT assay:

The MTT assay will be carried out with breast cancer cell line (MDA MB-468), which was cultured in Leibovitz's L-15 medium + 10% Fetal Bovine Serum (FBS) and 1% Antibiotic Antimycotic Solution (cL-15) 37 ± 0.5°C and 5 % CO2. The assay was carried out by treatingthe cell line with five (5) different concentrations of each test item (PLEE and PLWE) to find out the cytotoxicity or the anticancer activity. This result was useful to find out 50% inhibitory concentration (IC50) of the test item on cell line.

The vitality of the cells or potential cytotoxic effects of the test item was assayed by mitochondrial enzymes which reduce the MTT reagent to formazan, which is insoluble form; this was dissolved with a solubilizing reagent to quantify by measuring absorbance using a multi-mode plate reader at 570nm.

Determination of cytotoxicity through mtt assay:

Study design:

Total 9 samples was prepared for each extract to check the Cytotoxicity. Five different concentration of test sample was used in study as per table 1.

Table 1: Test Sample Information

Sample	Treatment	Test Concentration
S1	Blank	Test concentration was used between 300 ppmto 5000 ppm.
S2	Cell Control	
S3	Positive Control I	
S4	Positive Control II	

S5	Test item Concentration-1
S6	Test item Concentration-2
S7	Test item Concentration-3
S8	Test item Concentration-4
S9	Test item Concentration-5

Experiment Procedure:

The cells were removed from the culture flask by enzymatic digestion (Trypsin/EDTA) and centrifuged at 300 x g for 5min. The cell pellet was resuspended in complete L-15 media and3 x 104 cells in 100 µL of cL-15 was distributed to each test concentration wells, cell control wells and positive wells. For blank wells 100µL cL-15 only was distributed. Cells seeded plate was incubated for 48 h in 5 % CO2 incubator, at 37°C with > 90 % humidity.

After 80% confluent monolayer of MDA MB-468 cells were attained, the entire growth medium was aspirated from wells and 100 µL of fresh complete growth medium was added. Then 100µL of five different test concentrations of two different extracts (Ethanol and Water) and the reference items were added into labelled wells. For cell control wells and blank wells, 100 µL of fresh cEMEM were be added. The plate was then incubated for 66h in CO2 incubator (5% CO2, 37°C, > 90% humidity).

Post 66 hours of treatment, each wells were examined under the inverted phase contrast microscope to observe the cell growth and changes in the morphology of the cells due to cytotoxic effects of the test item. The entire volume from the each well was pipetted out, and replenished with fresh 100 µL of cL-15 media. From the MTT freshly prepared stock (5mg/mL) solution, 10 µL was added to each well to attain the final concentration of 0.5 mg/mL and the plate was further incubated for 4 h at 37 °C. After incubation, 100 µL from each well was decanted and 100 µL of DMSO was added and incubated for 20 minutes. Thecompletely dissolved blue-violet color solution in the each well was read by Multi-Mode plate reader at 570 nm.

RESULTS AND DISCUSSION

The uniform cells seeding in the each well was ensured and 80% confluence monolayer observed. Cell growth and morphology were observed from the day one till end of the experiment. All the observation was performed under the phase contrast inverted microscope.

The cytotoxicity activity of Ethanolic and Water extract was carried out by using MTT assay. MDA MB-468 cells were free from any kind of bacterial and fungal contamination. Percentage cell viability of the cell line was determined by using Trypan Blue dye exclusion technique. The % viability of MDA MB cell line was 74.7%, which was suitable to perform cytotoxicity study.

Result of Ethanolic Extract:

The MDA MB-468 cells were treated with five different test concentrations of the ethanolic extract and determined the percentage of cell viability at 5000 ppm (1.23%), 2500 ppm (20.86%), 1250 ppm (55.19%), 625 ppm (71.89%) and 312.5 ppm (87.96%). One-way ANOVA was analyzed with the t-test method for significance P-values determination. The significance in terms of P-values are $P \leq 0.0001$ (at 5000 and 2500 ppm), $P \leq 0.01$ (at 1250 ppm) and insignificant at the lower two concentrations (at 625 and 312.5 ppm) in comparison to the untreated cell control. The IC_{50} of the ethanolic extract was determined to be 1458 ppm.

Results of the absorbance readings (Mean± SD) and % Viability of MDA MB-468 cell line of the ethanolic extract solution are represented in the graphically represented in Figure 1 with One-way ANOVA (Analysis of variance) by GraphPad Prism. The normalized absorbance percentage plot (GraphPad Prism) is represented in Figure 2.

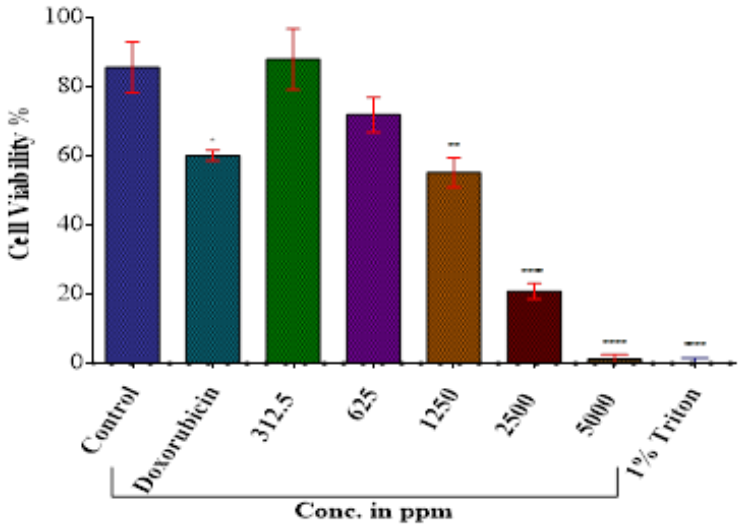


Fig.1: Results of the absorbance readings (Mean± SD) and % Viability of MDA MB-468 cell line of the ethanolic extract solution with One-way ANOVA (Analysis of variance) by GraphPad Prism

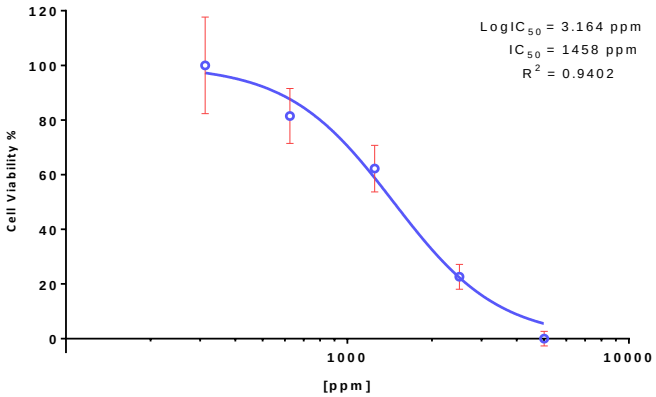


Fig.2: The normalized absorbance percentage plot of ethanolic extract

Result of Water Extract:

The MDA MB-468 cells were treated with five different test concentrations of the water extract and determined the percentage of cell viability at 5000 ppm (36.96%), 2500 ppm (41.92%), 1250 ppm (61.25%), 625 ppm (73.29%) and 312.5 ppm (71.58%). One-way ANOVA was analyzed with the t-test method for significance P-values determination. The significance in terms of P-values are $P \leq 0.001$ (at 5000 and 2500 ppm) and insignificant at the lower three concentrations (at 1250, 625 and 312.5 ppm) in comparison to the untreated cell control. The IC50 of the ethanolic extract was determined to be 1514 ppm.

Results of the absorbance readings (Mean± SD) and % Viability of MDA MB-468 cell line of the Water extract solution are represented in the below graphically represented in Figure 3 with One-way ANOVA (Analysis of variance) by GraphPad Prism. The normalized absorbance percentage plot (GraphPad Prism) is represented in Figure 4.

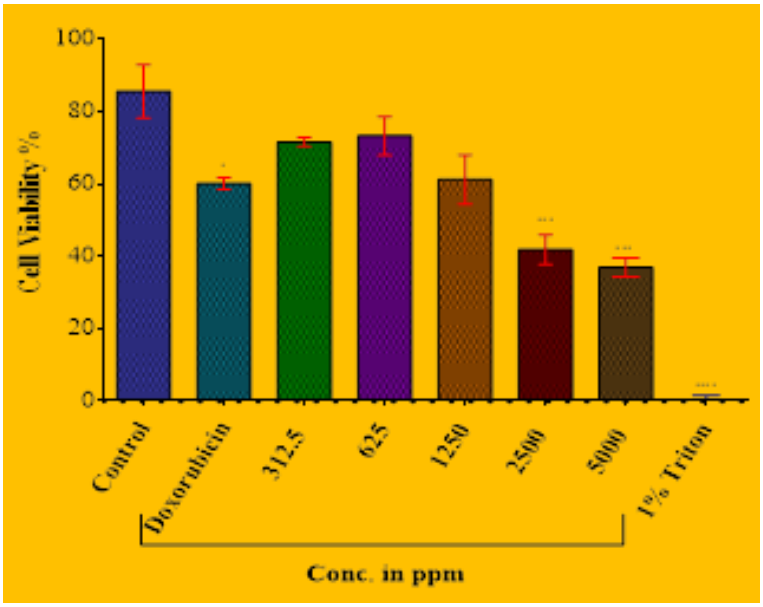


Fig.3: Results of the absorbance readings (Mean± SD) and % Viability of MDA MB-468 cell line of the Water extract solution with One-way ANOVA (Analysis of variance) by GraphPad Prism

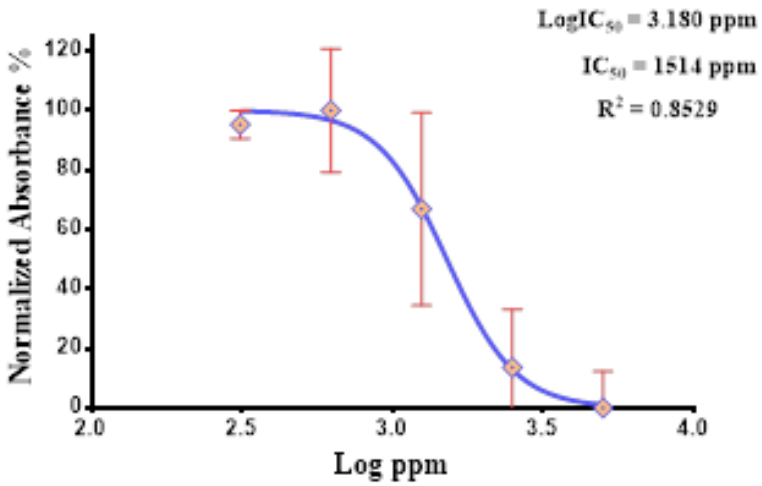


Fig.4: The normalized absorbance percentage plot of water extract

Summary of % Cell Inhibition:

% Cell Inhibition comparison summary of Ethanolic and Water extract in below table 2. and find the graphical presentation in Figure 5.

Table 2: %Cell Inhibition Summary

Sr. No.	Concentration inppm	EthanolicExtract	Water Extract
		% of Cell Inhibition	% of Cell Inhibition
1	5000	98.75	56.57
2	2500	75.61	50.17
3	1250	34.80	26.99
4	625	14.79	18.63
5	312.5	7.07	16.91

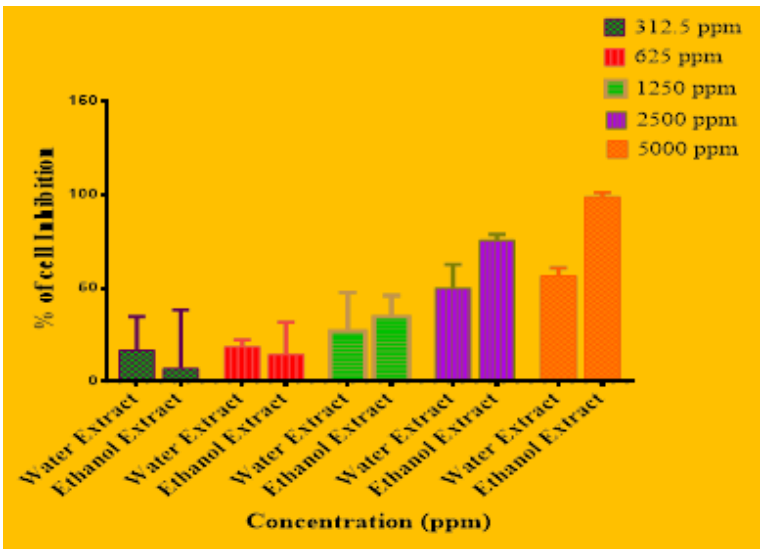
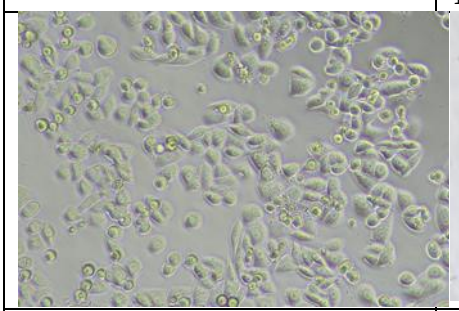


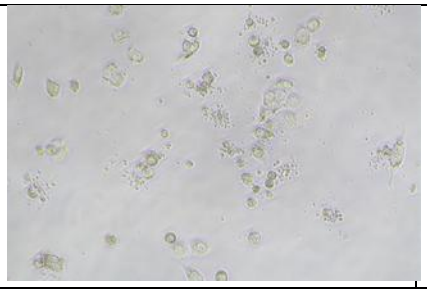
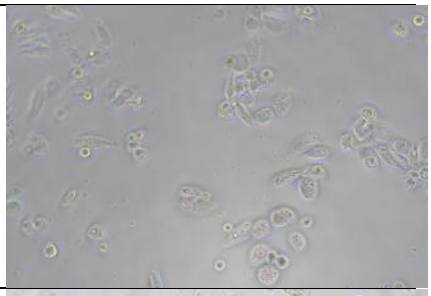
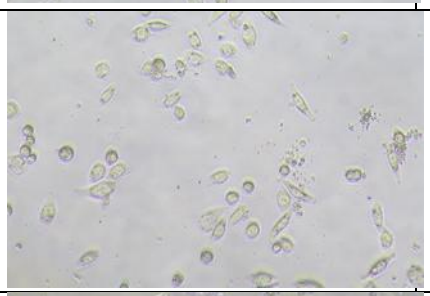
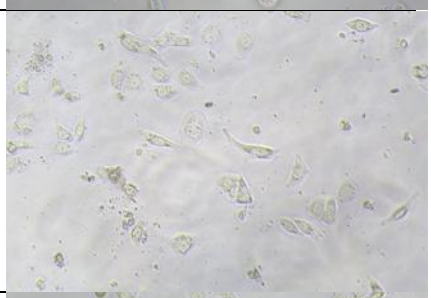
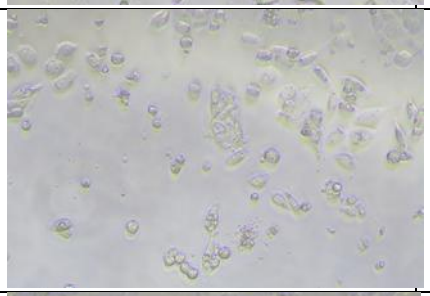
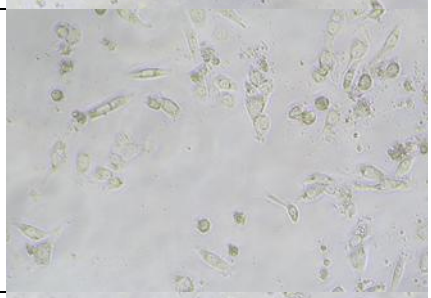
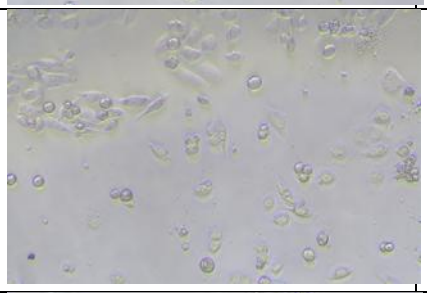
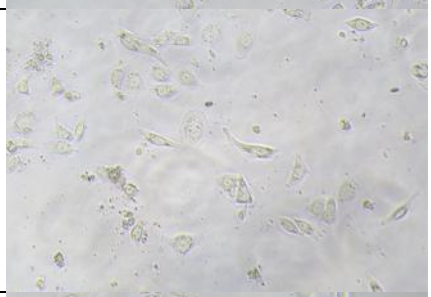
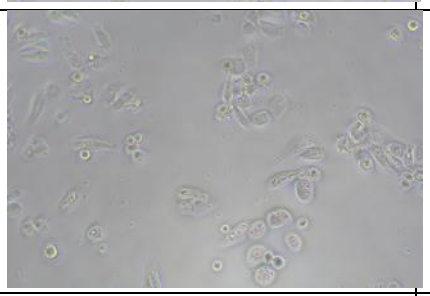
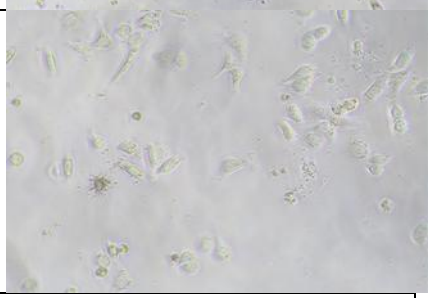
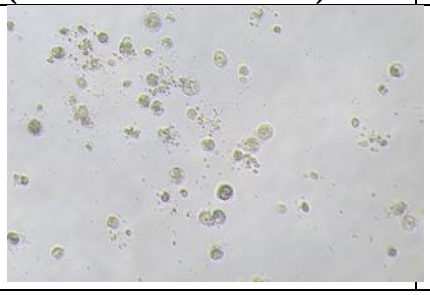
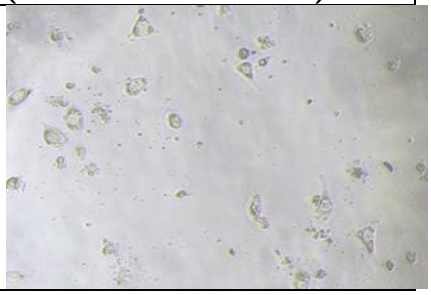
Fig.5: % Cell Inhibition comparison of Ethanolic and Water extract

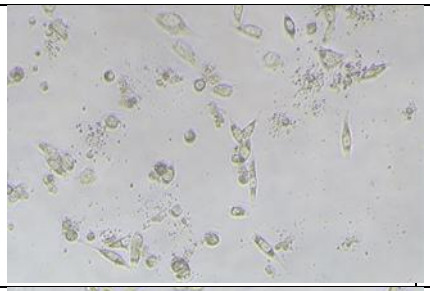
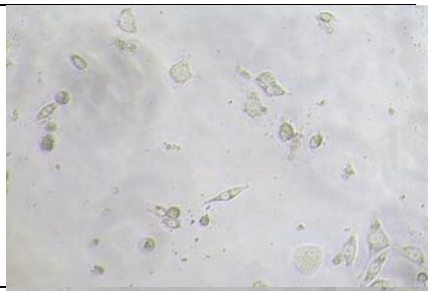
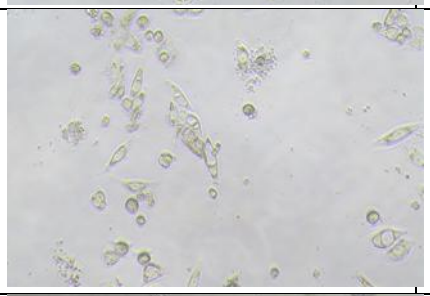
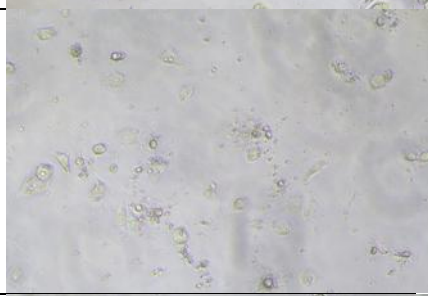
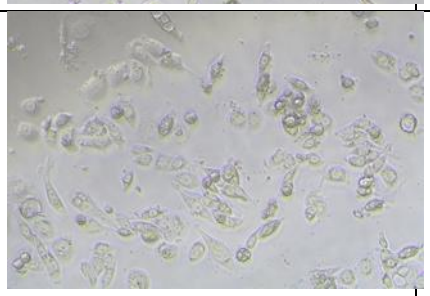
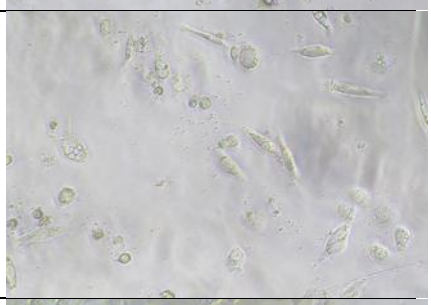
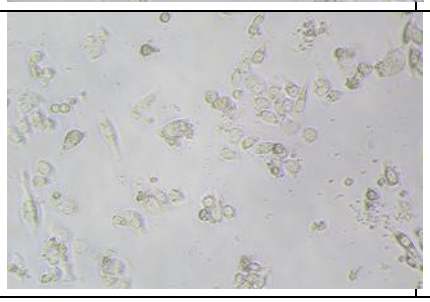
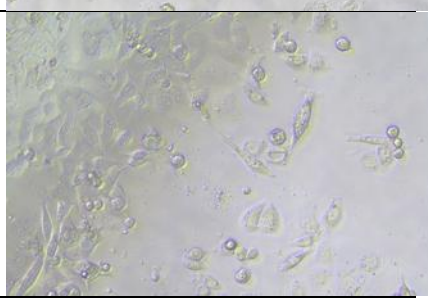
CONCLUSION

An MTT assay was performed to determine the anti-cancer activity of ethanolic extract and water extract with individual test items. Prominent morphological aberrations were clearly noticed, and this is indicative of cancer cell growth inhibition and cell death after 66 h with ethanolic extract when compared to the untreated (control) cell and water extract. The morphological image in below table 3.

Table 3: The Morphological Image of Ethanol and Water Extract

Control Cell	After 24 Hrs Treatment with Triton X 100	After 66 Hrs Treatment with Triton X 100
		
Concentration	**Ethanolic Extract (After 24 Hrs Treatment)**	**Water Extract (After 24 Hrs Treatment)**

5000 ppm		
2500 ppm		
1250 ppm		
625 ppm		
312.5 ppm		
Concentration	**Ethanolic Extract (After 66 Hrs Treatment)**	**Water Extract (After 66 Hrs Treatment)**
5000 ppm		

2500 ppm		
1250 ppm		
625 ppm		
312.5 ppm		

Ethanolic Extract:

An MTT assay was performed to determine the cytotoxic potential of the ethanolic extract onthe breast cancer cells. From the results in above section there was a clear dose-dependent response of the test item at the tested concentrations and was highly significant at the highest two dose whereas moderate at middle concentration and insignificant at the lowest two dose. It is evident that the ethanolic extract is more effective in causing cytotoxicity tothe ER –ve MDA MB-468 breast cancer cells as compared to the untreated cell control and its IC50 concentration was 1458 ppm.

Water Extract

Similarly, the cytotoxic potential of the water extract was determined on the breast cancer cells. From the results in above section there was a dose-dependent response of the test item at the tested concentrations and was moderately significant at the higher dose 5000 ppm and 2500 ppm whereas it was insignificant at subsequent lower concentrations It is evident that the Water extract is comparatively lesser effective in causing cytotoxicity to the ER –ve MDA MB-468 breast cancer cells as compared to the untreated cell control and its IC50 concentration was 1514 ppm.

As per above study and data comparison, conclude that Ethanolic extract is more effective compare to Water extract on breast cancer cells.

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