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Enhancement Activity of Anticancer and Cytotoxicity in Combination of Synthetic Drug and Ethanolic Extract From Ficus Religiosa (Peepal Tree) byUsing Cancer Cell line Culture (Breast Cancer)

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Abstract
Herbs and Plants have been the most important form of medicine in India since ancient times. the plant kingdom is a goldmine of potential drugs and active molecules. Medicinal plants played a vital role in manufacturing human health and improving the quality of human life for thousands of years. During last decade, use of traditional medicine has expanded globally and gained popularity. Medicinal plants have healing 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 diseases. The present work is an endeavor to improve the effectiveness of synthetic drug with combination with plant extracts.

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

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I. 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 usage as individual or whole plant. [1.]

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.[2] Ficus religiosa has a proven substance to use in Aryuveda and multiple therapeutic effect.

II. Objective of Research

The objective of this study is enhancement of the synthetic drug effectiveness with combination with plant ethanolic extract. To verify the effectiveness activity, perform the in-vitro cytotoxic activity (using MTT assay) of Synthetic drug, plant ethanolic extract from the leaves of Peepal (Ficus religiosa) tree and combination of both on cancer cell line (using Breast Cell Line).

III. 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 in 1977 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 DMEM (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), Cyclophosphamide was used in the study.

IV. Methods

4.1 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 through Soxhlet Assembly by using Ethanol. Plant extract was in brownish colour.Plant extract was filtered through filter media by vacuum pump. After filtration, Ethanol extract was concentrated through Rotary Evaporator (Make-Heidolph) under reduced pressure. Extracted concentrated mass was stored at ambient temperature.

4.2 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 treating the 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 (IC_{50}) of both test items and with or without combination of both test items to find out the anti cancer activity on 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.

4.3 Determination of Cytotoxicity through MTT assay

4.3.1 Study Design

Total 5 different concentrate sample was prepared for each test item (Synthetic Drug – Cyclophosphamide, Ethanolic Extract and combination of both) to check the Cytotoxicity.

Test Item 1 – Plant Ethanolic Extract (Table-1)

Sample	Treatment*	Test Concentration
S1	Blank	Final concentration between 2500 ppm to 156.25 ppm.
S2	Vehicle Control	
S3	Positive Control (Triton X-100)	
S4	Test item I Concentration-1	
S5	Test item I Concentration-2	
S6	Test item I Concentration-3	
S7	Test item I Concentration-4	
S8	Test item I Concentration-5	

Test Item 2 – Synthetic Drum – Cyclophosphamide (Table-2)

Sample	Treatment*	Test Concentration
S1	Blank	Final concentration 20 mM to 1.25 mM
S2	Vehicle Control	
S3	Positive Control (Triton X-100)	
S4	Test item II Concentration-1	
S5	Test item II Concentration-2	

S6	Test item II Concentration-3	
S7	Test item II Concentration-4	
S8	Test item II Concentration-5	

Test Item 3 – Combination of Plant Ethanolic Extract (Test Item 1) + Synthetic Drum (Test Item 2) **(Table-3)**

Sample	**Treatment***	**Test Concentration**
S1	Test item I Concentration-1 + Test item II Concentration-1	Final combination of concentration 2500 ppm + 20mM to 156.25 ppm +1.25 mM
S2	Test item I Concentration-2 + Test item II Concentration-2	
S3	Test item II Concentration-3 + Test item II Concentration-3	
S4	Test item I Concentration-4 + Test item II Concentration-4	
S5	Test item II Concentration-5 + Test item II Concentration-5	

4.3.2 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 DMEM media and 3 x 10^4 cells in 100 µL of cDMEM was distributed to each test concentration wells, cell control wells and positive wells. For blank wells 100µL DMEM only was distributed. Cells seeded plate was incubated for 24 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 Test Item 1 and 2 (Ethanolic extract and Cyclophosphamide) was added to respective wells. And for combination of test item 1 and 2, from each concentration 100 -100 µL taken from both test items and add torespective labelled wells. For cell control wells and blank wells, 100 µL of fresh cDMEM were be added. For reference items, 100 µL 1% Triton X -100 was added in respective cells. The plate was then incubated for 48h in CO2 incubator (5% CO2, 37°C, > 90% humidity).

Post 48 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 DMEM 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. The completely dissolved blue-violet color solution in the each well was read by Multi-Mode plate reader at 570 nm.

V. Results

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 extract and Cyclophosphamide 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 95%, which was suitable to perform cytotoxicity study.

5.1.1 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 2500ppm (15.1%), 1250ppm (41.7%), 625ppm (61.9%), 312.5ppm (85.5%) and 156.25ppm (92.6%). 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 2500, 1250 and 625ppm), and insignificant at the lower two concentrations (at 312.5 and 156.25ppm) in comparison to the untreated cell control. The IC50 of the ethanolic extract was determined to be 1458 ppm.

5.1.2 Result of Cyclophosphamide Extract

The MDA MB-468 cells were treated with five different test concentrations of tcyclophosphamide and determined the percentage of cell viability at 20 mM (15.6%), 10 mM (34.8%), 5 mM (81.1%), 2.5 mM (81.5%) and 1.25 mM (83.0%). 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 20 mM and 10 mM) and insignificant at the lower three concentrations (at 5, 2.5 and 1.25 mM) in comparison to the untreated cell control. The IC_{50} of the Cyclophosphamide was determined to be 8.526mm.

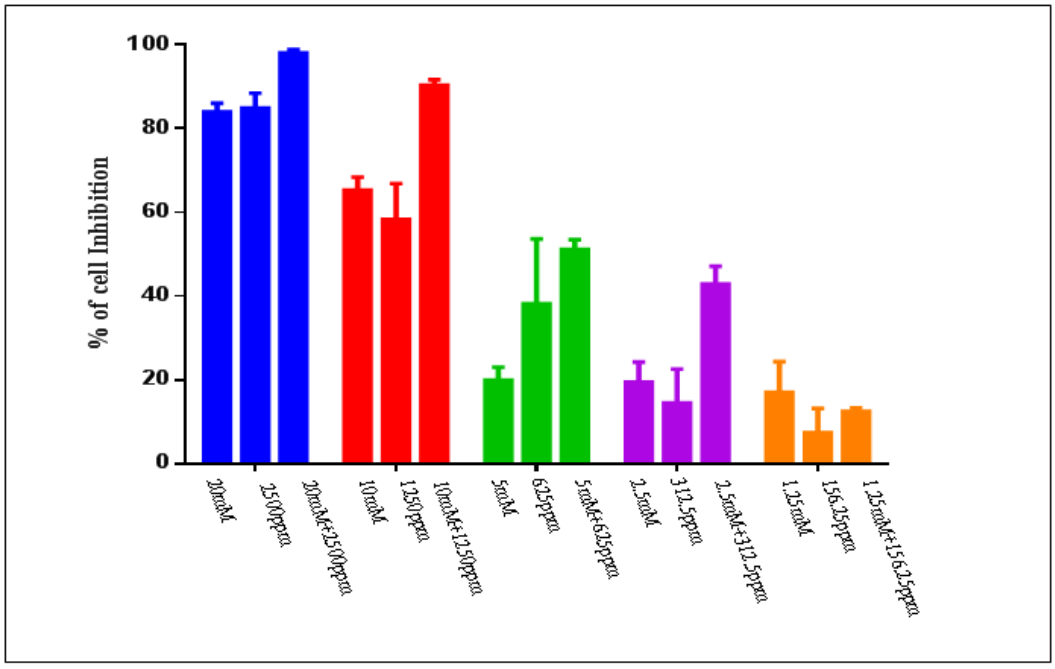
5.1.3 Results of Combination of Ethanolic extract and Cyclophosphamide:

The MDA MB-468 cells were treated with each combination of five different test concentrations of ethanolic extract and cyclophosphamide and determined the percentage of cell viability at 20 mM + 2500 ppm (1.9%), 10 mM + 1250 ppm (9.7%), 5 mM + 625 ppm (48.9%), 2.5 mM + 312.5 ppm (57.1%) and 1.25 mM + 156.25 ppm (87.5%). One-way ANOVA was analyzed with the t-test method for significance P-values determination. The IC_{50} of the Cyclophosphamide was determined with combination of ethanolic extract at 4.415mm.

5.1.4 Summary of % Cell Inhibition: (Table-4)

Sr. No.	Ethanolic Extract		Cyclophosphamide		Ethanolic Extract + Cyclophosphamide	
	Concentration in ppm	% of Cell Inhibition	Concentration in mM	% of Cell Inhibition	Concentration in ppm+mM	% of Cell Inhibition
1	2500	84.9	20	84.4	2500 + 20	98.1
2	1250	58.3	10	65.2	1250 + 10	90.3
3	625	38.1	5	19.9	625 + 5	51.1
4	312.5	14.5	2.5	19.5	312.5 + 2.5	42.9
5	156.25	7.4	1.25	17.0	156.25 + 1.25	12.5

Figure-1



VI. Discussion and Conclusion

An MTT assay was performed to determine the anti cancer activity of combination of ethanolic extract and cyclophosphamide compared with individual test items. Prominent morphological aberrations were clearly noticed, and this is indicative of cancer cell growth inhibition and cell death after 48 h of ethanolic extract with cyclophosphamide treatment when compared to the untreated (control) cell. From the results in above section there was a clear dose-dependent response of the ethanolic extract test item at the tested concentrations and was highly significant at the highest two dose where as moderate at middle concentration and insignificant at the

lowest two dose. For ethanolic extract, this results was found same compared to previous study of cytotoxicity by MTT (Ic 50 -1458 ppm). It is evident that the ethanolic extract is more 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 1458 ppm.

The anti cancer activity of cyclophosphamide was determined with five different concentration on the breast cancer cells. From the results in above section there was a dose-dependent response of the cyclophosphamide at the tested concentrations and was highly significant at the higher dose 20 mM and 10 mM whereas it was insignificant at subsequent lower concentrations. N Singh et al (2009) reported that the cyclophosphamide causing cytotoxicity to the MDA MB-231 and MCF-7 breast cancer cells and IC50 of CPA was 10 mM in MCF-7 cells and 12.5 mM in MDA-MB-231 cells. In combination of ethanolic extract with cyclophosphamide, results indicate that the significant observation at 20, 10, 5 and 2.5 mM of CPA in combination with 2500, 1250, 625 and 312.5 ppm ethanolic extract is more effective in inducing cytotoxicity in cells. Compared to individual treatment of test items, Combined doses induced more cytotoxicity at 20mM+2500ppm, 10mM+1250ppm, 5mM+625ppm and 2.5mM+312.5ppm (as per data mention point no. 5.1.4). Moreover, from the results showed that IC50 value of cyclophosphamide also decreased from 8.526 mM to 4.415mM with ethanolic extract combination.

Above result conclusion and data is indicate that ethanolic extract help to induced cytotoxicity with cyclophosphamide and increase the % inhibition. In other ward, Ethanolic extract + Cyclophosphamide combination is more effective with comparison of individual test item of Ethanolic extract and Cyclophosphamide.

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