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DIFFERENTIAL CYTOTOXICITY EVALUATION OF *AEGLE MARMELOS* (L.) LEAF EXTRACTS ON CANCER CELL LINES.

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ABSTRACT

Aegle marmelos (L.), a pharmacologically useful shrub is a native to central Asia. Due to the richness of therapeutically active ingredients, it holds significance in conditions such as cardiovascular diseases, osteoporosis, neurodegenerative disorders, cancer, and inflammatory disorders. These properties are attributed to the presence of various phytoconstituents present in the plant such as coumarins, alkaloids, tannins, phenols, flavonoids. This study focuses on studying the leaf extracts of Bael on different cell lines: MCF7, MDA-MB-231, HepG2, and A549. The cytotoxicity of the leaf extracts was studied against these cancer cell lines using MTT (3- [4,5- dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. The toxicity of the extracts against cancer cell lines was evaluated by determining the IC_{50} (Half maximal inhibitory concentration) value and the results were as follows, the IC_{50} against HepG2 cell line was 129.1µg/ml, for MDA-MB-231 it was 130.34 µg/ml, for A549 and MCF7 it was 239.5 µg/ml and 234.49 µg/ml respectively. This indicates the effectiveness of *Aegle marmelos* leaf extracts against liver, lung, and breast cancer cell lines. To verify the potency of this extract as an anti-cancer agent, the standard doxorubicin was used to compare the results. The evaluation of the extract shows the medicinal importance of this plant in cure of multiple diseases. It can be used as a combinatorial therapy with other drugs to verify the synergy in the cure of cancer.

Keywords: *Aegle marmelos* (L.), cytotoxicity, MTT, HepG2, MCF, MDA-MB-231, A549.

INTRODUCTION

Aegle marmelos (L.) is one of the most nutritionally packed therapeutic plant found in the Eastern ghats and Central India. This shrub belongs to the family of Rutaceae and its excellent medicinal properties are attributed to the varied phytochemicals and minerals present in them (Sharma et al., 2022). As per the Hindu mythology, Bael is a sacred plant with many pharmacological properties. It is a blend of tradition and medicine that makes its contribution significant to the field of ethnomedicine (Khanal & Kiran Dawadi, 2020). Different parts of this plant contain different phytoconstituents such as leaves that are rich in Marmesinin, Geline, Cuminaldehyde, Citral, Eugenol, Lupeol, Marmeline, fruits that are rich in imperatorin, tannin and psoralen, seeds that are rich in cineol, citral, citronellal (Monika et al., 2023). Coumarins, a phytoconstituent majorly found in plants of Rutaceae and Apiaceae family are polyphenolics

belonging to oxygenated heterocyclic compounds. These coumarins are categorized as: Simple coumarins, furanocoumarins, pyranocoumarins and pyrone substituted coumarins (Akkol et al., 2020).

Coumarins due to their pharmacologically significant role as anti-cancer, anti-inflammatory, anti- fungal, anti-viral, anti-malarial, neuroprotective, and anti-convulsant agent are the targets for medicinal therapies (Sharifi-Rad et al., 2021). These coumarins interfere with different processes in the cell such as apoptosis, cell cycle regulation, and angiogenesis that can lead to prevention of cancer. Coumarins regulate apoptosis through control over expression of apoptotic and anti- apoptotic proteins such as Bax, caspase-3 and Bcl2. Coumarins are also capable of altering expression of matrix metalloproteases (MMPs) and Vascular endothelial growth factors (VEGF) that aid in angiogenesis in case of cancer. p53 and MDM2 (Mouse double minute 2 homolog) are regulators of cell cycle. To treat cancer, it is important to destabilize the p53-MDM2 complex. Coumarins are used to develop compounds that can disturb these interactions and render p53 free for the regulation of cell cycle (Flores-Morales et al., 2023). To investigate the role of these coumarins in cancer, different cell line systems have been used. According to (Gupta et al., 2019) the derivatives of coumarins have shown anti-proliferative properties against HeLa, HepG2, HUVEC (Human umbilical vein endothelial cells) and SW620. Not only pure coumarins, but the hybrids of coumarins are used to study their anti-cancer activity on different cell lines. Coumarins- benzimidazole hybrids were studied using A549, H460, HT29, MKN-45, U87MG, and SMMC- 7721 cell lines. Coumarin-pyrazole hybrids were tested on HepG2 and MCF7 cell lines (Majnooni et al., 2019). Coumarins derived from *Rhizophora mucronata* leaves were tested on different cell lines like HeLa, HL-60, K562. These coumarins showed anti-proliferative effect against the cell lines with IC_{50} lower than the standard. This proved the potency of coumarins being anti- cancerous against various cell types (Majnooni et al., 2019).

MATERIALS AND METHODS

Collection of plant samples

The plant specimen was collected from Valsad with a longitude of 72.90953° or 72° 54' 34" east and latitude of 20.60839° or 20° 36' 30" north. The plant samples were collected in the month of April-May.

Collection of cell lines

The cell lines named HepG2, A549, MCF-7 and MDA-MB 231 were collected from NCCS, Pune, Maharashtra. The cell lines came with a report of contamination check and an authentication report with STR mapping.

Preparation of crude extract

The crude extract was prepared using the Soxhlet based extraction method. This Soxhlet based extraction is based on the continuous reflux and siphoning that helps in extracting the crude from the plant using appropriate solvent systems (Zhang et al., 2018). The extraction was performed using different solvent systems covering a range of non-polar to polar solvents. The solvents used were Petroleum ether, Diethyl ether, Methanol, Ethanol and Water. The extraction procedure started with the assembly of Soxhlet apparatus with the round bottom flask being fitted in the heating mantle and the temperature adjusted as per the boiling point of

the solvent systems used. The assembly of Soxhlet apparatus is shown in the image below:

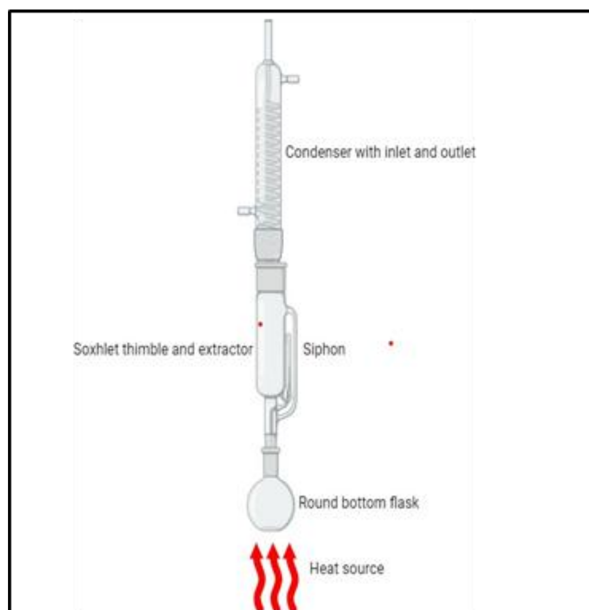


Figure 1: Soxhlet extractor for the extraction of crude from plant materials.

The powdered form of each plant sample was weighed to about 10 grams and packed in a muslin cloth then adjusted in the Soxhlet extractor. The volume of the solvents used for extraction was about 250ml (about 8.45 oz). The extraction with each solvent system was performed for about 35 cycles. The yield at the end of the extraction procedure was calculated using the following formula:

Yield Percentage (%) = (W2-W1/W0×100)

Where W0 = Weight of Initial Dried Sample
W1 = Weight of Blank Container
W2 = Weight of extract with Container Culturing the cells

The cell lines were cultured in complete DMEM (Dulbecco's minimal essential media) media (catalogue number: 10569010) with Fetal Bovine Serum (Catalogue number: 10270106) supplied by Gibco. These cell lines were procured at different passage numbers as mentioned in table below:

Sr. No.	Name of Cell line	Passage Number
1.	HepG2 (Hepatocellular carcinoma)	23
2.	A549 (Lung cancer)	54
3.	MCF7 (Breast cancer)	27
4.	MDA-MB (Breast cancer)	46

Table 1: Name of the cell lines used for cytotoxicity evaluation with their passage number.

The cell lines were checked every day for morphology, turbidity, and color change of the media to check for contamination and media change if required. The cells were cultured in T25 Flasks supplied by Corning. These cells were subcultured when 80% confluency was attained. The split ratio for the flask was determined using population doubling time as criteria. The enzymatic method was used for dislodging the cells at the time of subculture. 0.25% trypsin (Catalogue number: 25200-056) supplied by Gibco was used to break the adherence of cells from the surface of the flask. The flasks were incubated in humidified atmosphere at 37°C in CO_2 incubator (CellXpert® C170) by Eppendorf (Elufioye et al., 2017).

The cells were then seeded in 96 well plates (Tarsons) to perform MTT assay in triplicates and a single time point study.

Cytotoxicity Assay

The cytotoxicity evaluation of the samples was performed on the HepG2 (Hepatocellular carcinoma), A549 (Lung cancer), MCF-7 and MDA-MB 231 (Breast cancer) using MTT (3-[4,5- dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. The MTT assay is based on the fundamentals of conversion of MTT dye to formazan crystals. This conversion of MTT to formazan crystal is based on the activity of oxidoreductases that are present in mitochondria of living cells. The living cells are capable of this conversion. However, a dead cell does not possess active oxidoreductases and prevents conversion of MTT to formazan. The amount of formazan formed reflects the viability of cells. The amount of formazan formed is measured at 570nm using multiwell plate reader (Razak et al., 2019).

The MTT stock was prepared at a concentration of 1mg/ml. The leaf extract of *Aegle marmelos* (L.) was diluted from the stock and working solutions were prepared with the final concentrations of 50µg/ml, 100µg/ml, 250µg/ml, 500µg/ml, and 700µg/ml. The cells were counted using trypan blue assay and 1×10^5 cells/ml were seeded in 96 well plates. The cells were then treated with the leaf extracts of *Aegle marmelos* (L.) at working concentrations. After 24 hours of incubation, the media was discarded, and fresh media was added to the wells. MTT was added to the wells on day 3 at a volume of 10µl and incubated for 4 hours. Later the formation of formazan crystals was microscopically observed and DMSO (Dimethyl sulfoxide) was added to the wells at a volume of 100µl. The wells were then scanned using Epoch multimode reader (BioTek) at 570nm and readings were exported to Microsoft Excel for data analysis. The assay was performed in triplicates with 5 concentrations and single time point study (LI et al., 2015).

%Viability= Absorbance of test/Absorbance of control ×100

%Growth inhibition= 100-%Viability

Following formula was used to determine the % Viability and % Growth inhibition:

RESULTS

The cytotoxicity evaluation of *Aegle marmelos* (L.) leaf extracts on different cell lines was performed using MTT assay. The methanolic extracts of Bael were tested on cell lines and the results obtained are shown below. These results were compared to that of Doxorubicin, a

standard anti-cancer drug. IC_{50} (Half maximal inhibitory concentration) represents the concentration of the test compound at which 50% mortality results. The lower the value of IC_{50}, the more potency it holds. In case of Doxorubicin, IC_{50} was evaluated to be 99.11µg/ml. The IC_{50} for the compound on HepG2 and MDA-MB 231 was 129.1µg/ml and 130.34 µg/ml respectively which is close to that of Doxorubicin indicating the anti-cancer role of *Aegle marmelos* extracts against Liver and Breast cancer cell lines. However, the IC_{50} in case of A549 and MCF7 was 239.5 µg/ml and 234.49 µg/ml indicating their less potency to combat cancer. This indicates that Leaf extracts of *Aegle marmelos* do not work well with Lung cancer and primary breast cancer. All the results presented below as mean ± Standard deviation. Linear regression analysis was implemented to determine the value of co- relation co-efficient and to state $p<0.05$ to be statistically significant. The absorbance value along with % viability and % growth inhibition is shown in the table below. The equation is mentioned in the graph and IC_{50} value was determined using the equation where Y was substituted as 50 and Co-relation co-efficient is mentioned in the graphs below.

I) Cytotoxicity evaluation on MCF7 cell lines.

Concentrations (µg/ml)	Absorbance (570nm)	%Viability	%Growth Inhibition
50	0.65	71.42	28.58 ± 0.65
100	0.53	58.24	41.76 ± 0.57
250	0.42	46.15	53.85 ± 0.46
500	0.21	23.07	76.93 ± 1.69
700	0.14	15.38	84.62 ± 1.78

Table 2: Result for MTT assay on breast cancer cell line (MCF7)) showing % viability and % Growth inhibition.

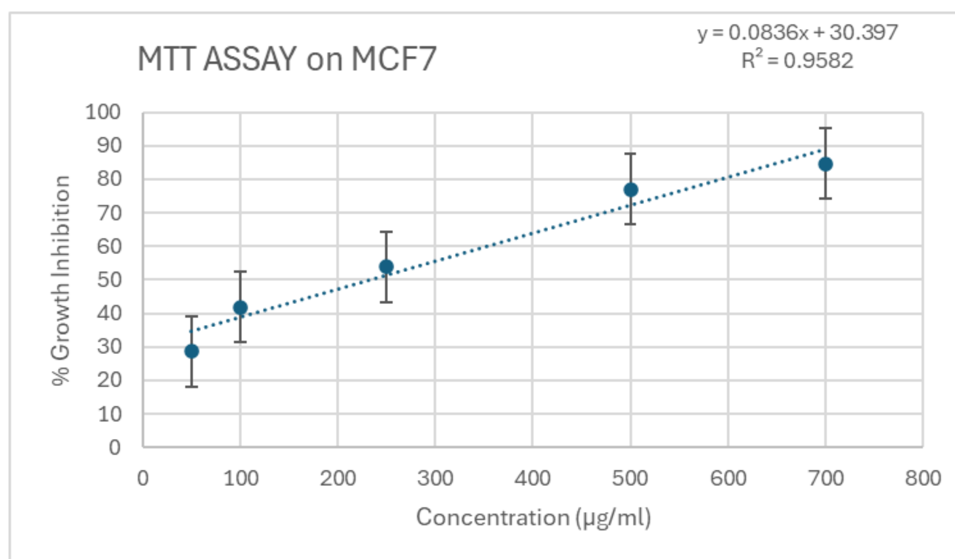


Figure 2: Graphical representation of MTT assay done on MCF7 cell line.

II) Cytotoxicity evaluation on HepG2 cell lines.

Concentrations (µg/ml)	Absorbance (570nm)	%Viability	%Growth Inhibition
50	0.58	63.74	36.26 ± 0.270
100	0.45	49.45	50.55 ± 0.32
250	0.32	35.16	64.84 ± 0.84
500	0.15	16.48	83.52 ± 0.51
700	0.08	8.79	91.21 ± 0.83

Table 3: Result for MTT assay on Liver cancer cell line (HepG2) showing % viability and % Growth inhibition.

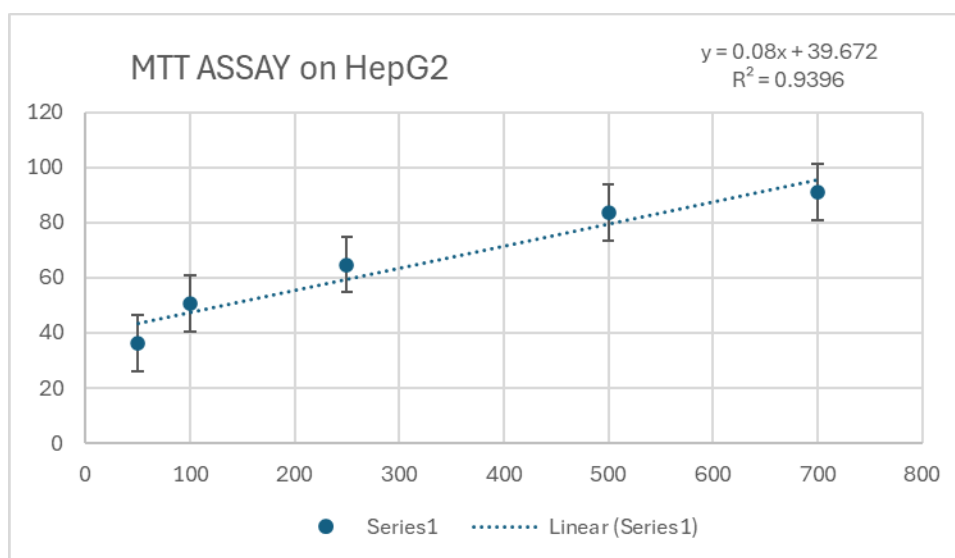


Figure 3: Graphical representation of MTT assay done on HepG2 cell line.

III) Cytotoxicity evaluation on A549 cell lines.

Concentrations (µg/ml)	Absorbance (570nm)	%Viability	%Growth Inhibition
50	0.61	67.03	32.97 ± 0.19
100	0.54	59.34	40.66 ± 0.24
250	0.48	52.74	47.26 ± 0.56
500	0.21	23.07	76.93 ± 0.26
700	0.13	14.28	85.72 ± 0.11

Table 4: Result for MTT assay on Lung cancer cell line (A549) showing % viability and % Growth inhibition.

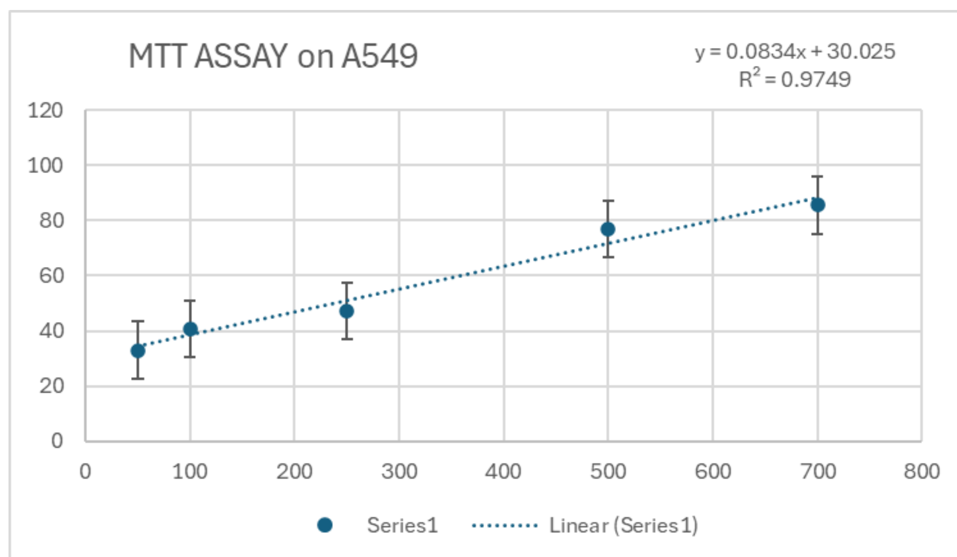


Figure 4: Graphical representation of MTT assay done on A549 cell line.

IV) Cytotoxicity evaluation on MDA-MB cell lines.

Concentrations (µg/ml)	Absorbance (570nm)	% Viability	% Growth Inhibition
50	0.54	59.34	40.66 ± 0.15
100	0.47	51.65	48.35 ± 0.98
250	0.34	37.36	62.64 ± 0.72
500	0.21	23.07	76.93 ± 1.23
700	0.11	12.08	87.92 ± 1.04

Table 5: Result for MTT assay on Breast cancer cell line (MDA-MB-231) showing % viability and % Growth inhibition.

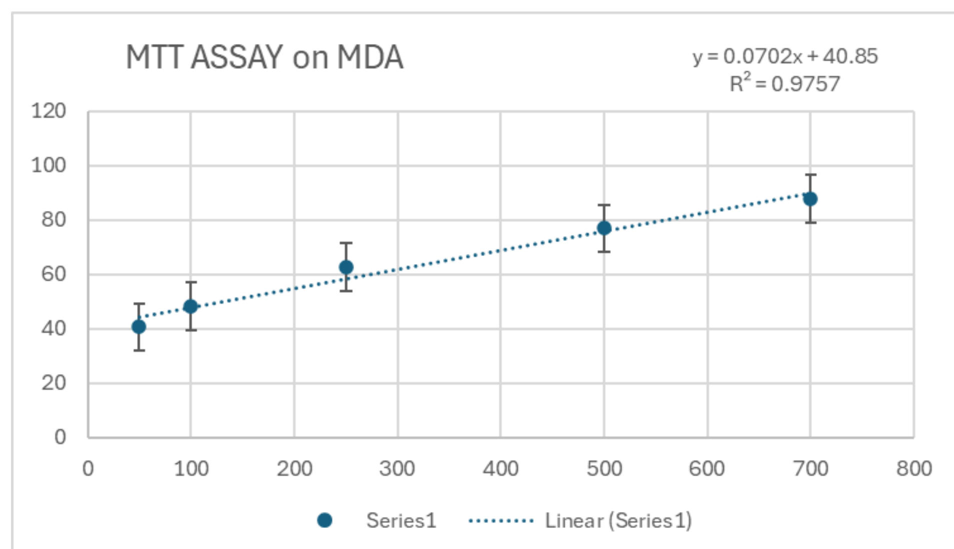


Figure 5: Graphical representation of MTT assay done on MDA-MB-231 cell line.

V) Cytotoxicity evaluation using Doxorubicin as standard on HepG2 cell lines:

Concentration (µg/ml)	Absorbance (570nm)	%Viability	%Growth Inhibition
50	0.52	57.14	42.86 ± 0.12
100	0.45	49.45	50.55 ± 0.22
250	0.32	35.16	64.84 ± 1.56
500	0.19	20.87	79.13 ± 1.23
700	0.09	9.89	90.11 ± 1.12

Table 6: Result for MTT assay using Doxorubicin showing % viability and % Growth inhibition.

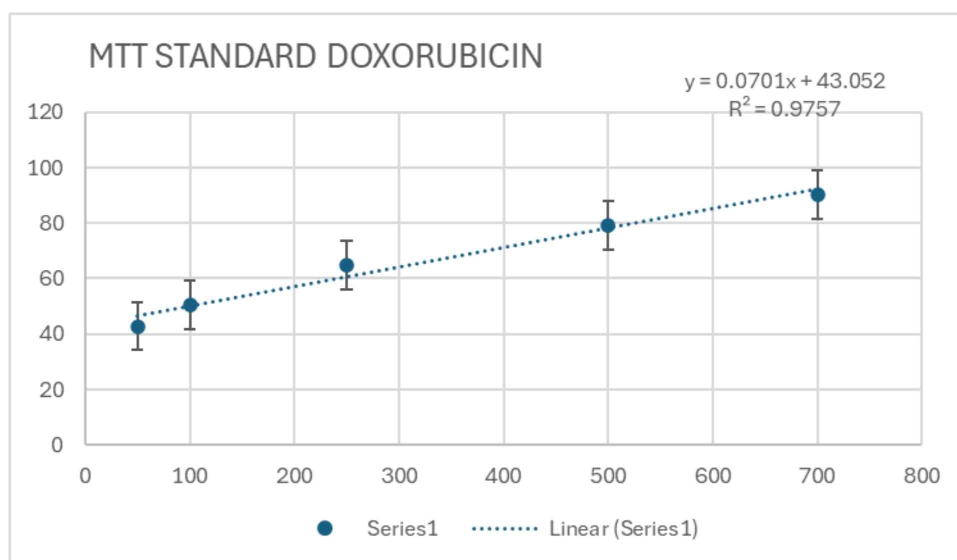
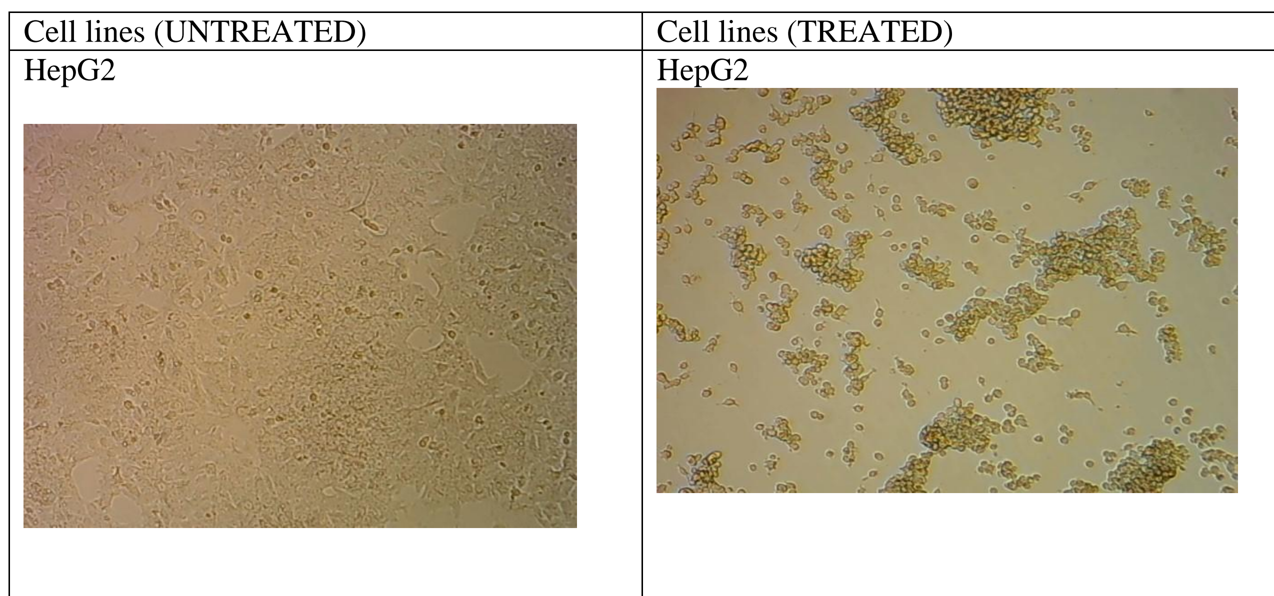


Figure 6: Graphical representation of MTT assay using Doxorubicin.

Cell lines (UNTREATED)	Cell lines (TREATED)
HepG2	HepG2



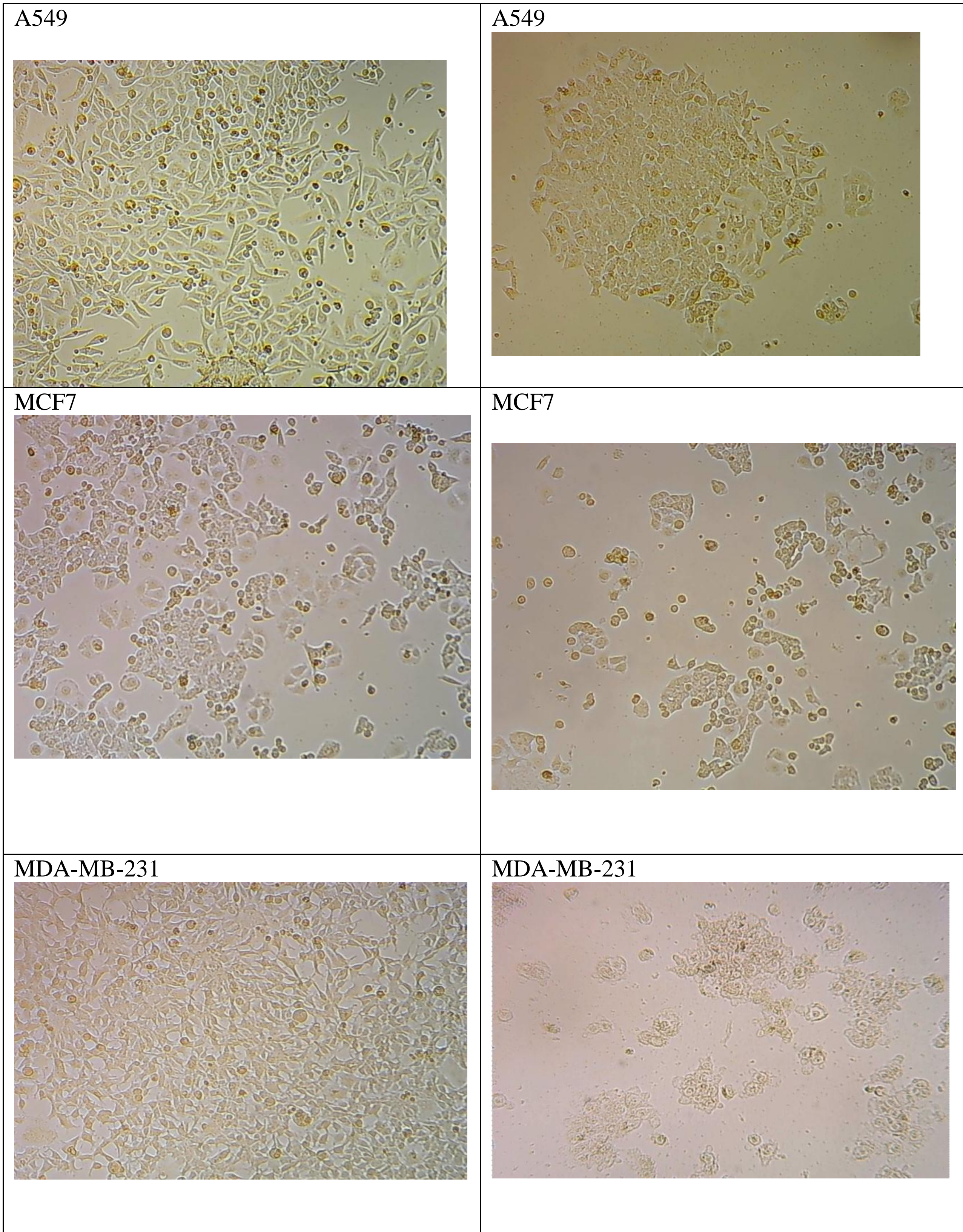


FIGURE 7: Images showing cell lines (UNTREATED) and cell lines (TREATED).

DISCUSSION

In this study, the efficiency of *Aegle marmelos* leaf extract to inhibit the proliferation of cells was studied on different cell lines including HepG2 (Liver cancer), MCF-7 (Primary Breast cancer), MDA-MB-231 (Breast cancer) and A549 (Lung cancer). The evaluation was performed using MTT assay that emphasizes on the conversion of tetrazolium salt to insoluble purple colored formazan crystals by the active dehydrogenases present in the mitochondria of live cells (Kulprachakarn et al., 2020).

Evidence of a compound being anti-proliferative comes from its tendency to control cell multiplication. This control over cell proliferation can be due to multiple mechanisms such as induction of apoptosis, autophagy, or necrosis (Green & Llambi, 2015). Moreover, there are signaling pathways such as MAPK pathway, RB pathway, Wnt and Notch pathways that are very likely to play a role in cell proliferation (Duronio & Xiong, 2013). In addition to this, there are certain molecules that are required to regulate and forward the cell cycle such as Cyclins, CDKs (Cyclin dependent kinases), p53, p38, Rb (Barnum & O'Connell, 2014). Adding to it, processes such as angiogenesis that involves *de novo* synthesis of blood vessels also depends on cell proliferation, an important feature of cancer progression (Nishida et al., 2006). Thus, to prove the role of a compound, and its efficiency as an anti-cancer agent, pathways and molecules related to cell proliferation and cell death should be targeted. A compound having ability to induce the cell death in any form can be a potent anti-cancer agent. Cell death corresponds to a smaller number of viable cells that becomes evident by using cell proliferation assays such as MTT, XTT, Resazurin reduction assay, ATP assay, protease viability marker assay (Riss et al., 2004)

In this study the compound was tested against different cell lines such as HepG2 and MCF7 showing IC50 comparable to that of doxorubicin, However, the potency of this extract was weaker against MDA-MB-231 and A549 cell lines. This indicates the leaf extracts to be effective against liver and breast cancer but weakly potent against lung cancer. *Aegle marmelos* being rich in phytoconstituents exerts such inhibitory effect against cancer through various mechanisms. Phytoconstituents from Aegle marmelos such as marmelin, Citral, lupeol, eugenol and limonene possess chemo preventive properties by minimizing the expression of IL-8 (Interleukin -8), VEGF (Vascular endothelial growth factor), and COX (Cyclo-oxygenase), AKT that are important for angiogenesis, tumor growth and metastasis. They are also responsible for reduction of MMPs expression (Matrix metalloproteases) that are responsible for degradation of extracellular matrix (ECM) and detachment of cells leading to metastasis (Katram et al., 2021). The bioactive components of *Aegle marmelos* leaf extracts also possess the ability to induce apoptosis in cancer cell lines by upregulating the expression of apoptotic factors such as Bax, caspase-3/9 and suppressing the expression of Bcl-2, an anti-apoptotic protein. This supports the cytotoxicity of *Aegle marmelos* leaf extracts against cancer cell lines and their role as a therapeutic agent in cancer. The differential properties of these extracts against lung cancer cell line refers to the difference in phytoconstituents of *Aegle marmelos* leaf extracts with a possibility of variation due to geographical location. Also in this study, we have used methanolic extracts of leaf which have been shown to be active against hepatocellular carcinoma and breast cancer whereas hydroalcoholic extracts were shown to be active against human lung cancer indicating the absence of phytoconstituent from the methanolic extract that interacts with human lung cancer cell line to cause cytotoxicity (Bhowmick & Bhowmick, 2024). Also, though the leaf extracts of AM (*Aegle marmelos*) show

cytotoxic effect against MDA-MB-231 but it is not that potent against MCF7. This difference in the potential of *Aegle marmelos* extracts to cause cytotoxicity against MCF7 and MDA-MB-231 can be attributed to MDA-MB-231 being triple negative breast cancer cell line indicating the difference in the receptors that are present on them against MCF7. (Razak et al., 2019). The difference in passage number of cell lines can be an indicative of the difference in potency as with the increasing passage number, heterogeneity may result thus causing uncertainty in the proliferation index of the cells (Ferraretto et al., 2007).

CONCLUSION

Aegle marmelos (L.), a shrub belonging to the family of Rutaceae, is an important plant of Dashamoola. This plant is sacred and its leaves are offered to Lord Shiva representing its mythological value. It possesses phytochemicals that are of pharmacological importance as they exhibit anti-inflammatory, anti-cancer, anti-microbial, anti-oxidant, anti-viral properties. They also hold therapeutic value as they have significant role in cardiovascular diseases, hepatic disorders, bone related disorders such as osteoporosis, neurological disorders such as Alzheimer's and Parkinson's. Our recent study indicates the potency of leaf extracts of Indian Bael against liver, lung, and breast cancer cell line. This potency of leaf extracts is mainly due to presence of coumarins and alkaloids making them therapeutically available. A channel is required to translate these therapeutic properties of this plant to medical cure.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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