Cell cytotoxicity and pro-apoptosis on MCF-7 cells using polyherbal formulation, MAT20

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SUMMARY

Today, standard of care cancer treatment includes chemotherapy, radiation, and surgery, which have serious side effects. A different option for medication could be using standard of care treatments in conjunction with herbal adjuvants. This integrated approach reduces the dosage, and the side effects of chemotherapy could lead to a better prognosis. The purpose of this study was to test the anti-cancer properties and pro-apoptotic effects of the polyherbal formulation MAT20 as a complementary treatment. *Moringa oleifera* (Moringa), *Phyllanthus emblica* (Amla) and *Ocimum sanctum* (Tulsi), these 3 herbs were used to formulate MAT20, which contain phytochemicals that are known to display anti-cancer properties. In this study, we hypothesized that MCF-7 breast cancer cells treated with MAT20 would show increased cytotoxicity compared to its individual plant extracts. MAT20 showed significantly lower IC50 values were found for both 24-hour (33.01µg/mL) and 48-hour (21.09 µg/mL) exposure compared to any of the other individual extracts for the given exposure duration. MAT20 did not show a significantly lower IC50 value compared to the chemotherapeutic drug Paclitaxel. Additionally, staining methods (DAPI, Hoechst, Dual staining) showed that MAT20 treated cells caused 49.6% cell death after 24 hours and 51% cell death after 48 hours of exposure, indicating successful drug activity in inducing apoptosis.

INTRODUCTION

Cancer is a deadly disease, killing nearly 10 million people in 2020 alone (1). Breast Cancer is the most prevalent type of cancer, affecting 1 in 8 women, which can be due to genetically inherited mutations, age, obesity and lifestyle, hormonal imbalances, and other factors. (2). Due to the widespread nature of this disease, many people have attempted to find methods to cure it. Although chemotherapy, radiotherapy and surgery are promising options for cancer treatments, currently 90% of failures in these therapies occur due to metastasis of cancers related to drug resistance, healthy tissue damage, recurrence, or relapse. (3-6). Therefore, in recent years complementary and integrative medicine (CIM) are gaining interest to overcome these side effects of chemo- and radio-therapy (7). Complementary and alternative medicine (CAM) is the terminology for numerous medical approaches that are not considered as part of conventional medicine. Integrative medicine is a method of treating patients that integrates conventional medicine with CAMs to find healthcare solutions (8). It is also approved to be safe and effective by the World Health Organization (9). Herbal medications are one form of CAMs that have been used to treat a variety of disorders since ancient times (10). In India, for example, a vast biodiversity of medicinal plants has been used individually or in the form of polyherbal formulations (11). Most of the currently available pharmaceutical drugs for treating cancer are of plant origin, for example, Paclitaxel from Taxane, and Vinblastine/ Vincristine from Vinca alkaloids. Herbal plants are a rich source of phytochemicals with medicinal properties like anti-cancer, anti-inflammatory, anti-oxidant, radioprotective, and analgesic (12). The phytochemical compounds present in herbals act through multiple molecular targets and signaling, reducing the likelihood of developing resistance, as can occur with synthetic chemical compounds. Herbals have also been found to kill only cancerous cells during treatment and not show toxicity to normal human cells (13). MAT20 is a polyherbal drug formulated using Moringa, Amla and Tulsi and has demonstrated anti-oxidative properties in previous studies (14). *Moringa oleifera* leaves are well known for having a variety of beneficial biological activities, including antioxidant and anticancer properties (15,16). *Phyllanthus emblica* contains polyphenols, tannins and flavonoids which exhibit anti-oxidant, cancer-preventative and antitumor properties (17,18). Holy Basil, also known as Tulsi or *Ocimum sanctum*, has also gained a lot of attention for its many health benefits, particularly anti-cancer characteristics (19,20). This study investigated the cytotoxicity properties of MAT20 and the mode of cell death it induces on the MCF-7 cell line. Furthermore, we compared MAT20 and the individual herbs regarding their effectiveness in inducing apoptosis in MCF-7 cells. Paclitaxel as a chemotherapeutic drug was used as a positive control to see it's cytotoxic effect on MCF-7 cell lines when compared to MAT20. We hypothesized that MAT20 would have higher cytotoxic effect than its individual herbal components and that the polyherbal would exhibit pro-apoptotic properties on the MCF-7 breast cancer cell line.

RESULTS

An MTT analysis was conducted to analyze cytotoxicity of MAT20 and determine the IC50 value of the formulation by determining cellular metabolic activity. IC50 is the required

	IC50 at 24 hours (µg/mL)	IC50 at 48 hours (µg/mL)
Moringa	100.5	94.7
Amla	99.5	51.8
Tulsi	67.5	29.5
MAT20	33.01	21.09

Table 1: IC50 Concentration (µg/ml) of individual extracts and MAT20 determined using MTT assay.

concentration of a drug to induce 50% cell death. The cells were further evaluated for apoptosis using Hoechst staining, 4',6-diamidino-2-phenylindole (DAPI) staining, and acridine orange/propidium iodide (PI) dual staining.

Cytotoxicity by MAT20

We used an MTT assay to determine the percent viability of MCF-7 cells after treatment with different concentrations of MAT20 and individual plant extracts, then used this data to calculate the IC50 values at 24 and 48 hours for each treatment (**Table 1**).

Also, MCF-7 cells were treated with the chemotherapeutic drug Paclitaxel as a positive control. At 24 and 48 hours, the IC50 of Paclitaxel was found to be 47.36 and 21.3 µg/mL respectively. There was no significant difference between MAT20 and Paclitaxel (**Figure 1**). MAT20 had a significantly lower IC50 value of 33.01 µg/mL (24 hours) and 21.09 µg/mL (48 hours) than any of the other individual component groups for the given exposure duration, indicating its effectiveness at low concentrations (p-value < 0.05) (**Table 1, Figure 1**). The IC50 values at 48 hours were lower than the IC50 values at 24 hours, except for the Tulsi extract. 10% of the total volume of the culture media, dimethyl sulfoxide (DMSO) was used as a vehicle control and showed 92% cell viability at 24 hours and 94% viability at 48 hours incubation (**Figure 2**).

Induced apoptosis by MAT20

Subsequently, we observed the induction of apoptosis through cell and nuclear morphology under the fluorescent
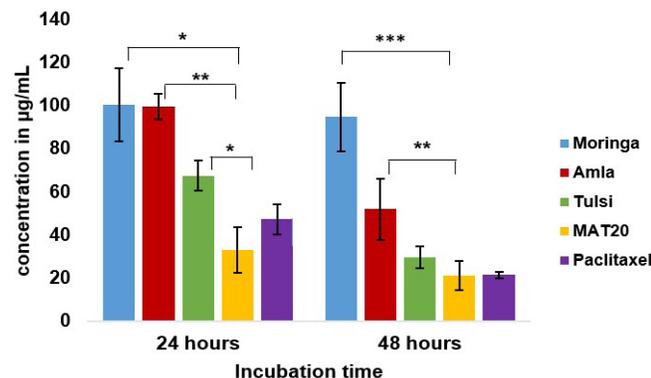

Figure 1: IC50 values of individual extracts, MAT20 and Paclitaxel drug. The MTT assay revealed IC50 for MCF-7 cells after 24 and 48 hours of incubation with Moringa, Amla, and Tulsi individually and compared with MAT20 polyherbal formulation. MAT20 showed lower IC50 value at 24 and 48 hours compared to individual plant extracts. MCF-7 cells were treated with Paclitaxel chemotherapeutic drug as a positive control. MAT20 did not show significant change in IC50 when compared with Paclitaxel. The data is presented as mean ± standard deviation, with n = 3. *p<0.05, **p<0.01 and ***p<0.001.
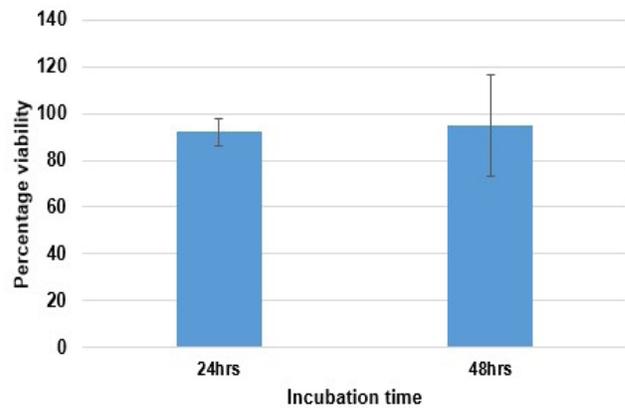

Figure 2: Percentage viability using DMSO as a vehicle control. MCF-7 cells were treated with 10% DMSO, which was used as a vehicle control. Cell viability at 24 hours 92% and at 48 hours incubation 94% was observed, (n=3).

microscope using dual staining with acridine orange and propidium iodide, Hoechst staining, and DAPI staining to determine the mode of cell death in MAT20 treated and untreated cells. We observed 51% cell death in the MAT20 treated group with prominent blebs (nuclear condensation that may form apoptotic bodies), and only 4% cell death in untreated group at 48 hours (p < 0.001) (**Figure 4**). Acridine orange stains live cells green and propidium iodide stains dead cells red (**Figure 3A-B**). Hoechst and DAPI staining allow for the detection of apoptotic cells as demonstrated by condensed nuclei and visualization of blebbing, which is
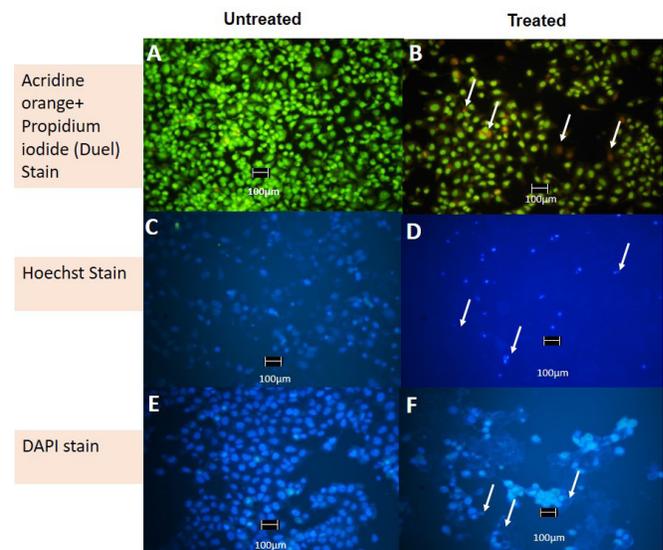

Figure 3: Apoptosis using MAT20 on untreated vs treated groups. Acridine orange and propidium iodide (dual) staining for MCF-7 untreated (A) and MAT20 treated cells (B). Hoechst staining for MCF-7 untreated (C) and MAT20 treated cells (D) and DAPI staining were used to observe MCF-7 untreated (E) and treated cells (F) under a fluorescence microscope. The data presented was done for one sample per stain (n=1). Arrows indicate fragmented nuclei with condensed chromatin. Apoptosis is indicated by the presence of fluorescent nuclear fragmentation and blebs, which are stained in Hoechst and DAPI staining. Also, stained orange to red in color showed cell death in acridine orange and propidium iodide (dual) staining. All images were taken using 20X magnification.

usually a common morphology of apoptotic cells (**Figure 3C-F**).

DISCUSSION

The presented findings demonstrate that MAT20 is an effective polyherbal formulation in the realm of integrative oncology based on the observed anti-cancer and pro-apoptotic properties of the MAT20. The cytotoxic effect of MAT20 with its individual extracts were assessed based on IC50 concentration which was found to be 33.01 ug/mL and 21.09 ug/mL at 24 and 48 hours respectively, which was found to be lesser than the IC50 values of individual extracts. (**Figure 1**). Since a smaller concentration of MAT20 was required to induce cytotoxicity, the formulation showed a considerably stronger anti-cancer property than its individual extracts. Paclitaxel, a chemotherapeutic drug, was used as a positive control to compare with MAT20. There was no significant difference between MAT20 and Paclitaxel (**Figure 1**). DMSO was used as vehicle control which demonstrated 92% and 94% cell viability at 24 and 48 hours respectively, indicating DMSO can be used as a solvent without inducing unintended cytotoxicity (**Figure 2**).

To study the mode of cell death using different stains (DAPI, Hoechst, dual staining), MCF-7 was treated with the IC50 MAT20. There is evidence of both living and dead cells in the treated group, suggesting the effectiveness of MAT20 in eliminating cancer cells through apoptosis (**Figure 4**). The results demonstrated that MAT20 treated cells induced 49.6% cell death at 24 hours and 51% cell death at 48 hours exposure, which was clear from the cell morphology pictures showing condensed nuclei and predominant blebbing in treated MCF-7 when compared to the untreated group (**Figure 3-4**). This validated our hypothesis that MAT20 had increased cytotoxic effect relative to individual plant extracts and also showed MAT20 has pro-apoptotic properties suggesting its potential as a supplementary or alternative approach to standard therapies. These preliminary results offer promise 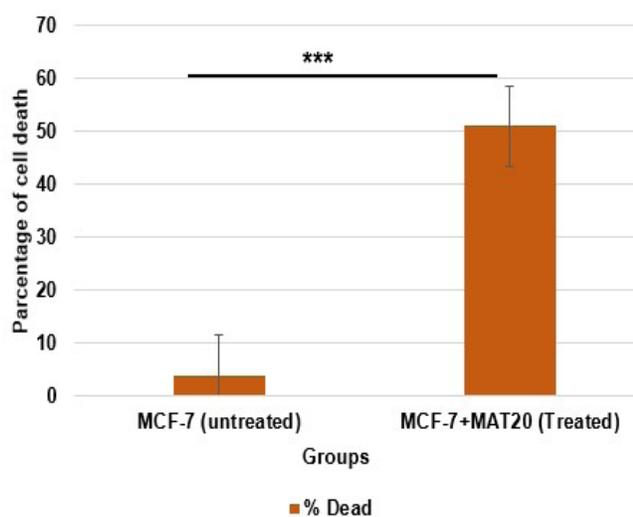and encourage further research on combining MAT20 with standard chemotherapeutic drugs as a complementary treatment for improving anti-cancer treatment capabilities while reducing the negative side effects of standard care.

Considering that these observations were made in *in vitro* conditions, these results must be extrapolated to animal models to study the bioavailability of the MAT20 formulation. This linear approach from *in vitro* analysis to in vivo experiments will further strengthen our in vitro findings. MTT assays help understand at a preliminary level the suggested doses where cells respond to treatment, which will subsequently help to formulate doses for *in vivo* studies.

Future research could study the pro-apoptotic gene expression levels to validate the mode of cell in cancer cells. Antibodies for cleaved caspase-3, a more accurate apoptotic marker that is triggered at the commencement of apoptosis, is one method that can be improved. Other pharmacological characteristics of MAT20, such as antibacterial, antifungal, and anti-inflammatory properties, might be explored in the future. In a similar way, other herbal plant species can be evaluated for their anti-cancer properties. MAT20 can also be mixed with other crude extracts to see whether they have any additional anti-cancer qualities. Other malignant cell lines might also be investigated, which could be an interesting approach in CAMs in the future.

MATERIALS AND METHODS
Formulation of MAT20

The optimum extracts of Moringa, Amla, Tulsi were selected based on qualitative and quantitative analyses of different phytochemicals (14). These herbal powders were purchased from an organics store. A combination of the three extracts was formulated by determining the weight before and after air-drying the extracts. Subsequently, we weighed 0.01 g of each dried extract and dissolved in 1 mL of DMSO (Qualigens). This final formulation was prepared using the combination of individual extracts with their respective IC50 concentration in the ratio of 1:1:1 and named MAT20. Then it is used to determine its anticancer and proapoptotic properties.

Cell Culture

MCF-7 cell lines were obtained from NCCS Pune and cultured in 10% Fetal Bovine Serum (FBS) containing Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) and maintained at standard cell culture conditions (37°C and 5% CO2). Adhered cells at 80% confluency were detached using 0.25% trypsin-EDTA (Hi Media) and were reseeded in T-25 Flask at a density of 3000 cells/cm2 with DMEM (10% FBS) for further studies.

Cytotoxicity assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) test settings were determined based on the presence of secondary metabolites (phytochemicals) in earlier phytochemical analysis where the essential groups of MAT20 were found to be of separate phytochemical groupings (14). The MTT assay procedure was adapted from Morgan's description (23). MCF-7 cells were counted using a hemocytometer and 3000-5000 cells/well were seeded in a 96-well plate in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and were maintained at 37°C with 5% CO2 for 24 hours. Tulsi extract (0, 1.57, 3.13, 6.25, 12.5, 25, 50, 100 μg/mL), Amla extract (0,1.57, 3.13,

Figure 4: Percentage of cell death. Bar graph showing dead cells quantified using fluorescence staining from dual staining, DAPI, and Hoechst on MCF-7 cells. Untreated (MCF- alone) vs. Treated (MCF + MAT20) groups were compared (n=3, mean±SD).

6.25, 12.5, 25, 50, 100 μg/mL), Moringa extract (0,1.57, 3.13, 6.25, 12.5, 25, 50, 100 μg/mL), and MAT20 (3.31, 6.62, 13.24, 26.5, 52.95, 105.9, 211.79 μg/mL) of respective concentration were used to treat the cells for 24 and 48 hours. Also, 10% DMSO was used as a vehicle control. Paclitaxel, a chemotherapeutic drug, was used as positive control with incubation periods of 24 hours and 48 hours. 20 μL of MTT was added to all the wells and the plates were incubated for 4 hours. After removing the medium from each well, 100 μL DMSO was added to dissolve the purple formazan crystals, the appearance of which meant that there were living cells present. In the spectrophotometer, the absorbance readings were taken at a wavelength of 545 nm (Thermo Scientific). To reduce mistakes, three different sets of experiments were carried out in triplicate.

The following formula was used to calculate the percent cytotoxicity:

$$\frac{Abs_{Control} - Abs_{Sample}}{Abs_{Control}} \times 100 = \% \text{ cytotoxicity}$$

The percent cytotoxicity graphs were plotted against the concentration of the treatment for all four groups. The IC50. tk software was used to determine the IC50 value for each of the four groups after both 24 and 48 hours of drug action.

Cell Staining and Microscopy

MCF-7 cells were grown on a 24-well plate with DMEM and 10% FBS (1 mL, 0.2 x 106 cells per well), maintained at 37ºC with 5% CO_2, and treated with MAT20 at the IC50 concentration obtained from the MTT experiment (21.09 μg/mL) along with untreated MCF-7 cells. The media was discarded after 48 hours of drug treatment, and the cells were rinsed with 1X PBS (pH7.4). Furthermore, cells were fixed with 4% paraformaldehyde (fixative agent) and incubated for 5 minutes. The cells were then rinsed with cold PBS and the cells were stained accordingly.

The dual staining method used comes from Mascotti *et al*. (24). 5 μL of 1 mg/mL propidium iodide and 1 mg/mL acridine orange were added at same time to the treated and untreated cells, which were observed under the fluorescent microscope immediately in a dark room 490 nm (excitation), 630 nm (emission).

The Hoechst staining method used comes from the protocol followed by Crowley *et al*. (25). 50μL of 0.5 μg/mL Hoechst stain was added to the treated and untreated cells and was observed under the fluorescent microscope in a dark room at 350–460 nm.

DAPI (4′,6-diamidino-2-phenylindole) was used to observe mode of cell death. 50 μL of 1000 mg/mL DAPI stain was added to untreated cells and the treated cells and was viewed under the fluorescent microscope in a dark room at 450 nm. To avoid exposure of the fluorescent stains to ambient light, they were stained in the dark and covered with aluminum foil.

An Olympus microscope with 0.34 numerical aperture, 20X magnification, and ProgRes® Capture Pro software was used to capture the images (26, 27).

Statistical analysis

The data was analyzed using Microsoft Excel software. The statistical difference between groups was examined using ANOVA with each experiment done in triplicate (n=3, p-value <0.05 was considered significant).

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