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Article

Cytotoxicity evaluation of *Amaranthus* extracts compared with AS20 on MCF-7 cancer cells

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SUMMARY

Advancement in oncology research is striving to find new and effective therapies for treating cancer, limiting the drawbacks of conventional treatments like chemotherapy, radiotherapy, etc. Many plant-derived active ingredients such as saponins, tannins, alkaloids, and polyphenols are used in cancer treatments which are FDA approved. Amaranthus is a traditional Indian medicinal plant used to treat a variety of diseases. It was previously found to contain phenols, tannins, flavonoids, and alkaloids among other bioactive phytochemicals. In the present study, we hypothesized that AS20, a polyherbal formulation derived from Amaranthus leaves and inflorescence has higher anticancer properties than individual extracts such as leaf methanol and inflorescence acetone extract on MCF-7 breast cancer cells. We also hypothesized that AS20 would induce higher levels of apoptosis in MCF-7 breast cancer cells. We discovered that AS20 had a considerably lesser maximal inhibitory concentration (IC50) compared to individual plant extracts of Amaranthus spinosus using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. AS20 had a lower IC50 than leaf methanol and inflorescence acetone extract respectively. Paclitaxel (a chemotherapeutic drug which can be used in cancer treatment) was used as a positive control and showed similar IC50 value like AS20. These findings indicated that AS20 did not show a significantly lower IC50 value compared to the chemotherapeutic drug Paclitaxel. Fluorescence staining methods such as Hoechst, 4′,6-diamidino-2-phenylindole (DAPI), and acridine orange with propidium iodide (dual staining) showed that AS20 caused 51% cell death in MCF-7 cells compared to 4% cell death in untreated MCF-7 cells after 48 hours of exposure, indicating successful drug activity in inducing apoptosis.

INTRODUCTION

Cancer is considered to be the second leading cause of death in India (1). For women worldwide, the second most frequent form of cancer is breast cancer, which in India specifically affects one in twenty-eight women at some point in their life (2, 3). The global prevalence of cancer has risen dramatically in recent years, prompting concerns about the effectiveness of current treatments (4). As a result, patients are looking for alternatives to established cancer treatments like surgery, radiation therapy, and chemotherapy to supplement or replace them (5). Complementary and alternative medicine (CAM) are a collection of medical and healthcare methods and products that are not currently regarded to be part of conventional medicine (6, 7). Plant-made drugs are gaining interest as an alternative treatment for cancer owing to their anti-proliferative activities on cancer cell growth without causing harm to non-targeted cells (8, 9). *Amaranthus* is one of the world's oldest crops. It is a tropical American staple crop which has natural anti-inflammatory properties. Due to the presence of phytochemicals, it has anti-inflammatory, antioxidant, anti-cancerous, anti-diabetic, anti-malarial, cardioprotective, and hepatoprotective qualities (10).

Phytochemical analysis of different plant parts of *Amaranthus* has indicated the presence of different active components like alkaloids, flavonoids, glycosides, phenolic acids, steroids, saponins, amino acids, vitamins, minerals, terpenoids, lipids, betaine, catechuic tannins, and carotenoids as major components (11). In vitro studies of *A. tristis* leaf ethyl acetate extract on human tumour cell lines AGS, A549, MCF-7, and COLO 320 DM showed significant cytotoxic effects and apoptotic cell death in a concentration-dependent manner (12).

Most of the commercially available drugs used for treating cancer in modern science originate from plant-derived active components, mainly secondary metabolites e.g., vinblastine, vincristine, taxol, elliptinium, and etoposide (13). Plant derived chemotherapeutic drugs target improperly expressed molecular factors, reduce oxidative stress, control cell growth factors, limit angiogenesis in malignant tissue, and trigger apoptosis in rapidly proliferating cells (12).

In our current study, we used MCF-7 cell lines, which are the most researched human breast cancer cell line in the world and was named after the Michigan Cancer Fund (MCF). MCF-7 cell lines were established from a 69-year-old female Caucasian who was suffering from breast adenocarcinoma (14). The aim of this study was to see how different concentrations of parts of *Amaranthus spinosus,* such as leaf and inflorescence extracts, would affect cell death and IC50 of MCF-7 cells while testing in different solvents. According to the FDA, IC50 denotes the medication concentration required for 50% inhibition in vitro (15). These results were then compared to experimentation done with AS20, a combination of *Amaranthus* leaf and inflorescence extracts in a polyherbal formulation prepared in the lab. Then, an MTT assay, a common method for assessing cell proliferation and drug response, was used to check the cytotoxicity of individual extracts of *Amaranthus* and AS20. The MTT assay is a colorimetric assay based on the measurement of mitochondrial activity by formazan crystal formation. During MTT assay, cells can be treated with different drug

concentrations to see the cell viability and drug cytotoxic effects (16).

One of the most important markers of cytotoxic anticancer drugs is the induction of apoptosis (17). Apoptosis, or programmed cell death, is a tightly controlled process that removes undesirable cells, as well as aged and injured cells which is obscured in cancer cells and cell death takes places through necrosis, an inflammatory form of cell death. Apoptosis does not harm other healthy cells as in necrotic cell death where cells leak nuclear material which can harm other healthy cells. As a result, in cancer-related research, apoptosis is preferred to necrosis (18, 19). Apoptosis-inducing phytochemicals have great promise as potential cancer treatments.

In our study we also hypothesized that AS20 is suitable to use as an anti-cancer and pro-apoptotic drug. To distinguish between living and dead cells during analysis, various staining techniques such as DAPI, Hoechst, and acridine orange with propidium iodide (dual staining) were used.

RESULTS

Cytotoxicity effect of AS20 on MCF-7 (breast cancer) cells

The MTT assay was used to investigate the cytotoxicity effects of *Amaranthus* leaf and inflorescence extracts singly and in combination (AS20) after 24 and 48 hours. Percentage inhibition was higher in AS20 polyherbal formulation compared to individual extracts (**Figure 1**).

IC50 is half maximal inhibitory concentration which measures the effectiveness of the compound in inhibiting biological function. At 24 and 48 hours after treatment, the IC50 values of methanol leaf extract were found to be 109.9 µg/mL and 93µg/mL, respectively. At 24 hours, acetone inflorescence extract had a higher IC50 value of 108.8 µg/mL, which was similar at 48 hours (107.2 µg/mL). At 24 and 48 hours, AS20 polyherbal formulation's IC50 was found to be the lowest, at 52.5 µg/mL and 26.7 µg/mL, respectively (**Figure 1**). Individual extracts of *Amaranthus* were statistically higher IC50 than AS20. The result demonstrates that the lower IC50 of AS20 was more effective at slowing cancer cell proliferation at lower doses. Paclitaxel, a chemotherapeutic drug commonly used to treat breast cancer, was used as a positive control. At 24 and 48 hours, Paclitaxel's IC50 was found to be 47.36 µg/mL and 21.3 µg/mL, respectively (**Figure 2**). DMSO was used as a vehicle control and showed 7-8% cell death at 24 and 48 hours.

Apoptosis of MCF-7 cells using AS20

In our study, we observed changes in nuclear morphology and mode of cell death using fluorescence staining such as acridine orange and propidium iodide (dual stain), Hoechst and DAPI (4′,6-diamidino-2-phenylindole) after treating with IC50 of AS20 to the cells. Propidium iodide stains dead cells red, while acridine orange stains live cells green **(Figure 3A-B)**. DAPI **(Figure 3C-D)** and Hoechst **(Figure 3E-F)** staining binds to dsDNA and fluoresces blue in color, which is typically the component of apoptotic cells. We also observed more blebbing and apoptotic bodies in the MCF7 cells in the AS20-treated group compared with untreated MCF7 cells. The arrows indicate cell death as well as apoptotic nuclei and cellular blebs of the AS20-treated group. Results were observed under a fluorescence microscope **(Figure 3)**.

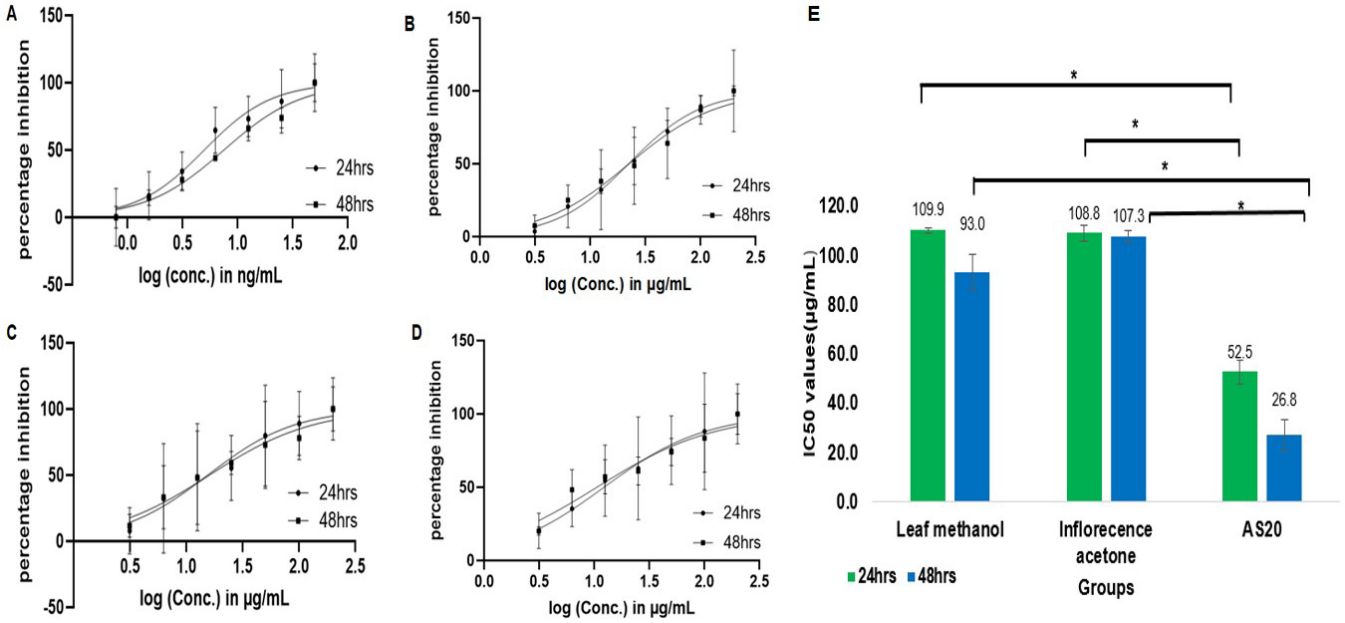

Figure 1: Percentage inhibition using different concentrations. Graph showing an increase in the percentage inhibition of MCF-7 cells as the concentration of *Amaranthus spinosus*-derived extracts increase and Paclitaxel was used as a chemotherapeutic drug control, as measured by the MTT assay: **(A)** Methanol extraction of leaves- (concentration-0, 3.13, 6.25, 12.5, 25, 50, 100, 200 µg/mL), **(B)** Acetone extraction of inflorescence-(concentration-0, 3.13, 6.25, 12.5, 25, 50, 100, 200 µg/mL), **(C)** AS20 (concentration-0, 3.13, 6.25, 12.5, 25, 50, 100, 200 µg/mL), and **(D)** Paclitaxel (chemotherapeutic drug)- (concentration-0, 0.78, 1.56,3.13, 6.25, 12.5,25, 50 mg/mL). **(E)** The MTT assay revealed IC50 for MCF-7 cells after 24 and 48 hours of incubation with leaf methanol, inflorescence acetone individually and compared with AS20 polyherbal formulation. AS20 showed lower IC50 value at 24 and 48 hours compared to individual plant extracts. The data is presented as mean ± standard deviation by mean, with n = 3, *p<0.05 is considered statistically significant.

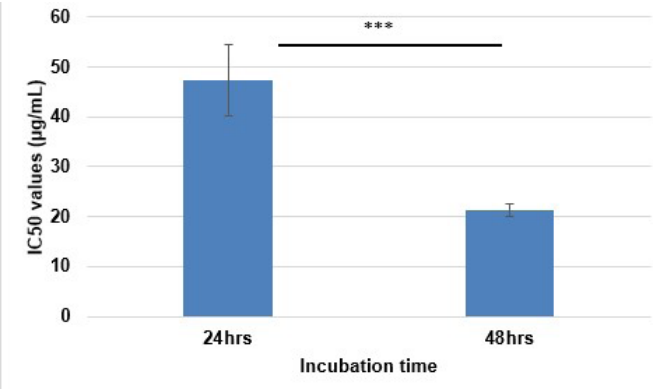

Figure 2: IC50 for Paclitaxel drug. Paclitaxel was used as a chemotherapeutic drug and IC50 values for 24 hours and 48 hours are shown using a bar graph. N = 3, ***p<0.001 is considered statistically significant.

Percent of cell death was quantified by the estimated cell count. 3.6% cell death was seen in control group of MCF-7 cells and 50.33% cell death was seen in AS20-treated MCF-7 cells, which showed significant impact on the IC50 of AS20 **(Figure 3G)**.

DISCUSSION

Our study demonstrates the anticancer and pro-apoptotic properties of AS20 poly herbal formulation compared to its individual extracts such as leaf methanol and inflorescence acetone extract on MCF-7 breast cancer cells. Secondary metabolites are abundant in most natural products exhibiting anti-cancer properties (20). They are a wide set of molecules that include several phytochemicals which include alkaloids, glycosides, amines, steroids, flavonoids, and related metabolites that may have antioxidant, antibacterial, or anti-diabetic activity, among other things (21, 22).

The cell cytotoxicity using MTT, showed that AS20 had a lower IC50 value compared to its individual extracts at 24 and 48 hours, suggesting anticancer potential as a lower concentration of AS20 is required to kill 50% of the cancerous MCF-7 cells. Additionally, there was a considerable variation in the IC50 value for 24 and 48 hours, demonstrating the major effect of AS20 exposure time on the death of cancerous cells. These findings are very fascinating because they show that an herbal extract has anti-proliferative effects on MCF-7 cells.

Certain morphological changes, such as cell rounding, detachment, blebbing, protrusion of apoptotic bodies, and nuclear fragmentation, are indicative of apoptosis (23, 24). In this study we have demonstrated the ability of AS20 in inducing apoptosis in MCF-7 cells by examining cell and nuclear morphology using different staining techniques, in control and drug treated groups using dual staining with acridine orange and propidium iodide (dual), Hoechst staining, and DAPI staining to identify a potential method of cell death. MCF-7 cells treated with AS20 showed 50.33% of cell death, whereas only 3.6% of MCF-7 cells died in the control group, demonstrating the considerable impact of AS20's IC50. Dual staining was used to visualize stages of apoptosis. Live cells were stained in green color, early apoptotic cells were stained a yellowish-green color, and late apoptotic bodies were stained orange in color (25). Hoechst and DAPI bind to dsDNA and visualize nuclear changes inside the cells (26, 27). From our results we can conclude AS20 at its IC50 concentration has the potential to induce cytotoxicity through apoptosis (**Figure 3**). Also, we have shown that AS20 has less IC50 value than the chemotherapeutic drug Paclitaxel.

A limitation of our study was to compare chemotherapeutic drug i.e., Paclitaxel's efficacy in apoptosis using staining technique. The fact that we had to manually count how many dead and living stains there were due to the staining methods we utilized was another limitation of our study. To confirm the findings, future investigation should study the levels of gene expression during apoptotic pathways. Future research may also focus on AS20's additional pharmacological properties, including its anti-inflammatory properties. Therefore, AS20 has potential to serve as an anti-cancer polyherbal formulation in the CAM platform.

MATERIALS AND METHODS

Cell cytotoxicity using MTT assay

MCF-7 cells were purchased from NCCS in Pune, India. MCF-7 cells were grown at 37°C with 5% CO2 in Dulbecco's Modified Eagle Medium (DMEM) along with 10% fetal bovine serum (FBS) (Himedia). 0.3 million cells were plated in a 96 well plate and incubated for 24 hours. The *Amaranthus* leaf in methanol, inflorescence extracts in acetone, and AS20 extracts in dimethyl sulfoxide (DMSO) were added to the 96 well plate with different concentrations of their respective solvents at 0, 3.13, 6.25, 12.5, 25, 50, 100, and 200 µg/mL. Paclitaxel drug was used as a positive control with concentrations at 0, 0.78, 1.56, 3.13, 6.25, 12.5, 25, and 50 mg/mL. This cell-treated plate was kept for 24 and 48 hours incubation to check cell cytotoxicity. To conduct the MTT assay, 20 µL of MTT was added to the well plate and incubated for 4 hours. The MTT assay was used to test the cell cytotoxicity (Himedia). To dissolve the formazan crystals, DMSO (10%) was added. The cell cytotoxicity figures were plotted using the optical density (OD) at 545 nm. The IC50 value was the concentration at which the cell death was precisely half. In pharmacological research, IC50 is often employed as a measure of drug potency since it allows one to determine the drug's potency.

Cell Staining and Microscopy

MCF-7 cells were cultured on a 24-well plate in DMEM with 10% Fetal Bovine Serum (FBS) (1 mL, 0.2 x 10^6 cells per well) and treated with AS20 at the IC50 concentration as determined in the MTT experiment. After 24 hours of drug activity, the media was discarded, and the cells were washed with 1x PBS (pH 7.4) solution before being incubated at room temperature for 5 minutes. The wells were then filled with 4% paraformaldehyde (fixative agent) and incubated for another 5 minutes. The cells were then washed once more with ice-cold PBS. The fluorescent stains were used in the dark and covered with aluminium foil to avoid light exposure.

Mascotti et al. employed a dual staining procedure, which we adopted (25). The cells were treated with 5 µL of (1 mg/mL) propidium iodide and 5 µL of (1 mg/mL) acridine orange, and the treated and untreated samples were viewed under a fluorescence microscope in a dark environment at 490 nm (excitation), 630 nm (emission).

The Hoechst staining procedure derived from Crowley et al.'s protocol, was used for treated and untreated cells, which

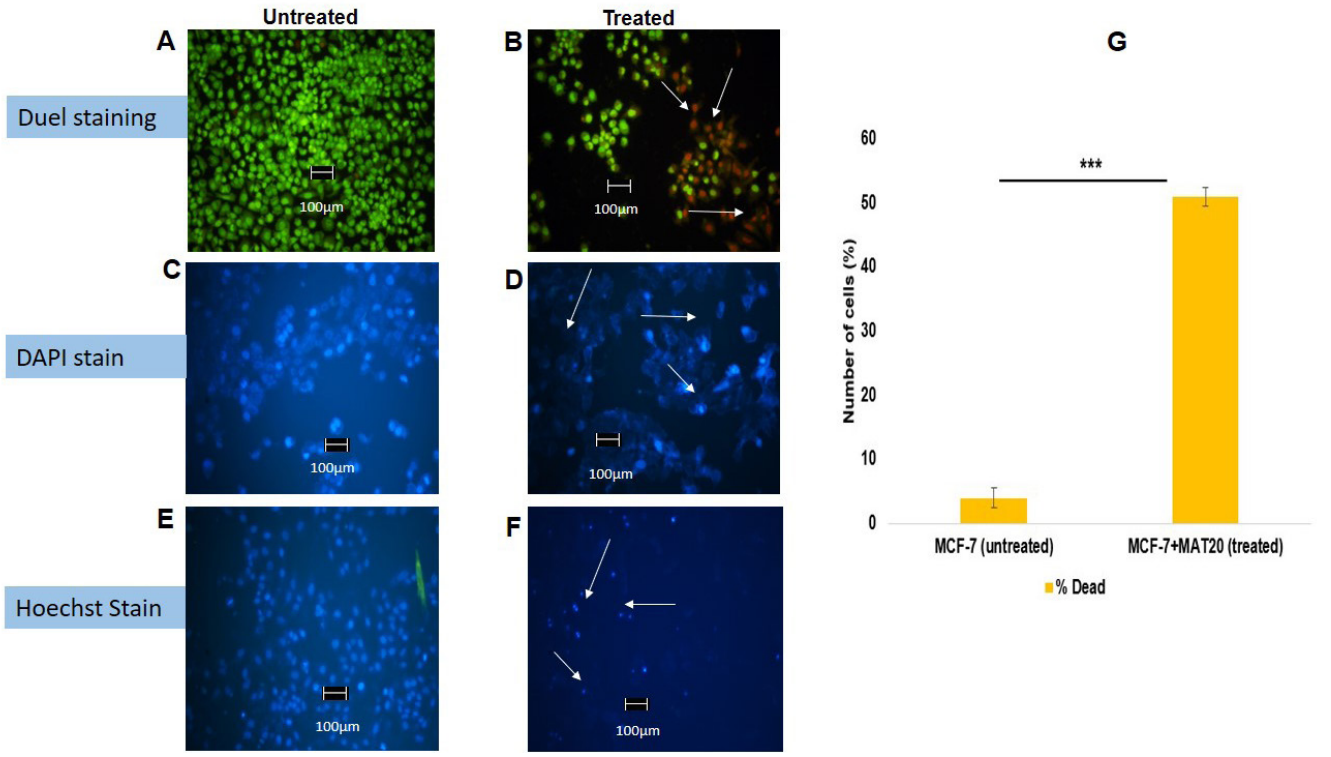

Figure 3: Apoptosis of MCF-7 cells using AS20 on control vs. treated groups. Dual staining **(A, B)**, DAPI staining **(C, D),** and Hoechst staining **(E, F)** (20x) were used to observe MCF-7 untreated and treated cells using IC50 value of AS20 under a fluorescence microscope. Apoptosis is indicated by arrows, the presence of fluorescent nuclear fragmentation and blebs, which are stained with Hoechst and DAPI. Stained orange to red in colour showed cell death in acridine orange and propidium iodide (dual) staining. Bar graph showing percent of dead cells obtained from fluorescence staining using dual staining, DAPI and Hoechst using MCF-7 cells **(G)**. Untreated (MCF-7 alone) vs. treated (MCF-7 + AS20) groups were compared (n = 3, mean ± SD).

were stained with 50 µL (0.5 g/mL) Hoechst stain then viewed under a fluorescence microscope in a dark room at 350–460 nm (26, 27).

DAPI staining was employed to observe the manner of cell death. 50 µL of DAPI stain (1000 mg/mL) was added to the untreated and treated cells, and immediately results were observed at 450 nm under a fluorescence microscope in a dark room.

The photos were captured using ProgRes® Capture Pro software and an Olympus microscope with a 0.34 numerical aperture and 20X magnification (28, 29).

Statistical analysis

GraphPad Prism was used to analyze percentage inhibition as well as IC50 values. ANOVA was used to investigate the statistical difference between groups. N = 3 and p-value < 0.05 was considered significant and denoted by a single asterisk. N = 3 and $p < 0.05$ was considered statistically significant with confidence interval for the IC50s of each sample set to 95%. Microsoft Excel was used to analyze the information for fluorescence staining for percent cell number.

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