Article

Anticancer, anti-inflammatory, and apoptotic activities of MAT20, a poly-herbal formulation.

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SUMMARY

Cancer is a disease that can be treated with surgery, chemotherapy, and radiation. Although these methods are very effective, they often result in micro-metastasis, drug resistance, and radio resistance. Disease relapse can be treated by escalating drug dosages, but this approach leads to drug resistance and drug toxicity. Complementary and Alternative Medicines (CAM) is a treatment which involves the the simultaneous administration of herbal formulations and chemotherapy, where the herbal formulations could mitigate the negative effects of chemotherapy, resulting in better outcomes. In this study, the efficacy of our formulation MAT20, a crude extract of the moringa, amla, and tulsi leaves, was investigated in terms of its cytotoxic and inflammatory properties. We hypothesized that MAT20, would induce higher HeLa cell death compared to other extracts of individual plants. Significantly lower IC50 values were found in 48 hours of exposure compared to any other individual extracts of the plant part. Also, we investigated the effects of MAT20 on the expression of COX2, a marker for inflammation on HeLa cells. Finally, to understand the mechanism of action, we studied the upregulation of the BAX gene and downregulation of the BCL2 gene by MAT20. Our study demonstrated significant cell cytotoxicity and DNA fragmentation upon the exposure of HeLa cells to the drug, substantiating our hypothesis. Treatment with MAT20 suppressed PMA-mediated induction of the COX2 gene. Our data is important for understanding the anti-cancer and anti-inflammatory properties of MAT20.

INTRODUCTION

Cancer is a disease where cells grow at an uncontrolled or abnormal rate and cause tissue damage. Global Cancer Observatory (GLOBOCAN) 2020 estimated that around 19.3 million new cancer cases and almost 10 million cancer deaths occurred in 2020 (1). Cervical cancer is the most common cancer among women worldwide with 604,237 women were diagnosed with cervical cancer globally in 2020 (2).
Cancer management is an area of intense research, and modern science has made important advances to provide some insights into it (3). Cancer is a multifaceted disease which affects the body in multiple ways. The tumor grows in the body by diverting vital nutrients from the other normal cells (4). In doing so, cancer cells are often in a state of

continuous growth, due to which their nutrition requirements are significantly higher than that of normal cells (5). Malignant cells also can secrete factors that allow them to colonize other organs by a process called metastasis, a complex biochemical process involving the invasion of basement membrane and cell migration, intravasation, survival in the circulation, and extravasation (6). Conventional cancer management includes surgery, chemotherapy, radiation, and immune therapy (7). These modalities are focused on targeting the tumor/tumor cells and thereby shrinking them to perform tumor resection surgeries. Although these measures are effective, they also make way for surgery-induced immune suppression, chemo resistance, radiation-induced immune suppression/resistance, and immune resistance. Cancer cells learn to adapt and evade the immune system resulting in immune editing, and the emergence of more aggressive clones which are not responsive to chemotherapy and immunotherapy (8, 9). Treatment outcomes emerging from such approaches are associated with poor quality of life and a high relapse rate (10).

Holistic approaches based on the "seed and soil" theory could be an inclusive approach for treating cancers (11). The seed and soil hypothesis claims that cancer cells will metastasize to a region where the biological microenvironment is favorable – much like a seed that only grows if it falls on fertile soil (12). Changes in the microenvironment due to treatment procedures could have long-lasting implications in the treatment outcome (13). Complimentary Alternative Medicine (CAM) is a viable approach which is comprised of modalities which address such issues (14). Natural products with medicinal properties are grouped under this umbrella, and our interest is to study and analyze these products for their anti-cancer effects, thereby extending their usage in the realm of Integrative Oncology alongside standard treatment procedures.

Existing studies rely on analysis based on a single peak obtained from High-Performance Liquid Chromatography (HPLC) analysis, following the basic drug discovery norms in the industry (15). Our present approach is based on the poly-herbal approach, which could serve as a complement to the existing standard approaches available to treat cancer (16, 17).

Medicinal plants play important roles in the medical system. Some anti-cancer agents are extracted from plant extracts because they cannot be synthesized chemically.
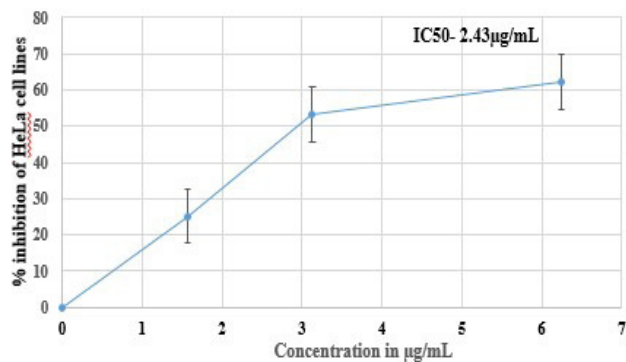
Figure 1: Cell cytotoxicity by CCK-8 assay. After 48-hour incubation with different concentrations of MAT20, there was different cell cytotoxicity on HeLa cells using CCK-8 assay kit. The data are presented as mean ± standard deviation by mean, n = 3.

Figure 2: DNA fragmentation. Treatment with poly-herbal drug MAT20 resulted in the degradation of genomic DNA into small fragments, which showed that MAT20 induced cell death via **apoptosis.**

Moringa has phenolic compounds such as tannins, saponins, flavonoids, terpenoids glycosides, and antioxidants (18). Moringa possesses key antioxidant effects which combat oxidative stress, which can convert fibroblasts to activated myofibroblasts via H2O2 production (19). Moringa has chemo-preventive and radio-preventive properties. Mature leaves have double the concentration of polyphenols (mainly quercetin and kaempferol) than tender leaves (20, 21). Quercetin has shown synergistic anti-cancer properties and potentiation of apoptotic effects of ionizing radiation on cervical cancer cells primarily in vitro and in some studies in vivo (22, 23). A reactive oxidation species (ROS)-dependent mechanism has again been implicated in radio-sensitivity mediated by some of the phytochemicals (20).

Oscimum sanctum, or tulsi, is an adaptogen. Its phytochemicals, including eugenol, rosmarinic acid, apigenin, myretenal, luteolin, β–sitosterol, and carnosic acid, have been shown to prevent various chemical-induced cancers (24). These phytochemicals also modulate side effects by increasing antioxidant activity, altering gene expressions, inducing apoptosis, and inhibiting angiogenesis and metastasis (25). Studies on tumor-bearing mice have shown that these flavonoids, along with eugenol, prevent clastogenesis (modifications in structure/ arrangement of chromosomes) (18). The free-radical scavenging, antioxidant, and anti-inflammatory effects may have shown the radio-protective effect (26).

Phyllanthus emblica, or amla, is also an adaptogen. Like the other extracts, it also has radio-modulatory, chemo-modulatory, and chemo-preventive effects due to free radical scavenging, antioxidant, anti-inflammatory, anti-mutagenic, and immuno-modulatory activities (27). Ascorbic acid acts as a chain-breaking antioxidant, impairing the formation of free radicals during the synthesis of intracellular substances, such as collagen, throughout the body (28, 29, 30). The flavonoid quercetin has shown potency in vivo to stop tumor growth in many xenografts (27). Analogues of quercetin designed as more specific PI3K inhibitors have observed to
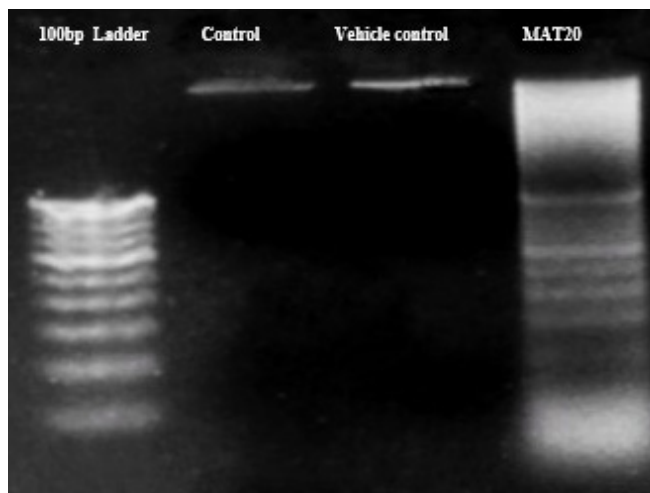
exhibit anti-proliferative activity (28). The high hydrolysable tannin content prevents mutagenesis and lipid peroxidation against carcinogens and ROS (29). Amla reduces the release of inflammatory cytokines and may prevent age-related hyperlipidemia. Alma also reduces nitric oxide synthase and COX2 protein levels by inhibiting NF-κB activation. NF-κB is an important survival factor in many cancers and is inflammatory in nature. Anti-cancer properties of amla include inhibition of this transcription factor binding with its cognate DNA-binding elements to cause apoptosis. 5-flurouracil (5FU) and phorbol 12-myristate 13-acetate (PMA) are a few drugs that induce COX2.

In this study, we have formulated MAT20 by using three herbal extracts: moringa, tulsi, and amla to investigate the anti-cancer properties. We hypothesized that MAT20, a polyherbal drug, induces cytotoxicity in HeLa cells, which was tested with an assay called cell cytotoxicity kit-8 assay (CCK-8 assay). The IC50 value of MAT20 was further used for assessing cell death by DNA fragmentation and expression of apoptotic genes by treating HeLa cells with MAT20. Our study demonstrated that MAT20 induced cell cytotoxicity leading to DNA fragmentation. Our findings substantiated the potency of phytochemical extracts against cancer cell growth, prompting further study.

RESULTS

Our study aimed to test the efficacy of MAT20, a polyherbal drug, against HeLa cells. Given that the extract comprised moringa, tulsi, and amla – extracts that have demonstrated anti-cancerous properties, we expected MAT20 to induce cytotoxicity. We aimed to test this using the CCK-8 assay, which would enable us to determine the IC50 value. Following this, we investigated DNA fragmentation between normal and compared it with the drug-treated groups.
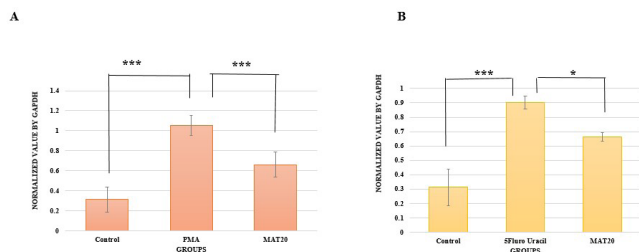
Figure 3: Normalized COX2 expression
A. When HeLa cells were treated with PMA, COX2 gene was significantly induced. When these groups were treated with MAT20, COX2 gene expression was reduced, n = 3. *p < 0.05, ***p < 0.001
 B. When HeLa cells were treated with 5FU, the COX2 gene was significantly induced, while in these groups with MAT20 treatment, COX2 gene expression was reduced, n = 3. *p < 0.05, ***p < 0.001

Figure 4: Normalized expression of BAX and BCL2 gene with GAPDH gene. HeLa as a control and MAT20. MAT20 group showed upregulation of BAX gene and down regulation of BCL2 gene. The data are presented as mean ± standard deviation by mean, n = 3. *p < 0.05, ***p < 0.001

MAT20 induced cytotoxicity as demonstrated by the CCK-8 assay. The IC50 value obtained from the CCK-8 assay at 450 nm was found to be 2.43 µg/ml by using IC50.tk software **(Figure 1)**.

DNA showed fragmentation in drug treated group, which was analyzed by the formation of a DNA ladder **(Figure 2)**. HeLa cells were treated with 2.85 ug/mL of MAT20 drug and incubated for 48 hours. DNA was extracted from treated and non-treated HeLa cells.

MAT20 reduced COX2 expression was induced by 5FU and PMA. The experiment showed that PMA, as well as 5FU, showed COX2 gene induction compared to the control group. When the group with PMA, as well as 5FU, was treated with MAT20, COX2 expression reduced notably **(Figure 3)**.

MAT20 downregulated BCL2 expression and upregulated BAX expression in HeLa cells. MAT20 significantly showed more expression of pro-apoptotic gene BAX and downregulated expression of anti-apoptotic gene BCL2 in HeLa cells **(Figures 4)**. These results suggest that BCL2/ BAX family members play a major role in MAT20-induced apoptosis in cervical cancer cells.

DISCUSSION

The rationale behind this work was to test the efficacy of a polyherbal drug MAT20, derived from various extracts of three plants under investigation: moringa (leaf), amla (pulp), and tulsi (leaf). This formulation induced cytotoxicity on HeLa cells at a concentration of 2.43 µg/ml.

The initial preparation of the formulation involved the dissolution of the plants in water, which was used given the significance of water as a phytochemical solvent (30). However, during standardization, the employment of different organic solvents elicited better results. Consequently, we dissolved the extracts as follows: tulsi (leaf) in methanol,
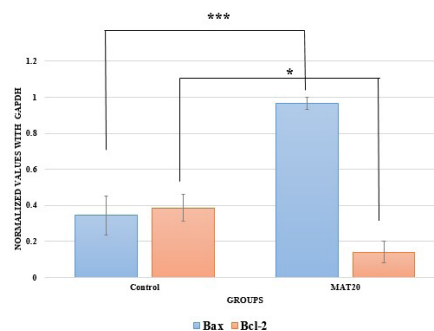
amla (pulp) in acetone, and moringa (leaf) in chloroform. The previous testing of the initial formulation yielded an IC50 value of 0.3 mg/ml while MAT20 has an IC50 of 2.43 µg/ml, which was arrived at using the CCK-8 assay. Consequently, this new formulation had a significantly lower IC50 value – 2.43 µg/mL. The herbal preparation induced remarkable cell death via apoptosis as indicated by DNA laddering with the induction of BAX gene and downregulation of BCL2.

CAM is considered a vital component required to supplement standard approaches to cancer treatment (31). Chemotherapy, surgery, and radiation, although effective, have their own limitations and side effects like fatigue, weight loss, oedema (swelling), thrombocytopenia (bleeding and bruising), and alopecia (hair loss). In late diagnosis, these forms of standard approaches do not lead to desired prognosis, and treatment may even be palliative. Alternative approaches to this could involve adopting herbal adjuvants to lead to better prognosis with reduced dosage of chemotherapy.

The anti-inflammatory properties of MAT20 were further elucidated by investigating its ability to downregulate COX2 expression induced experimentally by PMA. MAT20 was shown to downregulate COX2 gene expression. These results strongly suggest MAT20's strong anti-inflammatory capacities. MAT20 has a lower IC50 value than paclitaxel, which demonstrates IC50 values in the range of 2.5-7.5 µg/mL, demonstrating its potency as a potential CAM-supplement to conventional cancer therapies (32). Our exploration is substantiated by the existing literature which serves to prove that further studies to elucidate its mechanism of action and preclinical studies would further validate the role of phytochemical extracts in cancer management.

MATERIALS AND METHODS

Preparation of MAT20 poly-herbal formulation

Based on qualitative and quantitative analysis of different phytochemicals, the optimum extract of each plant was

chosen. A combination of the three plant extracts (organic market, Bangalore, India) was formulated by weighing 0.01 g of each dried extract and dissolved in 1 mL of dimethyl sulfoxide (DMSO). This final solution was named MAT20 and used for further studies.

Cell culture

Cryovials containing the HeLa cells were purchased from National Centre for Cell Science, Pune. Passage number four has been used for this study. After rapid thawing, cells were cultured in Dulbecco's Modified Eagle Media (DMEM/F-12) with 10% Fetal Bovine serum (Himedia). After centrifugation (1500 rpm for 10 minutes), cells were counted using a hemocytometer. The cells were cultured and maintained at $37^{\circ}C$ with 5% CO2.

CCK-8 protocol for cell cytotoxicity

The CCK-8 assay protocol was purchased from Sigma (Cat. No. 96992-100tests-F). We cultured 3×10^3 HeLa cells per well using 96-well plates. Cells were treated with different concentrations (1.57, 3.13 and 6.25µg/mL) of MAT20 and incubated for 48 hours along with the control cells without any treatment of MAT20. After the drug treatment, 10 µl of CCK-8 solution was added and incubated for 4 hours. Absorbance readings were taken at the wavelength of 450 nm using microplate reader (Lisaquant).

The percentage cell cytotoxicity was calculated by the formula:

$$\frac{Abs_{Control} - Abs_{Sample}}{Abs_{Control}} \times 100$$

From the absorbance readings obtained, the cell viability values were obtained for each concentration of each sample. The percentage viability graphs were plotted against concentration of sample for all four groups. Using the IC50. tk software (URL: www.ic50.tk/), the IC50 value for MAT20 using the CCK-8 assay was determined for each group after 48 hours of drug treatment (33).

DNA Fragmentation

DNA fragmentation was performed after 48 hours incubation with MAT20. Cells were harvested after trypsinization and lysed using 1% SDS. DNA was extracted using the phenol, chloroform, isoamyl alcohol (PCI) method and analyzed after gel electrophoresis (34, 35).

Gel Electrophoresis

Five µL of each DNA sample was mixed with 2 µL of 6X gel loading dye (Sigma) before pipetting into wells of a 1.2% agarose gel and 100 bp ladder was also loaded to observe the DNA fragmentation. The gel was run for one and half hours at 50 volts.

mRNA isolation

Control and treated cells were trypsinized and centrifuged at 1500rpm for 10minutes. To the pellet RNAiso plus (Takara: Cat.No.-9108Q) solution along with chloroform was added and vortexed. The cell suspension was incubated for 15minutes at room temperature and then centrifuged at 12,000rpm for 15minutes. Aqueous phase was collected into new tube and 500ul of 100% isopropyl alcohol was added to it. After 10minutes incubation suspension was centrifuged for 10minutes at 12,000rpm. After discarding the supernatant to the pellet 75% ethanol was added and kept for centrifugation at 7500rpm for 7minutes. After centrifugation supernatant was discarded and pellet kept for drying. After 45minutes to the pellet RNAse free water was added and stored at -200C for further use.

Quantification of RNA

Purity of RNA was calculated by using following formula,
Purity of RNA = absorbance @ 260 nm/ absorbance @ 280 nm
We quantified the RNA and calculated the concentration of each sample by using the standard formula:
Concentration of RNA (µg/ml) = absorbance @ 260 nm* dilution factor * 40

cDNA Synthesis

DEPC-treated tubes were used to prepare cDNA using Takara RT-PCR kit (Cat.No.-RR037A). Samples were incubated at 370C for an hour, and 1 min at 900C and then stored at -800C for further use.

Evaluation of apoptotic and anti- apoptotic genes:

After calculating the appropriate cDNA volumes, PCR reactions were set up with 6 µL of Jumpstart mix (Ct.No.-P2893-100RXN) and 1 µM of GAPDH, COX2, BAX, or BCL2

Table 1: Primers used for PCR to observe specific gene expression

Primers	Forward	Reverse
GAPDH	GGTCGGAGTCAACGGATTTGGTCG	CCTCCGACGCCTGCTTCACCAC
COX-2	CCACTTCAAGGGATTTTGGA	GAGAAGGCTTCCAGCTTTT
BAX	GCTGGACATTGGACTTCCTC	CTCAGCCATCTTCTTCCAC
Bcl-2	ATTGGGAAGTTTCAAATCAGC	TGCATTCTTGGACGAGGG

forward and reverse primers (Eurofins, **Table 1**).

Gel Electrophoresis for PCR product

Ten µL of each PCR product was mixed with 2 µL of 6X gel loading dye (Sigma) before adding the wells in a 2% agarose gel and 100 bp ladder was also loaded at the center well. The gel was run for 1 hour at 100 volts. Each gel image was exported and analyzed using ImageJ.

Data Analysis

To check experimental reliability, three independent sets of the same experiment were performed. COX2, BAX, and BCL2 mRNA levels were normalized with GAPDH levels. Statistical significance between the three groups was assessed by one-way ANNOVA using Excel software. P-value < 0.05 was considered significant. We used t-tests to determine statistical significance between the BAX and BCL2 groups, the values for which were all less than 0.05. Also, groups showed significance with a post-hoc test (Bonferroni or Tukey) to one-way ANOVA by Excel.

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