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Article

Anti-inflammatory and pro-apoptotic properties of the polyherbal drug, MAT20, in MCF-7 cells

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SUMMARY

Breast cancer remains a serious health issue with approximately 2.3 million women diagnosed with breast cancer in 2020. Standard treatment approaches include surgery, chemotherapy, and radiation. However, these treatments have side effects such as drug resistance, metastasis, and relapse. Therefore, it is necessary to investigate alternative therapy approaches that may mitigate the adverse effects of standard therapies. The emerging field of complementary and alternative medicine (CAM) presents an intriguing possibility of using natural products as therapeutics. The main objective of this study was assessing the anticancer efficacy of MAT20, a polyherbal formulation with extracts of moringa, amla, and tulsi. In this study, we examined the anti-inflammatory properties of MAT20, along with phorbol 12-myristate 13-acetate (PMA) and 5-fluorouracil (5-FU), on the MCF-7 breast cancer cell line. Specifically, we assessed the expression of the *COX2* gene, a key marker of inflammation. We hypothesized that the addition of MAT20, targeting *COX2* downregulation, may have a dual impact by not only reducing inflammation but also regulating programmed cell death pathways by apoptotic gene expression studies. Our results demonstrated that MAT20 decreased the PMA-induced *COX2* expression in MCF-7 cells. MAT20 treatment in MCF-7 cells showed upregulation of *BAX* and *BAD* (pro-apoptotic genes) and downregulation of *BCL2* and *BCL2L1* (anti-apoptotic genes) in MCF-7 cells. Overall, our results showed that MAT20 may have anti-inflammatory and pro-apoptotic effects on MCF-7 cells.

INTRODUCTION

Cancer is a major cause of death worldwide, accounting for one in six fatalities (1). Every 14 seconds a woman is identified with breast cancer making breast cancer the leading cause of death among women across the globe (2). In 2020, approximately 2.3 million new cases and 685,000 deaths from breast cancer were observed (3). In recent years, research has found that the main risk factors for breast cancer are aging, reproduction factors, and low parity (4,5).

Historically, the options for the treatment of cancer were limited to surgery, radiation, and chemotherapy (6,7). These modalities are focussed on targeting the tumour/tumour cells and thereby eliminating them from the body. Although these strategies are effective, they lead to issues such as immune suppression triggered by surgery, resistance to chemotherapy, and to radiation (8). However, in recent times, the discovery of several pathways involved in cancer has allowed the use of more targeted approaches or a combination of several approaches (9). These novel approaches to the use of cancer treatment include stem cell therapy, targeted drug therapy, gene therapy, and the use of natural antioxidants in the form of phytochemicals (10).

Complementary and alternative medicines (CAMs) are medicines that fall outside the conventional or mainstream medicine, such as homeopathy, naturopathy, mind-body medicines, chiropractic and massage therapy, bio-electromagnetic therapy, and most predominantly, the use of dietary supplements or polyherbal formulations (11). While some oncologists may be sceptical of CAMs, these methodologies have been shown to have positive effects in terms of overcoming various side effects of chemotherapy, including nausea, stress, anxiety, depression, and fatigue (12).

Moringa oleifera is predominantly found in Asia and South East Asia and contains several compounds with anti-oxidant and anti-cancer properties (13,14). *Phyllanthus emblica* fruit, often known as Indian gooseberry or amla extract, is high in polyphenols, which have been shown to have cytotoxic effects on cervical and ovarian cancer cells (15). *Ocimum sanctum*, or tulsi, belongs to the mint family, and its compounds have been found to possess several anti-cancer properties, radiation protection, and free-radical scavenging activities (16). Tulsi extract has also been shown to reduce the induction of the COX2 protein as well as three dimensional growth and morphogenesis of breast cancer cells (17).

MAT20 is a polyherbal formulation made of moringa (leaf), amla (pulp), and tulsi (leaf) (18). In our previous study, we showed cytotoxic effects and apoptotic cellular death of MCF-7 cells treated with MAT20 (19). In research, MCF-7 is a commonly utilized breast cancer cell line that is estrogen receptor/progesterone receptor (ER and PR) positive and belongs to the luminal A molecular subtype (20). MCF-7 has low aggressiveness and is considered to be a non-invasive cell line with low metastatic potential (21). We hypothesized that MAT20 has anti-inflammatory properties and that it regulates programmed cell death pathways by apoptotic gene expression changes in the MCF-7 breast cancer cell line.

Phorbol 12-myristate 13-acetate (PMA) and 5-flurouracil (5-FU) were used to induce *COX2* gene expression, an indicator of inflammation. COX2 was identified as a regulator of cell proliferation and apoptosis in solid tumors, specifically in colorectal, breast, and prostate cancers (22). We found that MAT20 was able to decrease COX2 expression in constitutive and induced conditions. MAT20 induced cell death via apoptosis, as observed by upregulation of BAX and BAD and

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downregulation of BCL2 and BCL2L1, suggesting its potential as a complementary polyherbal compound, which could be used with standard chemotherapeutic drugs.

RESULTS

Inflammatory gene expression

Increased expression of *COX2* has been linked to both cancers with a resistant phenotype and chronic inflammation (22). In our study, we hypothesized that MAT20 can downregulate *COX2* gene expression. To test our hypothesis, we examined *COX2* expression in MCF-7 cells after treatment with MAT20 or no treatment using semi-quantitative PCR and normalization to *GAPDH*. Our results showed that *COX2* expression was induced by PMA and 5-FU but reduced when treated with MAT20 at a concentration of 10.5 µg/mL, which is half of the IC_{50} for MAT20 (**Figure1**) (23).

In MCF-7 cells, treatment with PMA significantly upregulated *COX2* expression, while PMA+MAT20 treatment downregulated its expression compared to PMA alone ($p = 0.05$) **(Figure 1A)**. There was no significant difference between 5-FU and 5FU+MAT20 treatment **(Figure 1B)**. Treatment with DMSO, used as a control, did not result in a significant change in *COX2* expression. MCF-7 cells treated with MAT20 alone exhibited a decrease in *COX2* expression compared to untreated MCF-7 cells ($p = 0.05$) **(Figure 1C)**.

Pro- and anti-apoptotic gene expression

MAT20 treatment increased expression of the pro-apoptotic genes *BAX* and *BAD* ($p < 0.05$) (**Figure 2A**) and decreased expression of the anti-apoptotic genes *BCL2* ($p < 0.05$) and *BCL2L1* (**Figure 2B**). No significant change in the expression of any pro- or anti-apoptotic gene was seen in the DMSO treatment group.

DISCUSSION

Complementary and alternative medicine (CAM) techniques, such as acupuncture, herbal treatments, dietary modifications, and mind-body exercises like yoga or meditation, can lessen the negative effects of conventional cancer therapies (24, 25). Herbal treatments are frequently investigated as CAM in the treatment of cancer. Triphala, a polyherbal formulation containing three medicinal fruits, namely *Phyllanthus emblica* L. (*Emblica officinalis Gaertn*), *Terminalia chebula Retz*, and *Terminalia belerica Retz*, was investigated for its antiproliferative properties on three different cancer cell lines, namely SK-OV-3, HeLa (cervical cancer cell line), and HEC-1-B (endometrial cancer cell line) (26).

COX2 has been identified as a key factor for triggering cancer stem cell-like properties and fostering resistance to apoptosis in cancer cells (27). MAT20 is a polyherbal drug that was developed in earlier studies (22,23). This study focused on MAT20 and its anti-cancer properties on a breast cancer cell line. For this purpose, we tested the efficacy of MAT20 utilizing the MCF-7 cell line. This formulation decreased the expression of *COX2*, an inflammatory marker, in MCF-7 cells. The study also showed that MAT20 may have pro-apoptotic properties.

In conclusion, this investigation sheds light on the potential anti-inflammatory and pro-apoptotic effects of MAT20, offering a promising complementary therapeutic approach for breast cancer treatment. The study supports the further exploration of CAM formulations in combination with conventional therapies to enhance treatment outcomes and improve patients' quality of life. Future explorations could include expanding the scope of investigation to this formulation *for in vivo* studies: if MAT20 is applied directly to cells versus taken orally, the efficiency may vary greatly. *In vitro* (cell-based) studies can shed light on the drug's possible modes of action and preliminary safety information. To ascertain its effectiveness and safety when given systemically, however, it is important to move from *in vitro* investigations to *in vivo* investigations in animal studies, and eventually to human trials. However, transitioning to *in vivo* (animal) studies and eventually clinical trials in humans is necessary to determine its efficacy and safety when administered systemically.

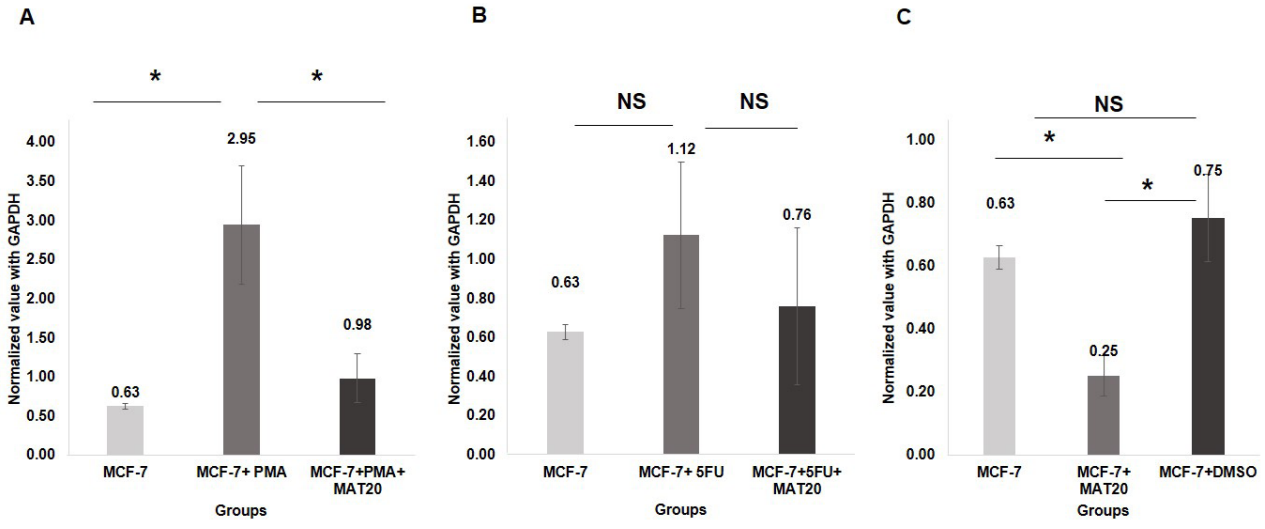

Figure 1: Impact of MAT20 on COX2 expression in MCF-7 cells. A) COX2 was significantly upregulated when MCF-7 cells were treated with PMA and lowered when this group was treated with MAT20. **B)** COX2 expression was induced when MCF-7 cells were treated with 5-FU and lowered when treated with MAT20, although it did not show statistical significance. **C)** COX2 was not significantly changed when MCF-7 cells were treated with DMSO (vehicle control). When MCF-7 was treated with MAT20 alone, COX2 expression was significantly decreased. All data presented as mean ± SEM, n = 3. *p < 0.05.

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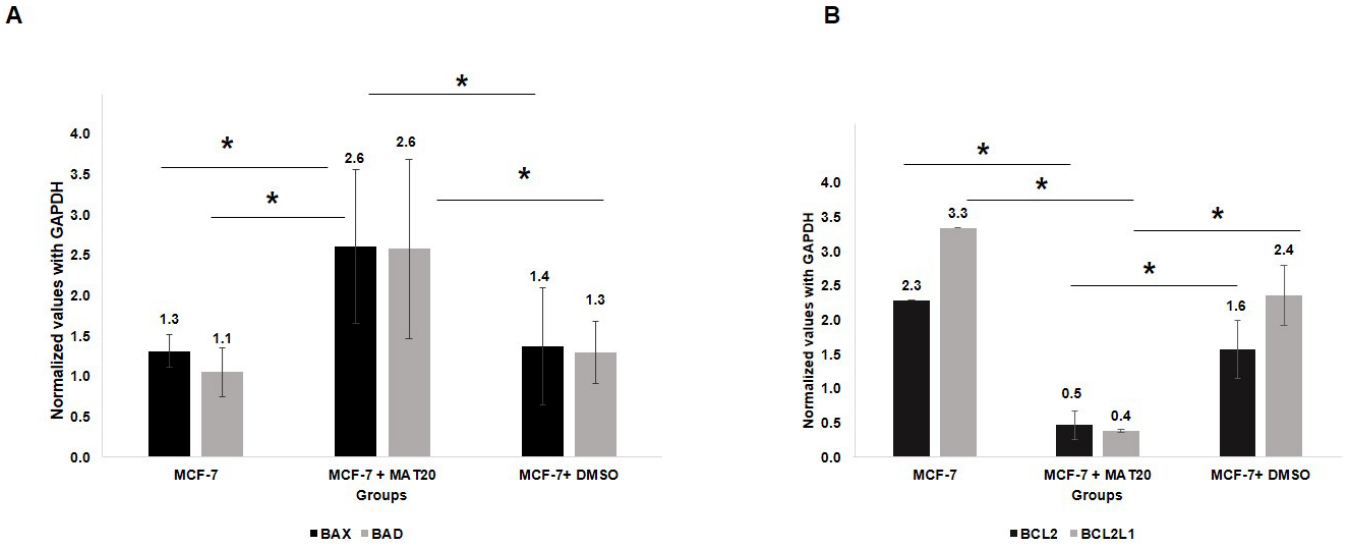

Figure 2: Differential regulation of pro and anti-apoptotic gene expression in MCF-7 cells treated with MAT20. MCF-7 cells as a control and MCF-7 cells treated with MAT20 were compared. The MAT20 group showed down-regulation of BCL2 and BCL2L1 **(A)** and upregulation of BAX and BAD **(B).** These four transcripts did not significantly change when MCF-7 cells were treated with the vehicle control, DMSO. The data are presented as mean ± SEM, n = 3. * = $p < 0.05$.

MATERIALS AND METHODS

Formulation of MAT20

MAT20 constitutes powders of moringa (leaf), amla (pulp) and tulsi (leaf) obtained from a local organic market in Bengaluru, India. A combination of the three extracts was formulated by determining the weight before and after air-drying the extracts. Subsequently, we weighed 0.1 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 IC_{50} concentration in the ratio of 1:1:1 and named MAT20 (22, 23).

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 (HiMedia) and were reseeded in T-25 flasks at a density of 3000 cells/cm2 for further studies.

Groups used for gene expression studies

MCF-7 cells were trypsinized, and counted. One million cells were cultured in a 6-well plate and incubated for 24 hours in 3 mL DMEM media. After incubation, PMA, 5-FU, and half of the IC_{50} value of MAT20 was added to the cell medium in respective groups and further incubated for 48 hours to observe the anti-inflammatory and pro-apoptotic effects of MAT20. Seven conditions were tested: MCF-7 (Control), MCF-7 + PMA (6.25 ng/mL), MCF-7 + PMA (6.25 ng/mL) + MAT20 (18.18 **µg**/mL), MCF-7 + 5-FU (2µg/mL), MCF-7 + 5-FU (2 **µ**g/mL) + MAT20 (10.54 **µg**/mL), MCF-7 + DMSO (vehicle control) (90 **µL/mL**) and MCF-7 + MAT20 (10.54 µg/mL).

mRNA isolation

Control and treated cells were trypsinized and centrifuged at 1500 rpm for 10 minutes. RNAiso plus (Takara: Cat.No.-9108Q) solution and chloroform was added to the pellet and vortexed. The cell suspension was incubated for 15 minutes at room temperature and then centrifuged at 12,000 rpm for 15 minutes. The aqueous phase was collected into a new tube and 500 uL of 100% isopropyl alcohol was added. After a 10 minutes incubation, the suspension was centrifuged for 10 minutes at 12,000 rpm. After discarding the supernatant, 75% ethanol was added to the pellet and kept for centrifugation at 7500 rpm for 7 minutes. After centrifugation, the supernatant was discarded and the pellet was kept for drying. After 45 minutes, RNAse-free water was added to the pellet, which was stored at -20°C for further use.

Quantification of RNA

Purity of RNA was calculated by using the following formula: Purity of RNA equals absorbance at 260 nm/ divided by absorbance at 280 nm. We quantified the RNA and calculated the concentration of each sample by using the standard formula: Concentration of RNA (µg/mL) equals absorbance at 260 nm multiplied by the dilution factor of 40, where, 40 equals one absorbance unit which equals 40 ug/ mL RNA at 260nm.

cDNA Synthesis

DEPC-treated tubes were used to prepare cDNA using the Takara RT-PCR kit (Cat.No.-RR037A). mRNA was diluted to 200 ng/uL using nuclease free water as per the kit instructions. To set up the reverse transcription reactions, 2 µL buffer, 0.5 µL oligo dT, 0.5 µL reverse transcriptase enzyme (RT), and 2 µL random hexamer were added to 5 µL of mRNA sample. Samples were incubated at 37°C for an hour followed by 1 min at 90°C, and then stored at -80°C for further use.

Semi-Quantitative Polymerase Chain Reaction (PCR)

Fifty ng of each cDNA sample was used to amplify the housekeeping gene (*GAPDH*), inflammatory marker *COX2*, and a set of pro-apoptotic and anti-apoptotic genes (*BAX, BAD, BCL2,* and *BCL2L1*) (**Table 1**). PCR was set up using

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Gene	Forward	Reverse
GAPDH	5' -GGTCGGAGTCAACGGATTTGGTCG -3'	5' -CCTCCGACGCCTGCTTCACCAC-3'
COX2	5' -CCACTTCAAGGGATTTTGGA -3'	5' -GAGAAGGCTTCCAGCTTTT-3'
BAX	5' -GCTGGACATTGGACTTCCTC -3'	5' -CTCAGCCCATCTTCTTCCAG-3'
BCL2	5' -ATTGGGAAGTTTCAAATCAGC -3'	5' -TGCATTCTTGGACGAGGG-3'
BAD	5' -CCTCAGGCCTATGCAAAAAG -3'	5' -AAACCCAAAACTTCCGATGG-3'
BCL2L1	5' -GGCTGGGATACTTTTGTGGA-3'	5' -AAGAGTGAGCCCAGCAGAAC-3'

Table 1: Forward and reverse primer sequences.

6 µL Jumpstart mix (Ct.No.- P2893-100RXN, Sigma) and 1 pmol (1 µL) of appropriate forward and reverse primers for each gene (Bioserve). The thermal cycling PCR conditions consisted of an initial denaturation step at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 51.7°C (*COX2*), 54°C (*BAX),* 51°C *(BAD),* 53°C *(BCL2)*, 49.5°C (*BCL2L1*), and 61.5°C (*GAPDH*) for 30 seconds, and extension at 72°C for 1 minute with a final extension at 72°C for 10 minutes. The PCR products obtained were resolved on a 2% agarose gel and were observed under a UV-transilluminator (Biobee Tech). The target gene expression was analyzed and quantified using ImageJ software and the results were normalized as a ratio to *GADPH. COX2, BAD, BAX, BCL2,* and *BCL2L1* mRNA levels were normalized with *GAPDH* levels.

Statistical Analysis

Three independent sets of the same experiment were performed. Statistical significance between two groups was assessed by one-way ANOVA with Tukey's test using Excel software. *p*-value < 0.05 was considered significant.

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