Research Article

Evaluation of *Trichosanthes dioica* Wall. Leaves against MDA-MB231 and MCF-7 Cells Along with Immunostimulant Property

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ABSTRACT

We evaluated the antiproliferative and immunostimulant activity of *Trichosanthes dioica* leaves (TDLE) on breast carcinoma cell lines (MDA-MB231 and MCF-7). The extract significantly inhibited cell viability in a time and concentration dependent manner in both the cell lines. Morphological study showed several signs of apoptosis and degraded DNA bands in carcinoma cells than control. We further elaborated the immunomodulatory effects of TDLE on RAW264.7 cell line. TDLE treated cells increases NO production. But when stimulated with rIFN-γ it showed marked enhancement in NO production. TDLE treated cells when pretreated with PDTC and NGMMA to the rIFN-γ ameliorated NO production as compared to the primed cells. TDLE acted as an accelerator in the activation of RAW264.7 cells by rIFN-γ via a process involving L-arginine-dependent NO production and elevated NO production via activation of NF-κB signaling pathway. These findings suggest that TDLE can be a potential anti-carcinoma therapeutic agent along with immunomodulatory activity.

Keywords: *Trichosanthes dioica*, leaves, breast cancer, cytotoxicity, immunomodulatory.

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INTRODUCTION

Breast cancer is one of the main life-threatening diseases that a woman may have to face during her lifetime [1]. Breast cancer is the uncontrollable cell growth of the breast cells. This affects both men and women, although it is most prevalent in women. It is the most common cancer among Indian women, with a prevalence of 25.8 per 100,000 women and a mortality rate of 12.7 per 100,000 women [2]. Breast cancer develops in many parts of the breast and might spread into various parts of the body including chest wall and skin. Triple-negative breast cancer is an aggressive form of breast cancer with limited treatment options [3]. Breast cancers are mostly treated with biological medicines other than surgeries using drugs such as Bevacizumab, Lapatinib, and Trastuzumab. However, these medications compromise our immunity, and show various comorbidity post curing of the disease. The immune system is developed to defend animals from infectious germs and cancer infiltrating their bodies. It can develop a vast array of cells and molecules capable of identifying and destroying a seemingly infinite number of foreign invaders. Immune regulation is a delicate balance between regulatory and effector cells, and any malfunction in the immune system can result in disease. By direct action or intercellular communication, nitric oxide (NO) is a primary secretory product of mammalian cells that begins host defence, homeostatic, and development activities. NO is produced when one of three isoforms of nitric oxide synthase catalyses a five-electron oxidation of the amino acid L-arginine [4]. NO is considered to activate regulatory proteins, kinases, and proteases that are regulated by reactive oxygen intermediates as a direct effector [5]. An increase in macrophage NO generation is linked to immune system activation. The concentration of NO is determined indirectly by assessing the quantities of nitrate and nitrite in the sample. Depending on the environmental circumstances and the redox state of the biological fluid, the relative quantities of nitrate and nitrite might fluctuate significantly. As a result, the most precise measurement of total nitric oxide production necessitates the quantification of both nitrate and nitrite. iNOS produces NO in macrophages in response to cytokines including interferon- γ (INF- γ), tumour necrosis factor- α (TNF- α), and interleukin-1 (IL-1), as well as microbial products like Lipopolysaccharide (LPS)[6]. NO expression has been connected to DNA damage, which stimulates the production of wild type p53 and eventually leads to apoptosis [7]. Auto-expression of iNOS can also cause NO to be produced in target cells, resulting in apoptotic cell death[8]. The increasing incidence of breast neoplasia reported over the last few decades has led to development of new anticancer drugs, drug combinations, and chemotherapy strategies by methodical and scientific exploration of enormous group of synthetic, biological, and natural products [9]. These medications may have a serious side effect, so we have used immunostimulant to

find the carcinogenic activity on the breast cancer cells. Although much pharmacological research has been done on various sections of *T. dioica*, certain other traditionally significant therapeutical applications such as, antipyretic, diuretic, cardiotonic, laxative, antiulcer, and so on may have been overlooked [10]. Vitamin A, vitamin C, tannins and saponins, as well as flavonoids and alkaloids, are chemical constituents of *Trichosanthes dioica* [11]. Despite the various claims about *Trichosanthes dioica* plant having medicinal uses, to our knowledge, no attempt has been made to scientifically confirm this plant's medicinal use in breast carcinoma cells. As a result, our current study aims to assess the anti-cancer and immunomodulatory efficacy of *Trichosanthes dioica* leaf extract in the breast cancer cells.

MATERIALS AND METHODS

Chemicals and reagents

DMEM and RPMI1640 (Gibco), fetal bovine serum(FBS), Trypsin (Gibco, USA), Penicillin-streptomycin (Biowest, Germany), Gentamycin (Nicholas, India), HEPES, L-glutamine, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide)] (Sigma), DMSO (dimethylsulphoxide), Acridine orange, Ethidium bromide, ethylene diamine tetra acetic acid (EDTA) Agarose (Puregene), Proteinase k (SRL), RNAse, Propidium iodide, rIFN-γ, PDTC, N^GMMA, Griess Reagent (Sigma), Chloroform, isoamyl alcohol, Methanol, and all other chemicals and reagents were of analytical grade and procured locally.

Cell culture

Breast carcinoma cell lines (MDA-MB231 and MCF-7) and murine macrophage cells RAW264.7 were obtained from National Facility for Animal Tissue and Cell Culture, Pune, India. The breast cancer cells were cultured and routinely maintained in DMEM medium, whereas murine macrophage cell line RAW264.7 was cultured and routinely maintained in RPMI1640 medium, and both the medium were supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100µg/ml), gentamycin (100µg/ml) and were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 inside a CO_2 incubator. Both cell lines were adherent in nature. During sub culturing of cells, the adherent property can be diminished by adding 1x trypsin solution in the cell.

Collection and identification of test samples

The leaves of *Trichosanthes dioica* were collected from Bankura, Bishnupur and Medinipur from the farmers during June 2011. The plant was authenticated by Dr. K. Karthigeyan, Scientist "C" Central National Herbarium, Botanical Society of India, Howrah, India, where a specimen was deposited for identification.

Extraction and preparation of *Trichosanthes dioica* leaves

One-kilogram leaves of *Trichosanthes dioica* plant were collected and then air dried for about 2 weeks. The leaves were powdered and extracted with methanol and concentrated in a rotary evaporator. The sticky residue was collected, and it was lyophilised to obtain a sticky extract green in colour. It was kept in a container, sealed with paraffin, and kept in room temperature. The extract was termed TDLE.

Cytotoxicity study by MTT assay

MDA-MB231 and MCF-7 cells ($1x10^5$) were seeded in 96-well sterile plates and were treated with different concentrations (10, 25, 50, 100 µg/ml) of TDLE for 24, 48 and 72 hrs. and with same concentration were grown in humidified atmosphere containing 5% CO_2 in an incubator at 37°C for 24 hrs. The untreated cells were considered as control. After 24, 48 and 72 hrs incubation 20µl of MTT (4-5mg/ml in PBS as a stock solution) was added to each well and incubated again for 4hrs at 37°C. The MTT assay is a colorimetric assay for assessing the metabolic activity of the cells or cell viability of NAD(P)H dependent cellular oxidoreductase enzymes and represents number of viable cells present [12]. These enzymes can reduce the tetrazolium dye MTT, which is yellow in colour, to insoluble purple coloured formazan. The intensity of the colour was measured at 570 nm by micro-plate manager (Reader type: Model 680 XR Bio-Rad Laboratories Inc.). The IC_{50} values were determined for the cells.

Fluorescence Microscopic Studies

MDA-MB231 and MCF-7 cells ($1x10^6$) treated with IC_{50} of TDLE for 24 h were observed using a fluorescence microscope for morphological changes. The untreated control cells and the TDLE treated cells were harvested separately, washed with PBS, and then stained with acridine orange (100 µg/ml) and ethidium bromide (100 µg/ml) (1:1). The cells were then immediately mounted on slides and observed under a fluorescence microscope for the morphological determination of the cells undergoing apoptosis.

Confocal Microscopic Studies

Breast carcinoma cells i.e., MDA-MB231 and MCF-7 ($1x10^6$) were treated with IC_{50} of TDLE for 24 hrs. After 24 hrs the untreated control cells and TDLE treated cells were harvested and washed with ice cold PBS. The cells were then stained with 10 µg/ml of propidium iodide separately for 5 min. After mounting on slides the cells were observed to see the differences in nuclear morphology between the untreated and the TDLE treated Breast carcinoma cells under confocal laser scanning microscope (Leica TCS-SP2 system, Leica Microsystem, Heidelberg, Germany) installed with an inverted microscope [Leica DM-7RB] as per Michell *et. al.*'s method [13]. Images for propidium iodide were acquired from argon/krypton laser and UV laser line using 590 nm long pass filter for propidium iodide and 450 nm band pass filter for UV images.

Detection of Apoptosis by DNA Fragmentation and Agarose Gel Electrophoresis

MDA-MB231 and MCF-7 cells were treated with IC_{50} dose of TDLE for 24 hrs. The cells were harvested and washed

twice with PBS. The cells were resuspended in 500 µl of lysis buffer (50 mM Tris-HCl, pH -8.0, 10 mM EDTA, 0.5% SDS), 100 µg/ml of proteinase K was added, and incubation was done at 55 °C for 1 hr and 37 °C overnight respectively. DNA extraction was done by following the general phenol-chloroform extraction procedure [14] and kept at -20 °C overnight. After centrifugation, DNA precipitates were washed with 70% chilled ethanol, dried and evaporated at room temperature and dissolved in TE buffer (pH 8.0) at 4 °C overnight. To detect the DNA fragments, the isolated DNA samples were electrophoresed overnight at 20 V in 1% agarose gel and stained with ethidium bromide. DNA fragmentation was observed in UV transilluminator.

Effect of TDLE on Nitric oxide (NO) production in non-primed (resting) and rIFN-γ primed (activated) RAW264.7 cells

To study the effect of TDLE on non-primed (resting) RAW264.7, the cells (1x10^6) were treated with 50, 100 and 200 µg/ml of TDLE separately for 24 hrs. Further in another set of experiment, to observe the effect of the extracts on rIFN-γ-primed mouse peritoneal macrophages, the cells (1x10^6) were activated with rIFN-γ (10 U/ml) for 6 hrs. at 37˚C in an atmosphere of 5% CO_2. The rIFN-γ-primed cells were then incubated with various concentrations of TDLE for another 18 hrs. at 37˚C in an atmosphere of 5% CO_2. NO synthesis was measured by a microplate assay plate [15]. 100 µl of each culture supernatant was allowed to react with 100 µl of Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine di-hydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance values were recorded at 540 nm.

Effect of PDTC on TDLE induced NO production in rIFN-γ primed RAW264.7 cells

It is well known that PDTC, an antioxidant compound, inhibits activation of NF-κB. As an approach to determine the signaling mechanism of the two extracts on NO production, the influence of PDTC, NF-κB inhibitor, on TDLE treated rIFN-γ primed mouse peritoneal macrophages was examined. The cells (1x10^6) were incubated in the presence of rIFN-γ (10 U/ml) with or without PDTC (100 µM) for 6 hrs. inside a CO_2 incubator. The cells were then treated separately with TDLE with various concentrations i.e., 50, 100 and 200 µg/ml for another 18 hrs. NO assay was performed using Griess reagent as described previously. The O.D values were read at 540 nm.

Effect of N^GMMA on TDLE induced NO production in rIFN-γ primed on murine macrophage cells RAW264.7

N^GMMA is the specific inhibitor of NO production in the L-arginine-dependent pathway [16]. To define if the signaling mechanism in TDLE induced NO production participates in the L-arginine-dependent pathway in mouse peritoneal macrophages, the cells (1x10^6) were incubated in the presence of rIFN-γ with or without N^GMMA (10 mM) for 6 hrs. inside a CO_2 incubator. The macrophages were then treated with 50, 100 and 200 µg/ml of each of TDLE for 18 hrs. NO assay was done and O.D values recorded at 540 nm were compared.

Effect of TDLE on Nitric oxide (NO) production in non-primed and rIFN-γ primed (stimulated) RAW264.7 cells

RAW264.7 cells (1x10^6) were seeded in 96-well plates and incubated for 24 hrs before stimulation and treatment. The cells were stimulated for 6 hrs in the presence of rIFN-γ (10 U/ml). rIFN-γ-stimulated cells were treated separately with TDLE with 50, 100 and 200 µg/ml for 18 hrs. In addition, to study the effect of the extracts on non-primed RAW264.7 cells, the cells (1x10^6) were treated with different concentrations of TDLE for 24 hrs without rIFN-γ-stimulation. Synthesis of NO was determined by assaying culture supernatants using Griess reagent as described earlier. The absorbance values of the assay samples were measured at 540 nm.

Effect of PDTC on TDLE induced NO production in rIFN-γ stimulated RAW264.7 cells

RAW264.7 cells (1x10^6) were seeded in 96-well plates for 24 hrs before stimulation. The cells were activated for 6 hrs in the presence of rIFN-γ (10 U/ml) with or without PDTC (100 µM). The cells were then treated with TDLE at 50, 100 and 200 µg/ml for 18 hrs. Culture supernatants were assayed for determination of NO production and the O.D values were recorded at 540 nm.

Effect of N^GMMA on TDLE induced NO production in rIFN-γ stimulated RAW264.7 cells

Before stimulation with rIFN-γ (10 U/ml), RAW264.7 cells (1x10^6) were seeded in 96-well plates and incubated inside a CO_2 incubator for 24 hrs. The cells were activated in the presence of rIFN-γ with or without N^GMMA (10 mM) for 6 hrs. The cells were then treated with TDLE with 50, 100 and 200 µg/ml, for 18 hrs. Synthesis of NO was determined by performing the NO assay with the culture supernatants. The O.D values were recorded at 540 nm with microplate reader and comparison was done.

Statistical Analysis

Percentage of cell growth inhibition was calculated by the following formula:
Percentage Cell Inhibition= 100 × (O.D of Control – O.D. of treated ÷ O.D. of Control)

O.D.= Optical Density. Percentage of cell viability was calculated as follows: Viable Cells (%) = (Total number viable cells per ml ÷ Total number of cells per 1ml) ×100.

RESULTS

Cytotoxicity Study and Cell Growth Inhibition Study

TDLE concentrations of 10, 25, 50, 100 µg/ml significantly inhibited the growth of MDA-MB231 cells compared with that of the control cells after 24, 48 and 72 hrs whereas the MCF-7 cells were compared with control cells for 24 and 48 hrs of treatment in a concentration-dependent manner. In the MTT assay, there was significant concentration-dependent reduction in the O.D values after treating the

MDA-MB231 as well as MCF-7 cells with 10, 25, 50, 100 µg/ml of TDLE. These observations provide proof for cytotoxic nature of TDLE on MDA-MB231 cells (Figure 1) and MCF-7 cells (Figure 2).

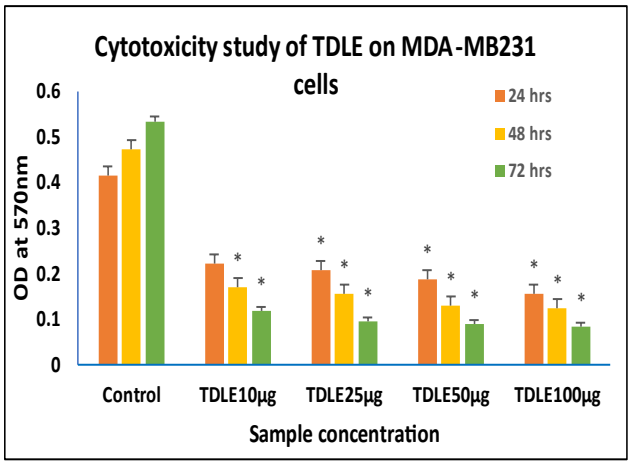

Figure 1: Cytotoxicity study of TDLE concentration of 10, 25, 50 and 100 µg/ml on MDA-MB231 cells. Data are mean ± S.E.M. * denotes significant decrease in cell count from control values p<0.05.

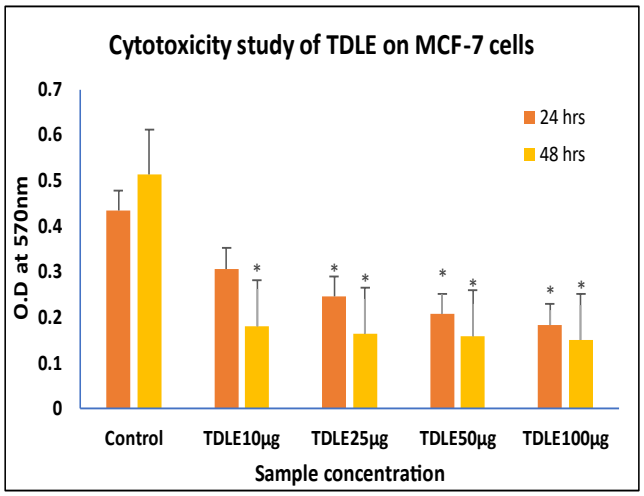

Figure 2: Cytotoxicity study of TDLE concentration of 10, 25, 50 and 100 µg/ml on MCF-7 cells. Data are mean ± S.E.M. * denotes significant decrease in cell count from control values p<0.05.

Fluorescence Microscopic Studies

Observations revealed that TDLE treated with both the breast carcinoma cells (MDA-MB231 and MCF-7) were stained with both acridine orange and ethidium bromide compared with that of the untreated control cells, stained with only acridine orange, indicating the fact that the treatment with TDLE brought about apoptotic changes in the cells like condensation of chromatin and nuclear fragmentation.

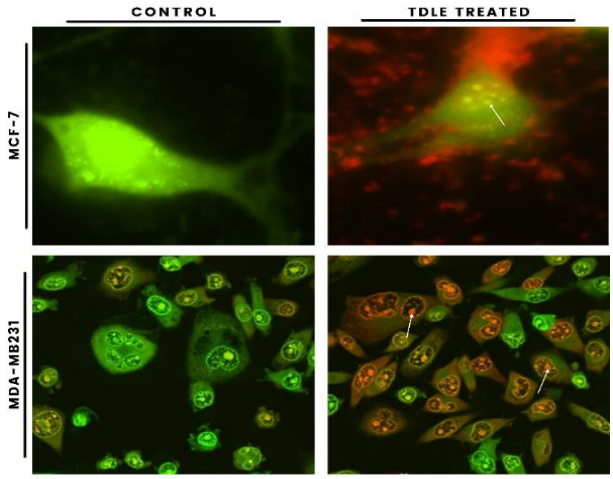

Figure 3: Fluorescence microscopic images of both the breast carcinoma cells and TDLE treated cells with IC50 dose. The control cells give a bright green fluorescence whereas the TDLE treated cells show an orange-red colour (indicated in white arrow), demarking the occurrence of apoptosis in breast carcinoma, MCF-7 and MDA-MB231 cells.

Confocal Microscopic Studies

TDLE induced apoptotic changes in all the breast carcinoma cells (MDA-MB231 and MCF-7) after 24 hrs of treatment showing chromatin disintegration and formation of apoptotic bodies whereas the untreated control cells were with intact nuclei.

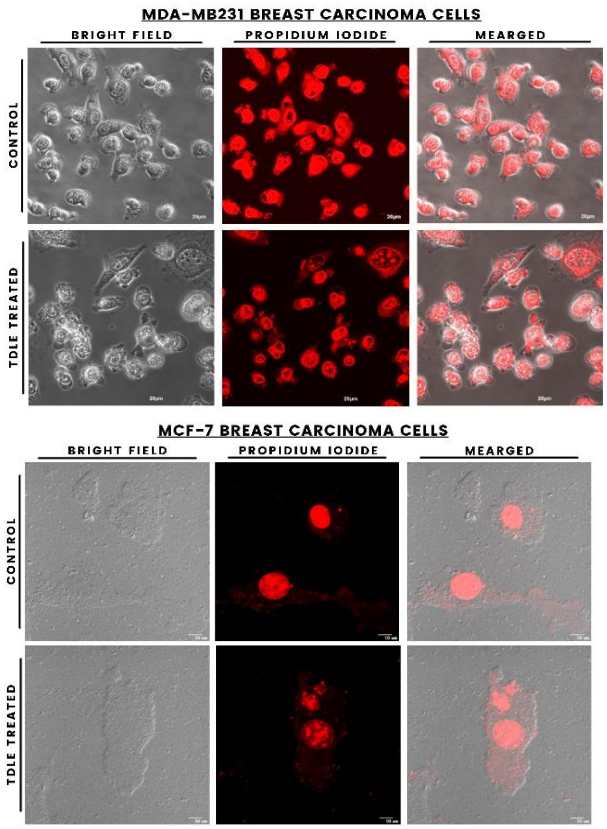

Figure 4: Confocal microscopic images of MDA-MB231 and MCF-7 cells showed apoptotic changes like nuclear disintegration and formation of apoptotic bodies for ethyl

acetate (ELA) fraction of TDLE treated cells stained with propidium iodide whereas untreated control cells showed intact nuclei.

Detection of Apoptosis by DNA Fragmentation and Agarose Gel Electrophoresis

The gel pattern of the DNA samples isolated from untreated control MDA-MB231, and MCF-7 cells showed intact DNA bands whereas the gel pattern of the DNA samples isolated from TDLE treated MDA-MB231 and MCF-7 cells showed degraded DNA bands in the form of ladders. So, the observations confirmed that the treatment with TDLE caused apoptosis in both the breast carcinoma cell.

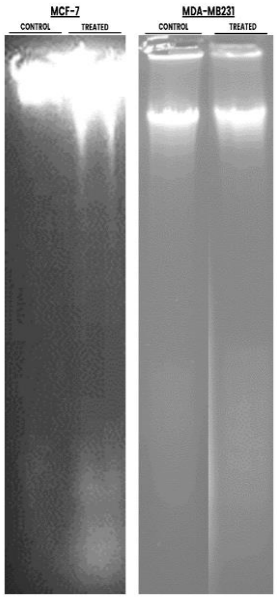

Figure 5: DNA fragmentation by agarose gel electrophoresis in MCF-7 and MDA-MB231 cell.

Effect of TDLE on Nitric oxide (NO) production in non-primed and rIFN-γ primed (stimulated) RAW264.7 cells

NO production was slightly elevated in resting RAW264.7 cells treated with TDLE.

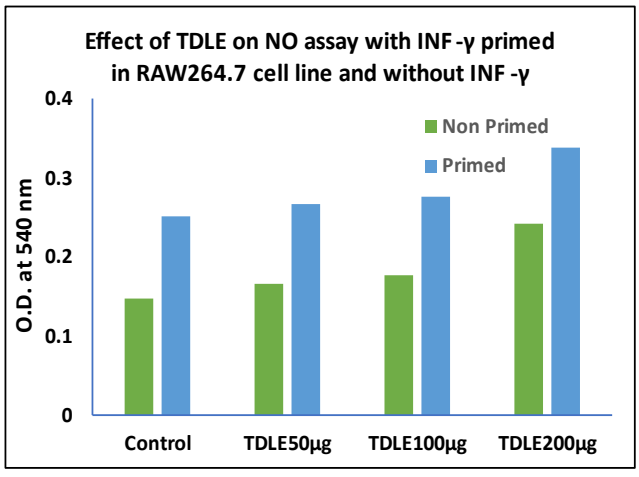

Figure 6: Effect of TDLE on NO production in resting and rIFN-γ stimulated RAW264.7 cell line.

However, when the cells were activated with rIFN-γ for 6 hrs. and then treated with TDLE, NO production was markedly enhanced compared with that of non-primed conditions.

Effect of PDTC on TDLE induced NO production in rIFN-γ stimulated RAW264.7 cells

Pre-treatment with PDTC to the rIFN-γ plus TDLE stimulated and to the rIFN-γ plus TDLE stimulated RAW264.7 cells caused significant block in the production of NO.

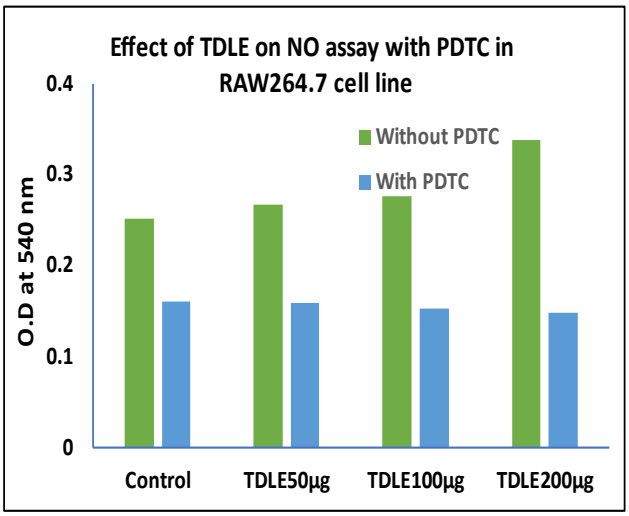

Figure 7: Effect of TDLE on NO production in rIFN-γ stimulated RAW264.7 cell line with and without PDTC.

Effect of N^GMMA on TDLE induced NO production in rIFN-γ stimulated RAW264.7 cells

The production of NO by rIFN-γ plus TDLE and rIFN-γ plus TDLE in RAW264.7 cells was significantly decreased due to the pre-treatment of N^GMMA.

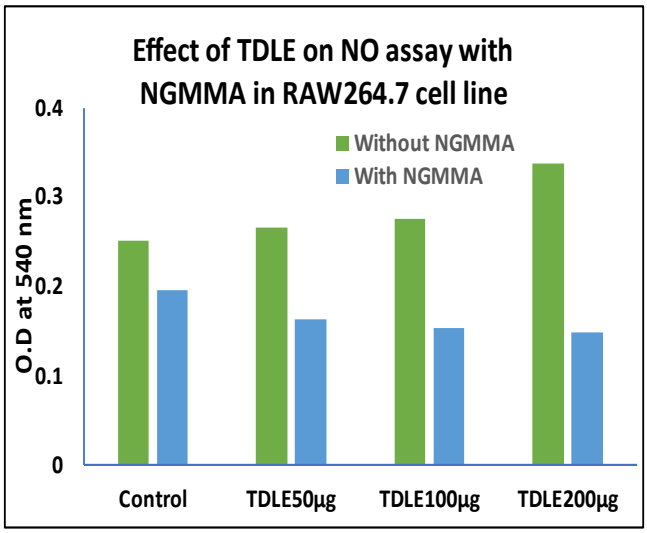

Figure 8: Effect of TDLE on NO production in rIFN-γ stimulated RAW264.7 cell line with and without N^GMMA.

DISCUSSION

This study reveals that the methanolic leaf extract of *Trichosanthes dioica* (TDLE) possesses apoptosis and immunostimulant activities. The cytotoxic activity was observed on human breast cancer (MDA-MB231 and MCF-7) cell lines. The extract showed significant inhibition in cell growth and metabolic activities of both the cell lines in a time and concentration dependent manner. IC_{50} dose of TDLE was 50.94 µg/ml on MDA-MB231 and 85.52 µg/ml on MCF-7 cells respectively. The effect of TDLE was further studied on cell morphology by fluorescence microscope and confocal microscope. Fluorescence microscopic images of both the cells clearly showed nuclear disintegration of TDLE treated MDA-MB231 and MCF-7 cells which were compared to the untreated control cells when stained with AO/EtBr and propidium iodide. Confocal microscopic images of both the cells showed chromatin condensation, nuclear fragmentation, and formation of apoptotic bodies. Further, the gel patterns of agarose gel electrophoresis of TDLE treated breast cancer cells showed ladder like disintegration supporting the apoptosis activity of TDLE.

The response of living tissues to trauma or injury is referred to as inflammation. It entails a complicated set of events, including enzyme activation, mediator release, fluid extravasation, cell migration, tissue destruction, and repair. Nuclear factor kappa (NF-kappa), a transcription factor, plays an important role in the regulation of immune and inflammatory responses. NF-κB proteins are involved in the activation of an exceptionally large number of genes in response to infection, inflammation, and other stressful situations. Excessive cytokine-mediated inflammation is likely behind several disease processes such as sepsis, ARDS, and many chronic inflammatory conditions [17]. Several studies have shown that NF-κB is needed for the endotoxin-induced transcription of genes coding to produce many proinflammatory cytokines: TNF-α, IL-1β, IL-2, IL-6, and IL-8. Intracellular adhesion molecules, ICAM-1, and E-selectin, also require NF-κB for transcription, as well as cyclooxygenase-2. The inducible form of nitric oxide synthase (iNOS) is also under the control of NF-κB [18,19]. Since observations showed that pre-treatment with PDTC blocked NO production by resting and rIFN-γ-stimulated, TDLE treated RAW264.7 cells, these findings might explain that TDLE influence NO production via the NF-κB signalling pathway. The results of this study strongly suggest that TDLE may provide a second signal for induction of NO production in RAW264.7 cells. The strong inhibition of NO production by N^GMMA indicates that the signalling mechanism in TDLE induced NO production participates in the $_L$-arginine-dependent pathway in RAW264.7 cells. Our results demonstrated that TDLE acted as an accelerator of RAW264.7 cells by rIFN-γ via a process involving $_L$-arginine-dependent NO production and elevated NO production via activation of NF-κB signalling pathway. In conclusion, it can be said that the immunomodulatory activity of TDLE might explain their beneficial synergistic effect in the treatment of cancer.

CONCLUSION

Our study demonstrated that the effect of TDLE on cancer cells showed apoptosis and extract was itself able to induce NO production in RAW264.7 cells and strong elevation of NO by rIFN-γ plus TDLE activated RAW264.7 cells. These cells were markedly inhibited by pre-treatment with PDTC, an inhibitor of NF-κB. N^GMMA, an analogue of $_L$-arginine, inhibited TDLE induced NO production in rIFN-γ-activated RAW264.7 cells.

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