

## Synergistic anticancer activity of *Curcuma longa*, *Tinospora cordifolia*, and *Moringa oleifera* (CTM) or MCF-7 Breast cancer cell line.

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### Abstract

Breast cancer is one of the most prevalent and lethal cancers affecting women worldwide, with limited treatment options in advanced stages. Natural plant-derived compounds have gained attention as potential anticancer agents due to their safety and efficacy. In this study, we investigated the anticancer effects of a synthesized compound, C.T.M., derived from *Curcuma longa*, *Tinospora cordifolia*, and *Moringa oleifera*, against the human breast cancer cell line MCF-7. The cytotoxicity of C.T.M. was assessed using an MTT assay, revealing a dose-dependent inhibition of cell proliferation, with a half-maximal inhibitory concentration (IC<sub>50</sub>) of 50  $\mu$ M after 24 hours. Further analysis showed that C.T.M. induced apoptosis in MCF-7 cells, as evidenced by morphological changes, an increase in PI-positive cells in the Acridine Orange/Propidium Iodide (AO/PI) dual staining assay, and Annexin-V/PI flow cytometry analysis. The apoptosis induction was associated with mitochondrial membrane potential ( $\Delta\Psi$ m) depolarization and increased reactive oxygen species (ROS) production. Co-treatment with N-acetyl cysteine (NAC), a ROS scavenger, significantly reduced ROS levels, confirming that ROS generation plays a critical role in C.T.M.-induced apoptosis. Additionally, the expression of caspase-3 and BCL-2 was altered, indicating activation of the caspase-3-dependent intrinsic apoptotic pathway. Our results suggest that C.T.M. significantly inhibits cell proliferation and induces apoptosis in MCF-7 breast cancer cells via a ROS-dependent mechanism, highlighting its potential as a therapeutic agent in cancer treatment...

**Keywords:** MCF-7, antioxidant, anticancer, *Curcuma longa* and *Tinospora cordifolia*, *Moringa oleifera* combination, GC-MS, AO-PI

### INTRODUCTION

Breast cancer is one of the most common and aggressive types of cancer, with significant morbidity and mortality worldwide. According to global cancer statistics, breast cancer accounts for approximately 25% of all cancer cases in women, and its incidence is expected to rise in the coming decades due to factors such as lifestyle changes and

improved detection methods (World Health Organization, 2020). Despite advances in early detection and treatment, including surgery, chemotherapy, and radiation, the development of resistance to conventional therapies remains a major challenge, necessitating the exploration of novel therapeutic approaches (Hanahan & Weinberg, *et al.*, 2011).

Phytochemicals derived from plants have garnered attention in recent years due to their potential anticancer properties. Several bioactive compounds from plants have demonstrated efficacy in modulating cancer cell growth and inducing apoptosis (Bhatia *et al.*, 2015). Among these, compounds from *Curcuma longa* (turmeric), *Tinospora cordifolia* (Guduchi), and *Moringa oleifera* (drumstick) have shown promise in preclinical cancer models. *Curcuma longa* contains curcumin, which has been reported to possess anti-inflammatory, antioxidant, and anticancer properties, including the ability to induce apoptosis and inhibit metastasis (Aggarwal *et al.*, 2003). *Tinospora cordifolia*, traditionally used in Ayurvedic medicine, has been shown to possess immunomodulatory and anticancer effects, potentially via modulation of the apoptotic pathways and antioxidant activity (Bandyopadhyay *et al.*, 2003). *Moringa oleifera*, often referred to as the “drumstick tree,” is another plant with a rich profile of bioactive compounds that exhibit anticancer properties, including the inhibition of cell proliferation and induction of apoptosis in various cancer cell lines (Fotsing *et al.*, 2018).

Recent studies have combined the bioactive components from these plants to form novel compounds, which are believed to synergistically enhance their therapeutic effects. A previous molecular docking study indicated that the combination of *Curcuma longa*, *Tinospora cordifolia*, and *Moringa oleifera* might offer promising binding affinities to cancer-related targets (Rathi *et al.*, 2019). Based on these findings, the present study aims to evaluate the anticancer potential of a synthesized compound, hereafter referred to as CTM, derived from these plants. The goal is to assess its ability to inhibit the growth and induce apoptosis in MCF-7 human breast cancer cells, with a particular focus on its mechanism of action involving oxidative stress and mitochondrial dysfunction.

## Materials and methods

### Material and chemicals

The analytical grade solvents used for extraction and chromatographic to separation were purchase from Cisco Research Laboratories Pvt. Ltd (SRL) Co. (Mumbai, India). DMEM grade percolated fetal bovine serum (SRL) (Hi Media) Hi Media pvt ltd. TLC plate (20–20 cm), silica gel F254, and RP the silica gel were purchased from Sigma-Aldrich Chemicals (Darmstadt, Germany). Did. Sulforhodamine-B (SRB) and doxorubicin purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile, methanol, formic acid (HPLC grade) were purchased from the Thermo Fisher Scientific, Waltham, MA, USA. Water was purified using the Indian Milli-Q system (Banaras Hindu University). Breast cancer MCF-7 cell line was purchased from National Institute of Cell Science, Pune, and Maharashtra. The MCF-7 breast cancer cell line and the 293 cell line donated by the Center of Excellence for Molecular Genetics of Cancer and Human Diseases (Centre for Genetic Diseases BHU). Ethanol, methanol, DMSO, and other chemicals used of the analytical grade.

### Plant material

Samples of *Curcuma longa*, *Tinospora cordifolia*, and *Moringa oleifera* (C.T.M) were collected from our fields in eastern India in January 2024. Collection procedures were performed in accordance with national and international guidelines legislation. Plant material was sourced and identified by Banaras Hindu University (BHU) staff and dried to obtain hexane extract. (C.T.M) powder was prepared by Shariya Tantra Laboratory, Department of Ayurveda (IMS BHU).

### Extraction procedure

Powder samples of *Curcuma longa* and *Tinospora cordifolia*, *Moringa oleifera* (C.T.M) were extracted by reflux method using a Soxhlet cellulose filter (Cytiva). Powder samples were refluxed for 6 h at a solid-liquid ratio of 1:60 (w/v) using *Curcuma longa* and *Tinospora cordifolia*, *Moringa oleifera* (CTM) 99.9% hexane and distilled water for CTM (Moldovan ML *et al.*, 2019). The extracted products were either extracted with hexane on a rotary evaporator or extracted with water using a freeze-drying method and evaporated. The yield of each CTM extract was calculated, and the extracts were stored in a –20 °C refrigerator for further use (Fauzantoro A *et al.*, 2017).

### Extracted sample preparations

Antioxidant and anticancer activity measurements were performed on three extracted samples containing three combinations. These are: *Curcuma longa* crude extract, *Tinospora cordifolia* crude extract, *Moringa oleifera* combination (*Curcuma longa* 50%: *Tinospora cordifolia* 50%: *Moringa oleifera* 50%). First, dried crude extracts of *Curcuma longa* and *Tinospora cordifolia* and *Moringa oleifera* were dissolved in DMSO (Sigma-Aldrich; Merck KGaA) to the generate stock solutions. Each stock solution was prepared at a concentration of 500 mg/mL. The extracted samples was mixed to produce the above solution. To prepare combinations, individual extracts was prepared from stock extracts of the same concentration. These combinations to the mixed and shaken vigorously

to obtain the desired combinations in their respective proportions. For the cell treatments (cytotoxicity, cell proliferation, and apoptosis assays), extract sample stocks were mixed in complete DMEM [DMEM mixed with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotic-antimitotic 100X freshly diluted to graded concentrations. (Penicillin, streptomycin, amphotericin B; Thermo Fisher Scientific, Inc.) Before the use. The final treatment medium contained approximately 0.1% DMSO. The negative control for cancer treatment was 0.1% DMSO (untreated cells) and the positive control was cells treated with 25  $\mu$ M cisplatin (Center for Genetic Diseases). All tests were run three times.

#### Physico-chemical analysis of curcuma longa and tinospora cordifolia, moringa oleifera mix extract

##### pH of curcuma longa and tinospora cordifolia, moringa oleifera mix extract

pH was measured at 30 °C in Department of Shalya Tantra, IMS (BHU). It was found at 6.49.

#### LC–MS analysis

##### Sample preparation

The LC-MS was performed by the SATHI (CDC) BHU

Samples from the Hexane extract and CH<sub>2</sub>Cl<sub>2</sub> fraction was prepared in MeOH (LC–MS grade) in the concentration of 50 mg/mL. The solution was then filtered and stored in the sealed glass vials until analysis.

The Liquid chromatography diode array detector-quadruple time-of-flight mass spectrometry (LC-DAD-QToF). Liquid chromatographic system was Thermo Fisher Scientific Series TSQ03 and separation was achieved on an Acquity UPLC™ HSS C18 column (100 mm × 2.1 mm I.D. 1.8  $\mu$ m). The mobile phase composed of water with 0.1 formic acid (A) and acetonitrile with 0.1 formic acid (B) at an inflow rate of 0.21 mL/min. The analysis of performed using the following grade elution 95 A/ 5 B to 35B in 15 min, in coming 5 min to 70B and eventually to 100 B in coming 2 min. A 3 min marshland followed each run with 100 B and a balance period of 5 min with 95 A/ 5 B. One microliter to the sample was fitted. The column temperature 40 °C.

The mass spectrometric analysis performed with a QTOF- MS – MS( Model# TSQ03, Thermo Fisher Scientific, India) equipped with an ESI source using the following parameters drying gas( N<sub>2</sub>) inflow rate, 13 L/ min; drying gas temperature, 300 °C; nebulizer pressure, 30 psig, sheath gas temperature, 400 °C; sheath gas inflow, 12 L/ min; capillary voltage, 4000 V; snoot voltage, 0 V; ladle, 65 V; Apr RF V, 750 V; and fragment or voltage, 150 V. All the operations, accession and analysis of data were controlled by thermo Fisher Chrome Leon 7.3.2 CDS Software Ver.A.10.1 and processed with Chrome Leon Qualitative Analysis software Ver.B.10.00. Each sample was analyzed in positive and negative modes over the range of m/z = 50–1100 and extended dynamic range of (flight time to m/z 1700 at 2 GHz accession rate). Accurate mass measures were obtained by means of reference ion correction using reference millions at m/z 121.0509 (protonated purine) and 922.0098 (protonated hexakis (1H,1H,3H- tetrafluoropropoxy) phosphazine or HP- 921) in positive ion mode, while at m/z 112.9856 (deprotonated trifluoroacetic acid- TFA) and 1033.9881 (TFA adducted HP- 921) were used in negative ion mode. Samples were analyzed in all- ion MS – MS mode, where trial 1 was carried out with collision energy of zero and trial two with a fixed collision energy of 45 Ev

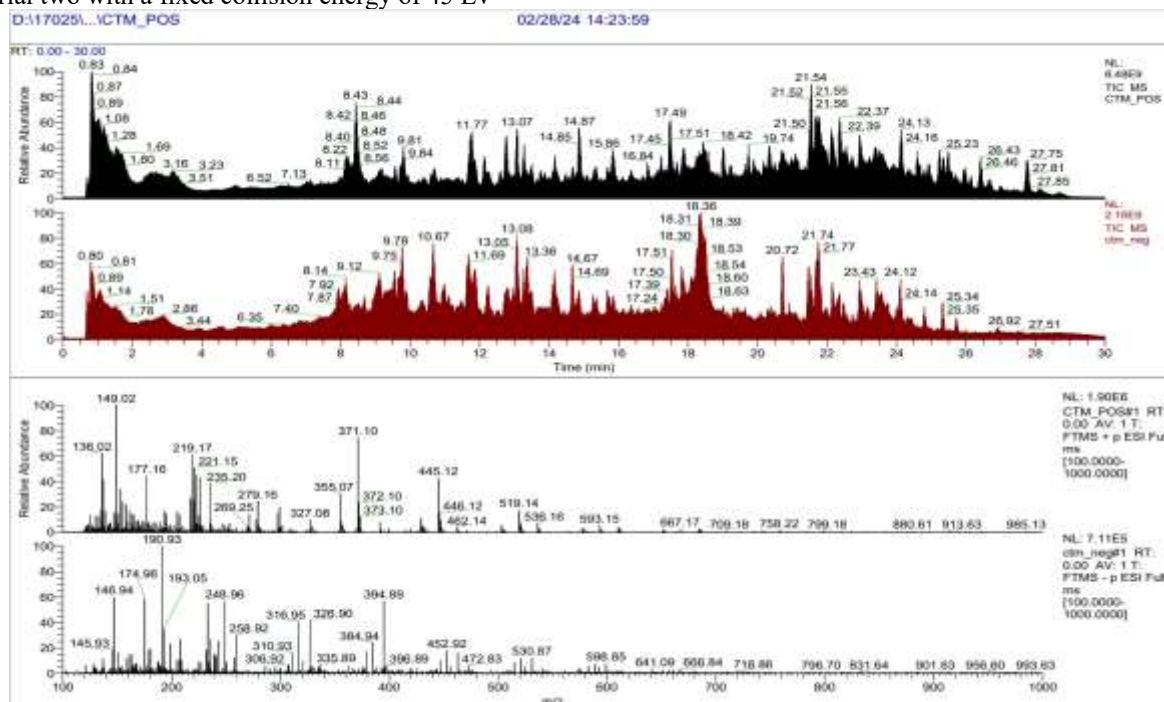


Fig:1.

| S.No | RT (min) | Compound Name                                                              | Molecular Formula  | M.W       |
|------|----------|----------------------------------------------------------------------------|--------------------|-----------|
| 01   | 0.746    | Glycerol 3-phosphate                                                       | C3 H9 O6 P         | 172.01361 |
| 02   | 0.765    | L-Aspartic acid                                                            | C4 H7 N O4         | 133.03741 |
| 03   | 0.804    | D-(-)-Quinic acid                                                          | C7 H12 O6          | 192.06331 |
| 04   | 0.828    | 3-(2-Fluorophenyl)-3H-spiro[2-benzothiophene-1,4'-piperidine               | C18 H18 F N S      | 299.11363 |
| 05   | 0.851    | Uridine                                                                    | C9 H12 N2 O6       | 244.06923 |
| 06   | 0.853    | Homovanillic acidsulfate                                                   | C9 H10 O7 S        | 262.01454 |
| 07   | 0.854    | Quebrachitol                                                               | C7 H14 O6          | 194.07875 |
| 08   | 0.873    | 3,4,5-trihydroxycyclohex-1-ene-1-carboxylic acid                           | C7 H10 O5          | 174.05275 |
| 09   | 1.026    | Microcyst Biopterin A                                                      | C18 H27 N5 O8      | 441.18437 |
| 10   | 1.039    | Citraconic acid                                                            | C5 H6 O4           | 130.02659 |
| 11   | 1.15     | N-Acetyl-DL-glutamic acid                                                  | C7 H11 N O5        | 189.06367 |
| 12   | 1.229    | (R)-2_3-Dihydroxy-3-methylbutanoate                                        | C5 H10 O4          | 134.05781 |
| 13   | 1.23     | 5-(3-nitrophenyl)-4-(prop-2-en-1-yl)-4H-1,2,4-triazole-3-thiol             | C11 H10 N4 O2 S    | 262.0516  |
| 14   | 1.253    | Guanosine                                                                  | C10 H13 N5 O5      | 283.09128 |
| 15   | 1.296    | Sangivamycin                                                               | C12 H15 N5 O5      | 309.1059  |
| 16   | 1.501    | Microcyst biopterin A                                                      | C18 H27 N5 O8      | 441.18458 |
| 17   | 1.594    | 2-Aminooctanedioic acid                                                    | C8 H15 N O4        | 189.10006 |
| 18   | 1.672    | Pyrogallol                                                                 | C6 H6 O3           | 126.03156 |
| 19   | 1.701    | N1-[4-(1H-pyrrol-1-yl)phenyl]-3,5-di(trifluoromethyl)benzene-1-sulfonamide | C18 H12 F6 N2 O2 S | 434.05198 |
| 20   | 1.996    | Citraconic acid                                                            | C5 H6 O4           | 130.02655 |
| 21   | 2.029    | Glutaric acid                                                              | C5 H8 O4           | 132.04219 |
| 22   | 2.064    | Trans-Aconitic acid                                                        | C6 H6 O6           | 174.0164  |
| 23   | 2.103    | Bendiocarb                                                                 | C11 H13 N O4       | 223.08424 |
| 24   | 2.335    | Penipacid C                                                                | C10 H10 N2 O4      | 222.0639  |
| 25   | 2.605    | Gentisic acid                                                              | C7 H6 O4           | 154.02664 |
| 26   | 2.611    | Citraconic acid                                                            | C5 H6 O4           | 130.02656 |
| 27   | 2.795    | Homovanillic acid sulfate                                                  | C9 H10 O7 S        | 262.01445 |
| 28   | 3.835    | Pantothenic acid                                                           | C9 H17 N O5        | 219.11053 |
| 29   | 3.926    | Tyramine glucuronide                                                       | C14 H19 N O7       | 313.11574 |
| 30   | 3.943    | Cordycdipeptide A                                                          | C10 H17 N3 O3      | 227.1268  |
| 31   | 4.252    | Aloesin                                                                    | C19 H22 O9         | 394.12619 |
| 32   | 4.979    | Isophthalic acid                                                           | C8 H6 O4           | 166.0266  |
| 33   | 4.993    | 2-Isopropylmalic acid                                                      | C7 H12 O5          | 176.0684  |
| 34   | 5.16     | Gentisic acid                                                              | C7 H6 O4           | 154.0266  |
| 35   | 5.507    | Salicylic acid                                                             | C7 H6 O3           | 138.0316  |
| 36   | 5.61     | Vanillin                                                                   | C8 H8 O3           | 152.0473  |

|    |        |                   |               |          |
|----|--------|-------------------|---------------|----------|
| 37 | 5.913  | Salicylamide      | C7 H7 N O2    | 137.0476 |
| 38 | 6.954  | Cathinone         | C9 H11 N O    | 149.084  |
| 39 | 7.125  | Isophthalic acid  | C8 H6 O4      | 166.0265 |
| 40 | 7.186  | Geniposide        | C17 H24 O10   | 388.1364 |
| 41 | 7.197  | Homovanillic acid | C9 H10 O4     | 182.0578 |
| 42 | 7.354  | Linamarin         | C10 H17 N O6  | 247.1054 |
| 43 | 7.356  | Caffeic acid      | C9 H8 O4      | 180.0422 |
| 44 | 7.366  | Ferulic acid      | C10 H10 O4    | 194.0579 |
| 45 | 7.436  | Tuliposide B      | C11 H18 O9    | 294.0947 |
| 46 | 7.489  | Thalictricoside   | C19 H27 N O12 | 461.1528 |
| 47 | 8.971  | Syringic acid     | C9 H10 O5     | 198.0536 |
| 48 | 8.992  | Flutimide         | C12 H18 N2 O3 | 238.1316 |
| 49 | 11.303 | Itaconic acid     | C5 H6 O4      | 130.0264 |
| 50 | 12.72  | Phaseolic acid    | C12 H22 O6    | 262.1412 |

### Biological Studies

Encouraged by the promising results from molecular docking studies, which indicated a strong binding affinity between *Curcuma longa*, *Tinospora cordifolia*, and *Moringa oleifera* derivatives with key cancer-related targets, the synthesized compound (C.T.M) was further investigated for its anticancer potential. The chemicals used in these studies were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified. The MCF-7 cell line, representing human breast cancer, was procured from the National Centre for Cell Science (NCCS), Pune, India.

### Preparation of Culture Medium and Working Concentrations of *Curcuma longa*, *Tinospora cordifolia*, and *Moringa oleifera* Derivatives

The cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/mL C.T.M). To prepare the working concentrations of C.T.M (5, 2.5, 1, 0.5, 0.25, 0.1 mg), stock solutions of C.T.M (10 mM in DMSO) were diluted in freshly prepared DMEM. The osmolarity ( $290 \pm 5$  mOsmol) of the culture medium remained stable after adding C.T.M. The medium without C.T.M was used as a control.

### MTT Assay

To assess the cytotoxicity of C.T.M, an MTT metabolism assay was conducted. The MCF-7 cells (10,000 cells/well) were seeded in 24-well tissue culture plates for 24 hours. The cells were then treated with varying concentrations of C.T.M (5, 2.5, 1, 0.5, 0.25, 0.1 mg) for another 24 hours in a CO<sub>2</sub> incubator. MTT working solution (5 mg/mL) was prepared in PBS, and 10  $\mu$ L was added to each well. After 4 hours of incubation, 100  $\mu$ L of DMSO was used to dissolve the formazan crystals. Absorbance was measured at 590 nm using a Bio-Rad 680 microplate reader. The effective concentration (EC<sub>50</sub>) was calculated using GraphPad Prism 4 (GraphPad Software, USA) via nonlinear regression analysis. Experiments were repeated at the least three times Fig:2. Cell survival was calculated using the formula:

Cell survival percentage = (Absorbance of control cells / Absorbance of treated cells)  $\times$  100

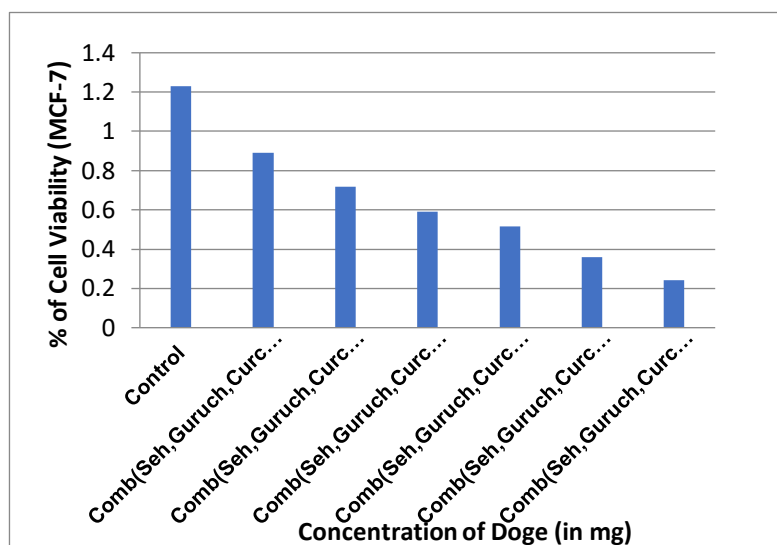


fig.2: MTT Assay

### Biological Studies and Apoptosis Induction

We investigated whether the synthesized compound (C.T.M) exerts an anticancer effect against breast cancer and, if so, through what mechanism. Breast cancer is one of the most prevalent cancers in women worldwide. Over the past decade, various medical interventions such as surgery, chemotherapy, and radiation therapy have been employed to combat this disease.

As shown in Figure 2, Curcuma longa, Tinospora cordifolia, and Moringa oleifera inhibited the metabolic activity of MCF-7 cells in a dose-dependent manner. We determined that the half-maximal inhibitory concentration (IC<sub>50</sub>) of (C.T.M) was 50  $\mu$ M after 24 hours of treatment.

To assess whether the cytotoxic effect of (C.T.M) on MCF-7 cells was associated with the induction of apoptosis, multiple tests were conducted. Figure 4 illustrates that Curcuma longa, Tinospora cordifolia, and Moringa oleifera induced morphological characteristics of apoptosis. This was evidenced by an increase in the population of PI-positive cells (early apoptosis) in the acridine orange/propidium iodide (AO/PI) double staining assay and a rise in PI-positive cells (late apoptotic cells) in the annexin V/PI FACS assay compared to controls.

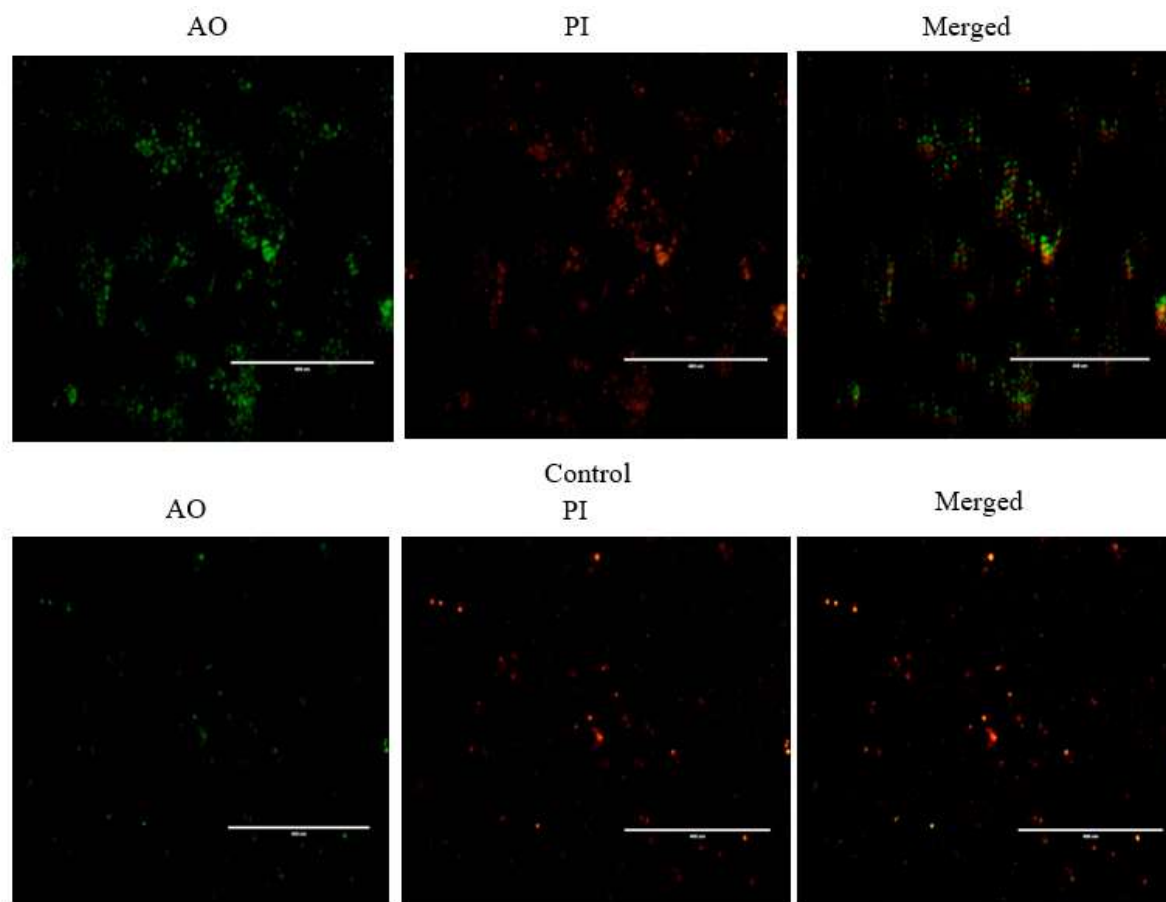
Our findings suggest that the synthesized compound exhibits a remarkable ability to inhibit cell proliferation and induce apoptosis in MCF-7 cells. To further evaluate mitochondrial membrane potential ( $\Delta\Psi$ m) in response to (C.T.M) treatment, cells were stained with the fluorochrome JC-1. Treated cells showed high fluorescence intensity, particularly an increase in green fluorescence of JC-1, indicating a depolarized  $\Delta\Psi$ m state of the mitochondria.

### Acridine Orange/Propidium Iodide (AO/PI) Dual Staining Assay

The AO/PI dual staining assay was utilized to evaluate morphological changes in MCF-7 cells after treatment with CTM. MCF-7 cells ( $1 \times 10^6$ ) were treated with or without an optimal effective dose of CTM (50  $\mu$ M) for 24 hours in a CO<sub>2</sub> incubator.

Following treatment, the cells were washed with phosphate-buffered saline (PBS) and incubated with 100  $\mu$ L of AO/PI staining solution. The staining solution was prepared by mixing equal volumes of 50  $\mu$ g/mL acridine orange and 50  $\mu$ g/mL propidium iodide in PBS.

After 30 minutes of incubation, the cells were washed again with PBS, and their morphology was analyzed using a fluorescence microscope (EVOS FL, Life Technologies). Viable cells with intact membranes emitted green fluorescence, whereas apoptotic cells, indicative of cell death, exhibited bright orange fluorescence. Images were captured using an inverted fluorescence microscope (EVOS FL, Life Technologies) at 20 $\times$  magnification.

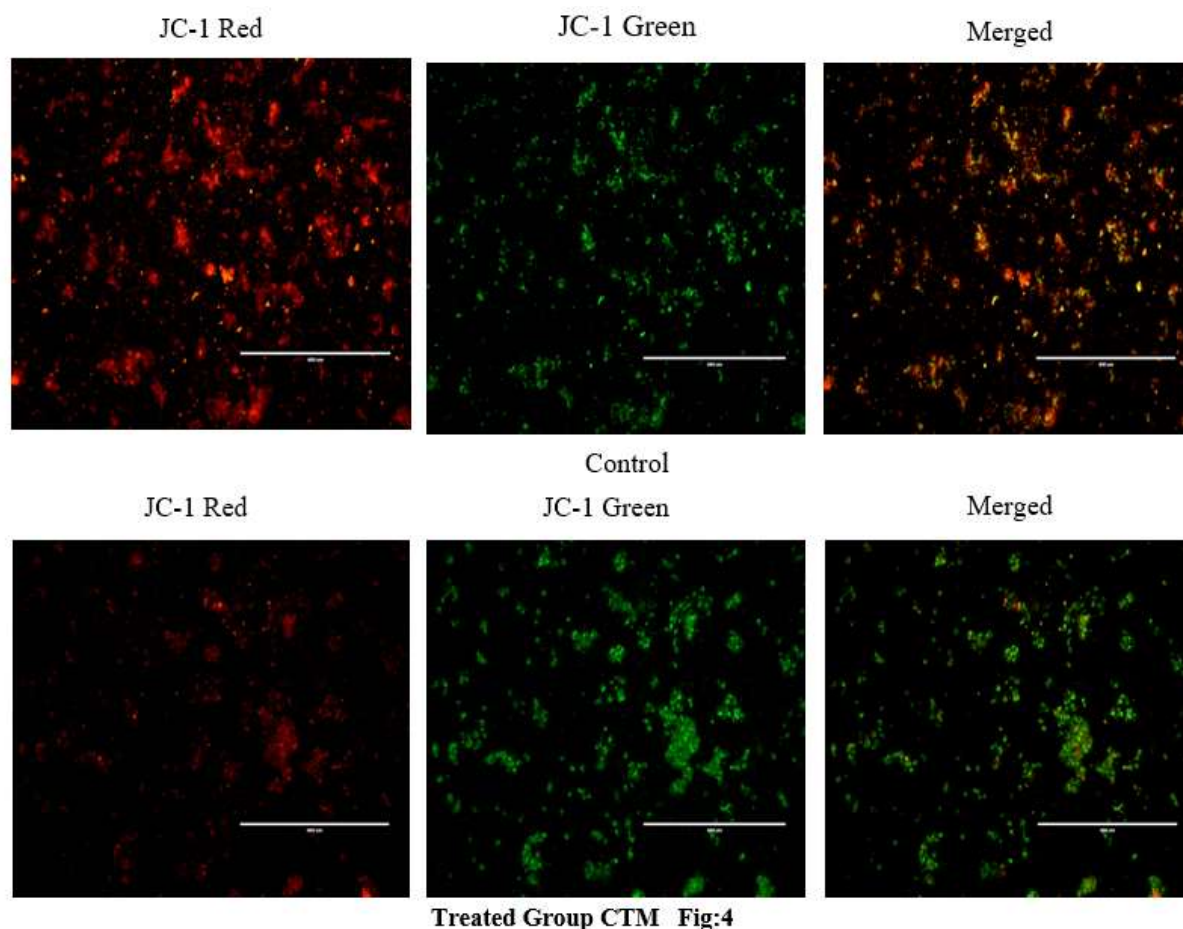


Treated Group CTM Fig:3

AO/PI staining and the flowcytometric analysis of MCF cells treated with the without CTM (A) The untreated cells showing green fluorescence of (AO)/representing live cells while treated cells are showing red fluorescence (PI) representing of dead cells (B) flow cytometry data are showing to the treatment of CTM (50  $\mu$ M) induces early apoptotic as well as late apoptotic in MCF cells. (Bar = 200  $\mu$ m).

#### Analysis of Reactive Oxygen Species (ROS) Levels

To investigate whether apoptosis induced by CTM is mediated by reactive oxygen species (ROS) production, MCF-7 cells were treated with CTM (50  $\mu$ M) for 24 hours. Following treatment, the cells were washed with phosphate-buffered saline (PBS) and incubated with 10  $\mu$ M dichlorofluorescein diacetate (DCFH-DA; Beyotime, Nantong, China) at 37°C for 30 minutes. After incubation, the cells were washed three times with PBS to remove excess dye. ROS levels were then assessed using a fluorescence microscope (EVOS FL, Life Technologies) following the manufacturer's instructions. The fluorescence of dichlorofluorescein (DCF), a product of ROS-mediated oxidation of DCFH-DA, was detected with an excitation wavelength of 488 nm and an emission wavelength of 525 nm using the inverted fluorescence microscope (EVOS FL, Life Technologies).



**Treated Group CTM Fig:4**

Representative photographs showing mitochondrial membrane potential in CTM treatment in MCF-7 cells.

Bar diagram shows CTCF analysis of fluorescence intensity of control and treated cells.

Three independent experiments were conducted to confirm the results. (Bar =100  $\mu$ m).

### Biological studies

We investigated whether the synthesized CTM demonstrates anticancer activity against breast cancer and explored the underlying mechanisms. Breast cancer remains one of the leading causes of cancer-related mortality among women worldwide. Over the past decade, various medical interventions, including surgery, chemotherapy, and radiotherapy, have been utilized to manage breast cancer (M.G. Ruo et al., 2002; K.R. Kutanzi et al., 2011).

In recent years, chemical compounds featuring N-heterocycles such as pyridine, triazole, pyrazole, and pyrimidine, as well as O-heterocycles like benzofuran, coumarin, and chromones, have received significant attention. These compounds are recognized for their safety, efficacy, cost-effectiveness, and therapeutic potential in the treatment of various cancers.

As shown in Fig3, CTM inhibited the metabolic activity of MCF-7 cells in a dose-dependent manner, with a half-maximal inhibitory concentration of (IC<sub>50</sub>) of 50  $\mu$ M after 24 hours. To investigate whether the cytotoxic effect of CTM in MCF-7 cells is associated with apoptosis induction, various assays were performed. Figure 4 demonstrates that CTM induced morphological features are apoptosis by increasing the population of PI-positive cells (early apoptosis) in the AO/PI dual-staining assay and elevating the number of Annexin-V/PI-positive cells (late apoptotic cells) in the FACS assay compared to controls.

These findings indicate that CTM significantly inhibits cell proliferation and induces apoptosis in MCF-7 cells. To assess changes in mitochondrial membrane potential ( $\Delta\Psi$ m) following CTM treatment, cells were stained with the JC-1 fluorochrome. Treated cells exhibited increased green fluorescence intensity, suggesting a depolarized  $\Delta\Psi$ m state (Figure 4).

This mitochondrial membrane depolarization implies that CTM-induced apoptosis may be associated with increased oxidative stress and/or reactive oxygen species (ROS) production. Cytotoxic agents and compounds are well-known to inhibit tumor growth via ROS-dependent activation of apoptosis (W. Davis et al., 2001). Additionally, elevated ROS levels in cancer cells in response to phytochemicals often culminate in cell death (M. Müller et al., 1998).

In this study, intracellular ROS levels were analyzed in CTM-treated and untreated cells using the fluorescent probe DCFH-DA. CTM treatment (50  $\mu$ M) significantly increased DCFDA fluorescence intensity compared to controls ( $P < 0.05$ ). Co-treatment with N-acetyl cysteine (NAC), a ROS scavenger, nearly suppressed CTM-induced ROS generation in MCF-7 cells. The reduced fluorescence intensity in the NAC + CTM group compared to CTM alone confirms that ROS generation is a critical mechanism underlying CTM-induced apoptosis (Figure 5).

Anticancer drugs elevate ROS levels through various mechanisms, including disruption of the mitochondrial electron transport chain, alterations in mitochondrial membrane potential, and dysregulation of antioxidant enzymes. Apoptosis is often mediated by mitochondrial ROS production, which can activate both intrinsic and extrinsic apoptosis pathways (M. Redza-Dutordoir et al., 2016; S. Galadari et al., 2017).

This study indicates that CTM significantly induces ROS generation in MCF-7 cells, triggering a ROS-dependent intrinsic apoptotic pathway. However, ROS-independent mechanisms of cell death have also been reported, suggesting that additional intrinsic pathways may contribute to CTM-induced cell death.

To further elucidate the molecular mechanism of apoptosis, the expression levels of BCL-2 and caspase-3 in CTM-treated MCF-7 cells were analyzed using immunocytochemistry and real-time PCR. The ratio of the pro-apoptotic gene Bax to the anti-apoptotic gene BCL-2 is a critical determinant of programmed cell death. Caspase-3, a key executioner protease, is activated during apoptosis, ultimately leading to DNA fragmentation (L. Zhang et al., 2016; S. Terashima et al., 2013).

Immunocytochemical analysis revealed significantly higher corrected fluorescence intensity of caspase-3 in CTM-treated cells compared to BCL-2. This suggests that CTM markedly suppresses BCL-2 expression while inducing caspase-3 expression in treated cells relative to controls. Quantitative RT-PCR analysis further confirmed an increase ( $P < 0.05$ ) in caspase-3 mRNA expression and downregulation of BCL-2 mRNA in CTM-treated MCF-7 cells. These findings suggest that CTM induces apoptosis in MCF-7 cells via a ROS-dependent, caspase-3-mediated mitochondrial intrinsic pathway (A.G. Porter et al., 1999).

## Discussion

In this study, we demonstrated that the synthesized compound CTM exhibits significant anticancer activity against MCF-7 breast cancer cells. The results reveal that CTM effectively inhibits cell proliferation and induces apoptosis, providing evidence of its potential as a therapeutic agent for breast cancer treatment.

CTM's anticancer effect was evident from its ability to decrease cell viability in a dose-dependent manner, with an IC<sub>50</sub> of 50  $\mu$ M. Apoptosis induction by CTM was confirmed through multiple assays, including AO/PI dual staining, FACS analysis, and mitochondrial membrane potential assessment. The observed depolarization of the mitochondrial membrane ( $\Delta\Psi$ m) highlights mitochondrial dysfunction as a key event in CTM-induced apoptosis. One of the critical findings of this study is the role of reactive oxygen species (ROS) in mediating CTM's apoptotic effects. CTM treatment significantly elevated intracellular ROS levels in MCF-7 cells, and this effect was attenuated by co-treatment with N-acetyl cysteine (NAC), a known ROS scavenger. This indicates that ROS generation is a primary mechanism through which CTM exerts its cytotoxic effects. Elevated ROS levels in cancer cells are known to disrupt mitochondrial integrity, activate pro-apoptotic signaling pathways, and ultimately lead to cell death. These findings align with previous reports suggesting that ROS-mediated apoptosis is a hallmark of many anticancer compounds (W. Davis et al., 2001; M. Müller et al., 1998).

Further, CTM induced significant changes in the expression of apoptosis-related proteins and genes. The downregulation of the anti-apoptotic protein BCL-2, coupled with the upregulation of the pro-apoptotic caspase-3, indicates that CTM triggers the mitochondrial (intrinsic) apoptotic pathway. This is consistent with the established role of caspase-3 as a key executioner of apoptosis, which leads to the cleavage of cellular components and DNA fragmentation (A.G. Porter et al., 1999; L. Zhang et al., 2016). The increased Bax/BCL-2 ratio further supports the activation of intrinsic apoptotic pathways.

Interestingly, although ROS generation appears to be a critical mediator of CTM-induced apoptosis, we cannot exclude the involvement of ROS-independent mechanisms. Cancer cells often exhibit alterations in multiple signaling pathways, and CTM may interact with additional targets to induce cell death. Future studies should explore these alternative mechanisms to provide a comprehensive understanding of CTM's mode of action.

The structural features of CTM, incorporating N-heterocycles and O-heterocycles, may contribute to its cytotoxic potential. Compounds with similar scaffolds have demonstrated significant therapeutic effects in previous studies, particularly through their ability to target key signaling pathways in cancer cells (M.G. Ruo et al., 2002; K.R. Kutanzi et al., 2011).

In conclusion, our findings suggest that CTM induces apoptosis in MCF-7 cells via a ROS-dependent, caspase-3-mediated intrinsic mitochondrial pathway. These results highlight the potential of CTM as a novel anticancer agent. However, further studies are required to validate these findings *in vivo* and to assess the safety and efficacy of CTM in preclinical and clinical models. Additionally, mechanistic studies involving proteomics and transcriptomics could provide deeper insights into the molecular targets and pathways affected by CTM.

**Conclusion**

In this study, we demonstrated that the synthesized compound CTM possesses potent anticancer activity against MCF-7 breast cancer cells. CTM effectively inhibited cell proliferation and induced apoptosis through a mechanism involving ROS generation and mitochondrial dysfunction. The downregulation of the anti-apoptotic protein BCL-2 and the upregulation of the pro-apoptotic protein caspase-3 highlight the activation of the mitochondrial intrinsic apoptotic pathway as a primary mode of action.

Furthermore, the attenuation of CTM-induced ROS generation by N-acetyl cysteine (NAC) underscores the pivotal role of oxidative stress in CTM-mediated cytotoxicity. These findings suggest that CTM induces apoptosis via a ROS-dependent, caspase-3-mediated mechanism, offering a promising strategy for targeting breast cancer cells.

CTM's structural composition, incorporating N- and O-heterocyclic scaffolds, underscores its potential as a novel therapeutic agent. However, additional *in vivo* studies and preclinical evaluations are necessary to confirm its safety, efficacy, and therapeutic applicability in cancer treatment.

Overall, CTM represents a promising candidate for anticancer drug development, and this study provides a foundation for further exploration of its potential in combating breast cancer.

**Authorship contribution**

CRediT authorship contribution statement Rashmi Gupta: Writing – original draft, Investigation, Formal analysis, Data curation. Rakesh Kumar Gupta: Writing – review & editing, Data curation. Pawan K. Dubey: Methodology, Investigation, Formal analysis. Gopal Das Gupta: Validation, Resources, Methodology. Anima Tripathi:

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Data availability**

No data was used for the research described in the article.

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**Author contribution**

R.B and PKD were responsible for study, concept, design and writing—review and editing R.B, S.H, R.K.G, A.R., A.D, S.A., and A.T were responsible for acquisition and interpretation of data, statistical analysis. All authors have read and agreed to the published version of the manuscript.

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