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ROS mediated pro-apoptotic effects of *Tinospora cordifolia* on breast cancer cells

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TABLE OF CONTENTS

1. ABSTRACT

The inevitable development of chemoresistance and unmanageable side effects are the major therapeutic challenges in management of breast cancer imposing an urgent need for identification of novel therapeutic agents. In the present investigation, we report anti-proliferative activity of chloroform fraction of *Tinospora cordifolia* (TcCF), an Ayurvedic medicinal plant, on breast cancer cells. We found that TcCF inhibited growth of breast cancer cells, MDA-MB-231 and MCF-7. More interestingly, we observed TcCF treatment increased intra-cellular ROS levels, altered expression of pro and anti-apoptotic genes, decreased colony formation ability and induced apoptosis in breast cancer cells. We also found that inhibition of ROS abrogated TcCF induced apoptosis in breast cancer cells, emphasizing the role ROS in TcCF induced breast cancer cell death. Furthermore, we identified the presence of pharmacologically active compounds like rutin and quercetin which account for the anti-cancer property of TcCF against breast cancer cells. These data show TcCF is a promising anti-cancer agent against breast cancer cells.

2. INTRODUCTION

Breast cancer is the most common cancer and the second leading cause of cancer related death among women worldwide. However, in Asian countries like India it continues to be the leading cause of cancer related

mortalities among women making it a major health issue of concern (1, 2). Where patients with early stages of breast cancer have many therapeutic strategies, patients of the advanced stage have limited or sometimes no treatment options (3, 4). Conventional drugs despite of their tremendous efficacy drastically reduce the quality of life of the patients undergoing chemotherapy (5). Furthermore inefficacy of these agents in the advanced stages of the disease and development of chemoresistance advocates the identification of novel therapeutic drugs with much safer toxicity profiles.

Previous research findings from pre-clinical data have shown promising effects of many natural agents in breast cancer, thereby emphasizing their clinical evaluation and further identification of other novel anti-cancer agents of natural origin (6, 7). Natural compounds inhibit cancer cell growth through inhibition of cell proliferation, reactive oxygen species (ROS) generation, DNA damage, cell cycle arrest, inhibition of pro-survival pathways and apoptotic induction (8-10). Cancer cells are known to harbor increased amounts of intracellular ROS than non-cancerous cells. Most of the natural agents despite of their antioxidant potential have shown to selectively increase the levels of ROS in cancer cells and consequently disrupt the redox homeostasis, induce genotoxic effects, mitochondrial dysfunction, activation of stress pathways and ultimately cancer cell death (9, 10). Therefore, generation of ROS by natural plant based products represents an interesting strategy to kill cancer cells by triggering the plethora of pro-apoptotic events.

Tinospora cordifolia (Wild.) Miers ex Hook. F. & Thoms. (Family: Menispermaceae) commonly known as "Guduchi" is an important Ayurvedic 'Rasayanas'. *T. cordifolia* have been reported to possess medicinal properties like anti-diabetic, anti-periodic, anti-spasmodic, anti-inflammatory, anti-arthritic, anti-oxidant, anti-allergic, anti-stress, anti-leprotic, anti-malarial, hepatoprotective, immunomodulatory as well as it has anticancer activity (11-16). *T. cordifolia* extracts have shown anti-tumor effects against prostate cancer, glioblastoma, liver cancer, neuroblastoma, cervical cancer and a variety of other cancer cell lines. Mechanistically extracts from *T. cordifolia* have shown to induce radiosensitization, DNA damage, differentiation, senescence, inhibit side population of cancer stem-like cells as well as chemopreventive effects on cancer cells (17, 18). Although *T. cordifolia* has gained acknowledgement for its tremendous medicinal properties, but its anticancer properties on breast cancer cells have not been fully understood.

In the present investigation, we report that chloroform fraction of *T. cordifolia* (TcCF) stem possesses anti-cancer activity against human breast cancer cells through ROS generation. TcCF treatment also decreased colony formation ability, increased intracellular ROS and induced apoptosis in these cells through regulation of anti and pro-apoptotic markers p21, p53, Bax and Bad. According to previous reports, detailed biochemical analysis of *T. cordifolia* extract has identified different classes of secondary metabolites such as alkaloids, steroids, diterpenoid lactones, aliphatics, and glycosides (19). In this study, however, we have found the presence of Quercetin, Rutin and other active compounds in TcCF that might be responsible for its anti-tumor property. Our study suggests that TcCF with pharmacologically active phytochemicals might have the potential to be developed as a chemotherapeutic agent for the management of breast cancer.

3. MATERIALS AND METHODS

3.1. Collection of plant materials and extraction

Stems of *T. cordifolia* were procured from an authorized dealer of Ayurvedic and Unani drug materials in Lucknow, India. Collected material was identified by Department of Botany, Shia P. G. College, Lucknow, India.

3.2. Preparation of extract and fractionation

Stems were shade air dried and grinded to a fine powder for the study. About 10.0 kg of dried plant material was extracted with methanol to obtain the methanolic crude extract. The crude extract was further partitioned into different fractions on the basis of polarity, i.e. hexane-chloroform-ethyl acetate and methanol. The solvent of each fraction was concentrated under reduced pressure with controlled temperature on a rotatory evaporator, final residues obtained were used for bioassay.

3.3. Total phenolics content

Total phenolics content was determined by the method based on Folin- Ciocalteu reagent and compared with Gallic acid equivalent as standard. Briefly, 0.5 mL (1mg/mL) solution of TcCF was mixed with 5 mL of Folin-Ciocalteu reagent and incubated for 3 minutes after that 4 mL of 10% Na_2CO_3 solution was added and stand for 15 min with intermittent shaking. The absorbance of the blue color produced was measured with the help of a double beam UV/Visible spectrophotometer (Systronics) at 765 nm. The Total Phenolics contents value was expressed in terms of Gallic acid, equivalent (GAE) mg/g.

3.4. High performance liquid chromatography (HPLC) analysis

Determination of quercetin and rutin were performed by an Agilent 1260 infinity Quaternary LC system consisting of 1260 infinity Diode Array Detector (DAD), quaternary solvent delivery system with thermostatted auto sampler, thermostatted columns compartment and equipped with an Agilent Zorbax C-18 (4.6mm × 250 mm, 5 µm) column as described previously (20). Briefly, gradient elution was performed at 25◦C with solution A (Water 0.3% HCOOH in water) and solution B (Methanol)

in the following gradient elution program: 0–1min— 90% of solution A and 10% of B, 1–5 min—30% of A and 70% of B, 5–7 min— 10% of A and 90% of B, 7-10 min— 90% of A and 10 of B, 10-11 min— 90% of A and 10% of B. Detection was conducted at a wavelength of 280 nm. Flow rate and injection volume were set at 1.0 mL/min and 5 μL, respectively.

3.5. Cell viability assay

The antiproliferative activity of extract was evaluated by 3-[4, 5-Dimethyl-2- thiazolyl]-2, 5-diphenyltetrazolium bromide (MTT) reduction assay. In brief, the cells ($5x10^3$) were seeded in 100 µL complete medium in each well of 96-well culture plate for 24 hrs at 37^oC and 5% CO_2. Stocks of analytes were prepared in DMSO and diluted to the desired concentrations in the complete growth medium and added to the wells in triplicate as per experimental design. After respective treatment time points (24, 48 & 72h), 10 µL of MTT (5 mg/mL) solution was added to each well and the plates were further incubated for 3 hrs at 37^oC until formazan blue crystal developed. Thereafter, DMSO was added to solubilize formazan crystals and absorbance was recorded at 540 nm by a microplate reader (BIORAD-680).

3.6. Clonogenicity assay

The colony formation assay was performed to evaluate the efficacy of extract on the survival and proliferation of cells. Briefly, cells at the initial density of $1x\ 10^3$ in 2 ml medium were seeded in 6-well plates and incubated for 16-18h. Cells were treated with different concentrations of TcCF for 24h. Thereafter media was removed and cells were grown in complete growth media for next 7-10 days until distinct colonies were formed. Colonies were then washed with PBS, fixed with chilled 100% methanol and stained with 0.05% Coomassie for 10 min. Plates were then washed and air dried and the number of colonies (group of ~50 cells) was determined by counting them under microscope.

3.7. Hoechst s]taining

Cells were grown to 50% confluence on pre-sterilized cover slip and left for 18-20h in the incubator until full morphology is attained. Next day, old media was replaced with fresh media and cells were treated with different concentrations of TcCF. After 24h cells were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton-X100 in PBS. Cells were then stained with Hoechst 33342 at a final concentration of (0.5µg/ml) in PBS and incubated for 5 min at 25°C. Images were captured at 20X magnification.

3.8. Measurement of intracellular ROS

Intracellular ROS generation was monitored using 2',7'-dichlorfluorescein-diacetate (CM-H2-DCFDA) dye (Molecular Probes, Grand Island, NY, USA). For fluorescence microscopy, cells were seeded on the pre-sterilized coverslips in 6 well plates. Cells were incubated with CM-H2-DCFDA in serum free media for 30 mins in dark and subsequently treated with TcCF (25 µg/mL). ROS generation was monitored at 0, 1, 3 and 6h under 20X magnification in a fluorescence microscope (Nikon Eclipse Ti-S). Time dependent ROS kinetics were determined by fluorimetry as described previously (6). In brief, semi confluent T25 flask was pre-treated with NAC (6mM) for 1hr followed by trypsinization and staining with CM-H2-DCFDA dye in serum free media for 30 min in the dark. Both NAC pre-treated and untreated stained cells were washed, re-suspended in serum free media and seeded in black well plate. Seeded cells were treated with TcCF (25µg/ml) and fluorescence intensity was measured upto 7h with 30 min time interval in the Multimode detector.

3.9. RNA isolation and semi-quantitative PCR

Total RNA from control and treated cells were isolated by Trizol method and cDNA was prepared from 1µg RNA using Verso cDNA synthesis kit (Takara). Semi-quantitative PCR was performed to amplify p21, p53, Bax, Bcl2, Bad and c-myc genes using specific primers. Beta actin was used as loading control.

3.10. Protein extraction and western blot analysis

Total protein was extracted using radio immune-precipitation assay (RIPA) buffer as described elsewhere (6). Briefly, cells were lysed in RIPA buffer and protein supernatant was extracted through centrifugation. Protein was estimated using Bradford reagent (Sigma Aldrich) and a total of 40µg of protein was separated on SDS-PAGE gel. Proteins were blotted on nitrocellulose membrane and incubated with primary antibodies against Poly ADP-ribose polymerase (PARP) and Cleaved Caspase-3 followed by incubation with HRP conjugated secondary antibody. Blots were developed using ECL Detection System (Millipore).

3.11. Statistical analysis

Data are presented as mean ± SEM of three independent experiments and were analyzed for statistical significance using analysis of variance, followed by Scheffe's test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference

4. RESULTS

4.1. Extraction, fractionation and purification of *T. cordifolia*

The stem of *T. cordifolia* was extracted with methanol and fractionated into different solvents. Different fractions obtained were tested for antiproliferative activity. The chloroform fraction was packed in normal phase silica gel chromatography and eluted with 2% methanol-chloroform mixture to dispel the impurities. Eluted purified fraction was concentrated on rotatory evaporator, named as TcCF and used for bioassay (Figure 1).

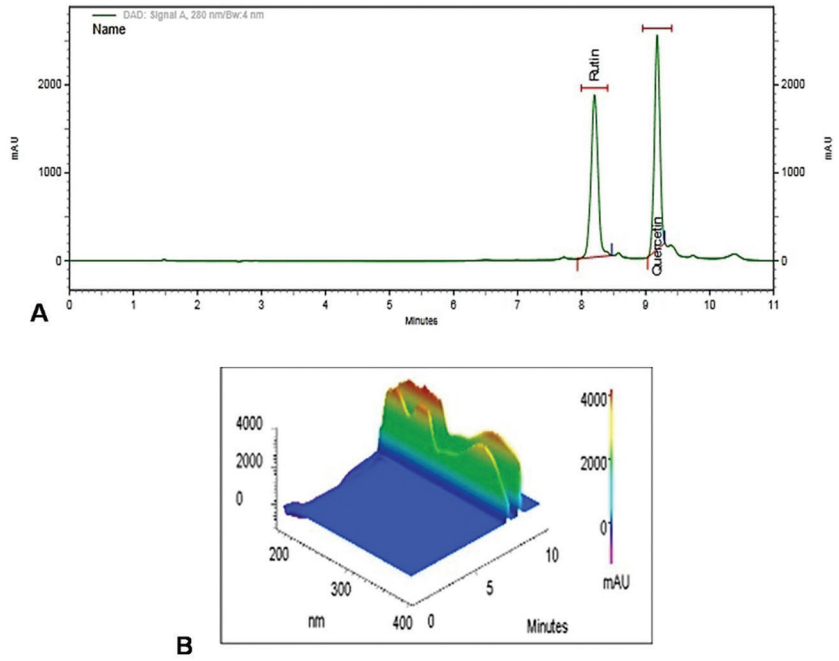

Figure 1. Extraction, fractionation and purification of *Tinospora cordifolia* stem. Air shade dried powdered stem was percolated with methanol. Methanol crude extract was further partitioned into hexane, chloroform, ethyl acetate and methanol fractions. Chloroform fraction was passed through normal phase column chromatography to dispel the impurities.

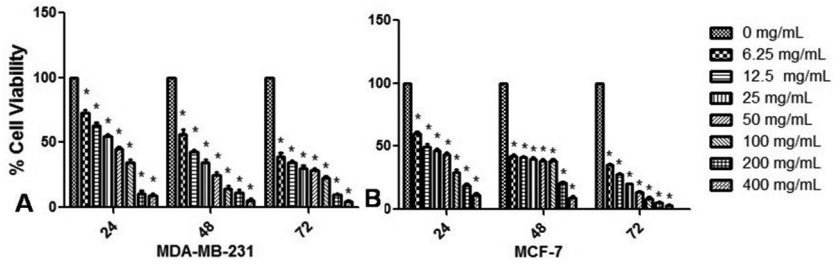

Figure 2. (A) HPLC chromatogram of rutin and quercetin standard and (B) 3D view of chromatogram. Detection at 280 nm with Zorbax Eclipse ODS C-18 column (4.6 x 150 mm, 5 µm), gradient eluents methanol/water, flow rate 1.0 mL/min with injection volume 5.0 µL.

4.2. TcCF contains high amount of polyphenols

Polyphenols are highly linked with antiproliferative activity. Therefore, we estimated presence of total phenolics contents in TcCF. We found that the total phenolic content of TcCF was 17.2± 2.26 mg Gallic Acid Equivalent of per gram dry extract. The results were calculated from standard Gallic acid calibration curve (R2 = 0.9980).

4.3. TcCF showed the presence of rutin and quercetin

Primary screening for total phenolics content showed that TcCF has significant amount polyphenols. Since, polyphenolic compounds are polar in nature, therefore, we performed HPLC analysis to identify polyphenols of TcCF. We identified the presence of two important molecules, rutin and quercetin, which could be linked with the antiproliferative and other activities of TcCF. HPLC ODS C-18 column separation of TcCF showed that TcCF has higher amount of rutin (mg/g of extract±SEM) 12.5±1.10 as compared with quercetin (mg/g of extract±SEM) 4.2±1.05 (Figure 2). Presence of rutin and quercetin offer additional support to anticancer activity of TcCF.

4.4. Effect of TcCF on growth and viability of breast cancer cells

In our preliminary screening experiment to determine the effect of methanolic extract and hexane, chloroform ethyl acetate and methanol fractions on breast cancer cell growth, we performed MTT assay. Breast cancer cells were treated with different concentrations of the crude extract and different fractions of *T. cordifolia* for 24, 48 and 72h. Our results demonstrated that chloroform fraction of *T. cordifolia* (TcCF) exhibited more potent growth inhibitory effect on both the cell lines as compared

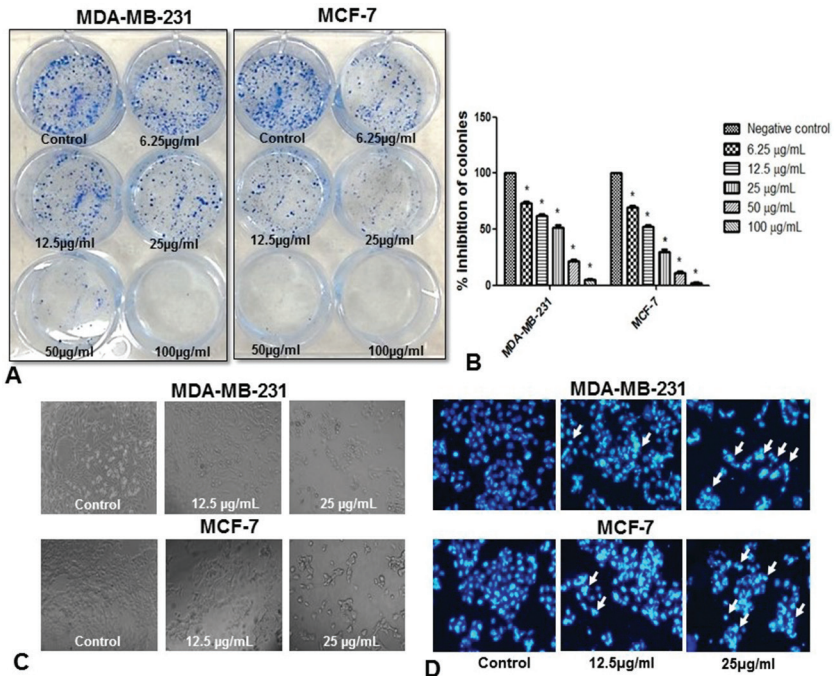

Figure 3. Antiproliferative effect of hexane, chloroform, ethyl acetate and methanol fractions against human breast cancer MDA-MB-231 cells.

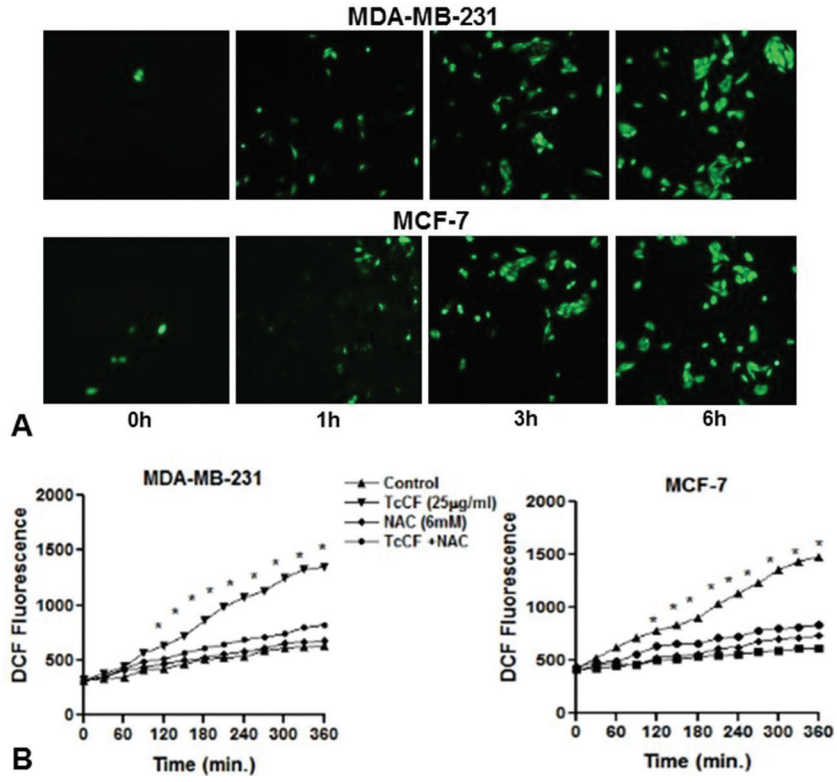

Figure 4. Antiproliferative effect of hexane, chloroform, ethyl acetate and methanol fractions against human breast cancer MCF-7 cells.

with other fractions (Figure 3 and Figure 4). IC_{50} values (Table 1) also indicated that TcCF inhibited breast cancer cell growth at much lower concentrations as compared to other parallel fractions. TcCF induced growth inhibition was

Table 1. IC_{50} values of different fractions of TcCF against MDA-MB-231 and MCF-7 cancer cells after exposure of 24h

Extract	IC_{50} Value (µg/mL)		
	MDA-MB-231 Cells	MCF- 7 Cells	HACAT Cells
Hexane fraction	47.5±2.53	37.2±2.77	178±5.66
Chloroform fraction	35.6±3.66	28.9±2.93	120.1±3.16
Ethyl Acetate fraction	56.7±3.78	35.6±3.25	157.6±4.96
Methanolic fraction	223±2.75	158±2.02	348.5±2.52

dose and time dependent as depicted in Figure 5A and B. Comparison of cell viability between control and treated cells revealed that the significant viability decline was initiated at 12.5 µg/mL concentration in MDA-MB-231 cells and 6.25 µg/mL concentrations in MCF-7 after 24 hour treatment with TcCF. Therefore, it indicated that TcCF exhibited differences in potency towards different breast cancer cells. The IC_{50} value for control HACAT cells were found to be much higher as compared to cancer cells, suggesting a safer toxicity profile of TcCF against normal cells. These results altogether indicate the growth inhibitory activity of TcCF on breast cancer cells. Since the TcCF was found to be most effective against breast cancer cells, therefore we selected this fraction for our further mechanistic experiments.

4.5. Effect of TcCF on colony formation of breast cancer cells

Cancer cells have the ability to proliferate indefinitely and form colonies from a single cell. Hence we sought to evaluate the effect of TcCF on proliferation and colony formation ability of breast cancer cells. As evident in Figure 6A, treatment of human breast cancer MDA-MB-231 and MCF-7 cells, with different concentrations of TcCF resulted in a significant decrease in growth and colony formation when compared with respective controls. A decrease of 44 to 3 colonies/field 73.56% to 5.04% was noted in MDA-MB-231 as compared to control (61 colonies/field) after 6.25 µg/mL to 100 µg/mL concentration exposures with TcCF. A similar decrease in 42-2 colonies/field, 69.56% to 2.0% inhibition of colony (control 62 colonies/field) was observed in MCF-7cells after treatment with TcCF (Figure 6B). These results suggested that TcCF possess anti-proliferative activity against breast cancer cells *in vitro*.

4.6. Effect of TcCF on nuclear morphology of breast cancer cells

In continuation of anti-proliferation properties, nuclear morphological changes were assessed to determine whether TcCF induces apoptosis in MDA-MB-231 and MCF-7 cancer cells, Hoechst 33342 staining was performed. Fluorescence microscopy of MDA-MB-231 and MCF-7 untreated cells verses TcCF treated cells revealed distinctive features of apoptosis, including blebbing, loss or cell membrane symmetry and attachment, cell shrinkage, nuclear fragmentation and chromatin condensation (Figure 6C, D). However, the untreated cells showed normal morphology of nuclei such as round, homogenous and intact chromatin (Figure 6C, D). Therefore, these results suggested that TcCF treatment inhibited breast cancer cell proliferation through induction of apoptosis, which is a potent feature of most of the chemotherapeutic agents.

4.7. Effect of TcCF on intracellular ROS generation in breast cancer cells

Many natural compounds tend to increase intracellular ROS levels as their innate mechanisms to induce cancer cell death (21). Therefore, we speculated that TcCF might increase intracellular ROS levels as preliminary event to induce cell death. To determine the levels of ROS in MDA-MB-231 and MCF-7 cancer cells, 2',7'-dichlorfluorescein-diacetate (DCFH-DA) was used. As represented in Figure 7A, treatment of MDA-MB-231 and MCF-7 cells with 25 µg/mL resulted in increase in DCF fluorescence as examined through fluorescence microscopy as early as 1h after treatment. For quantitative assessment of ROS generation in cancer cells, we performed slow kinetics through fluorimetry from 0 to 6h. Our results showed that TcCF induced significant ROS generation in a time-dependent manner in MDA-MB-231 and MCF-7 cancer cells after 2h of treatment which increased upto 6h (Figure 7B). However, treatment with ROS inhibitor NAC abrogated TcCF induced ROS generation back to levels as comparable to untreated control. Cumulatively, these results suggested that TcCF induced ROS generation in breast cancer cells, which were inhibited by NAC treatment.

4.8. Effect of TcCF induced ROS generation on breast cancer cell death

We next thought to confirm ROS generation to be a potential mediator of TcCF induced cell death in breast cancer cells through assessment apoptotic cell death. N-acetylcysteine (NAC) was used as a quencher of ROS to confirm the involvement of intracellular ROS in TcCF induced apoptosis in MDA-MB-231 and MCF-7 cancer cells. Both the cells were pre-incubated with NAC (6mM) for 1h followed by treatment with TcCF (25µg/ml) for 24h. As revealed in Figure 8A, pretreatment of both the cells with NAC reduced the extent of apoptosis as indicated by the percentage of cells in the early and late apoptotic quadrants analyzed through flow cytometry. However, NAC treatment only partially reversed the pro-apoptotic effect of TcCF on both the cells suggesting that there might be other pro-apoptotic signaling pathways that are responsible for TcCF induced cell death in these cells. The results demonstrated that TcCF induced

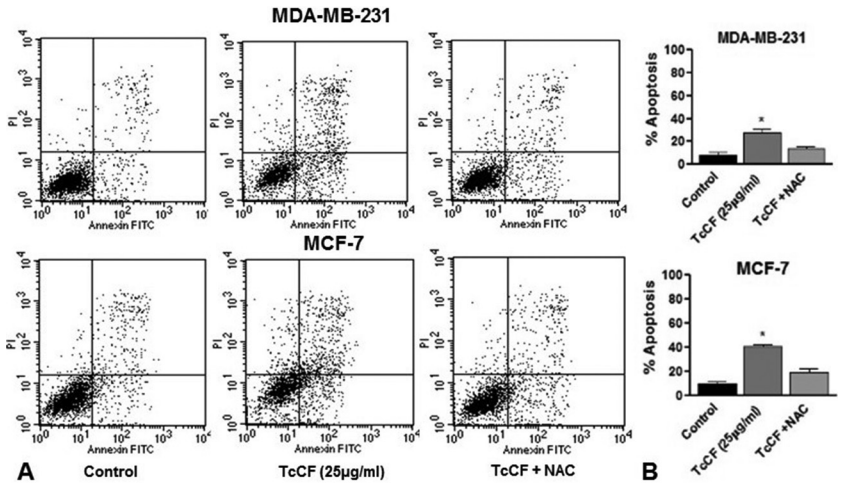

Figure 5. Anti-proliferative effect of TcCF against human breast cancer cells. (A-B) Human breast cancer MDA-MB-231 and MCF-7 cells were treated with various concentrations of TcCF for 24, 48 and 72h, and the cells viability was determined by MTT assay. Cell viabilities are shown as percentages, and the untreated cells were regarded as 100% viable. Data represent the means of three experiments conducted in triplicate and were significant (p≤0.05).

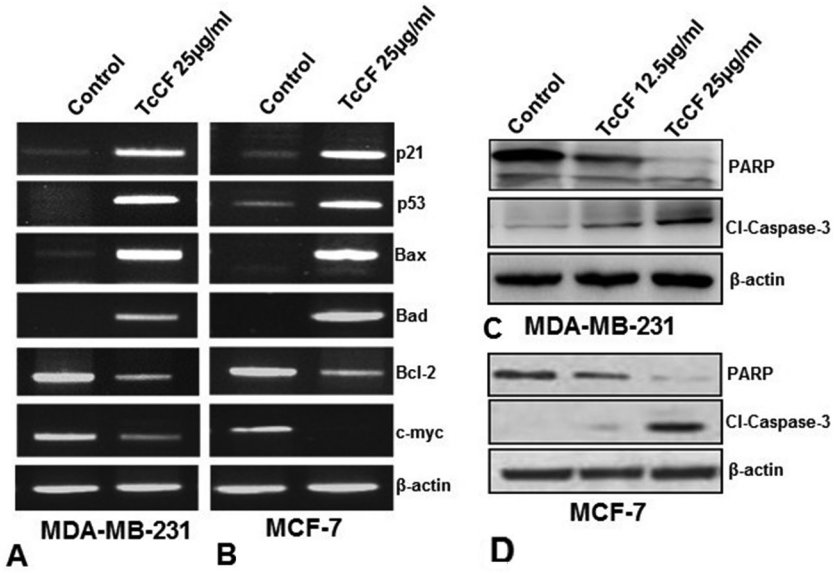

Figure 6. (A, B) Effect of TcCF on growth assay estimated by colony formation. Human breast cancer MDA-MB-231 and MCF-7 cells were grown in media along with different concentrations of the TcCF. The number of colonies was recorded after 7 days of treatment. Data represents the mean ± SEM of three different assays. *p ≤ 0.05 versus control. (C) Microscopic examination of human breast cancer MDA-MB-231 and MCF 7 cells, after treatment with TcCF for 24 h. (D). Induction of nuclear fragmentation by TcCF in MDA-MB-231 and MCF-7 cells: 2×10^4 cells/well were seeded in 6-well culture plate and allowed to grow for 24 h and then treated with different concentrations of TcCF for 24 h and stained with Hoechst 33342 stain following standard protocol and image was captured by inverted microscopy at 20X magnification. Arrows indicate the cells with DNA fragmentation and apoptotic nuclei.

apoptosis in MDA-MB-231 and MCF-7 cells are partially mediated by ROS generation.

4.9. TcCF alters the expression of pro and anti-apoptotic markers in breast cancer cells.

In our previous experiments we found that TcCF induced apoptosis as evident through nuclear condensation and percent apoptotic cells. We next sought to confirm that whether TcCF can alter expression of pro- and anti-apoptotic proteins or not. For this we treated MDA-MB-231 and MCF-7 cells with TcCF for 24h and subjected to total RNA isolation and cDNA synthesis. Both control and treated cells were analyzed for expression of p21, p53, Bax, Bcl2, Bad and c-myc genes. TcCF treatment increased the expression of tumor suppressor genes p21, p53, Bax and Bad and decreased the expression of anti-apoptotic genes Bcl2 and c-myc (Figure 9). We further confirmed our findings through western blotting analyzing the expression of PARP and cleaved caspase-3. We observed decrease in total PARP and the increase in

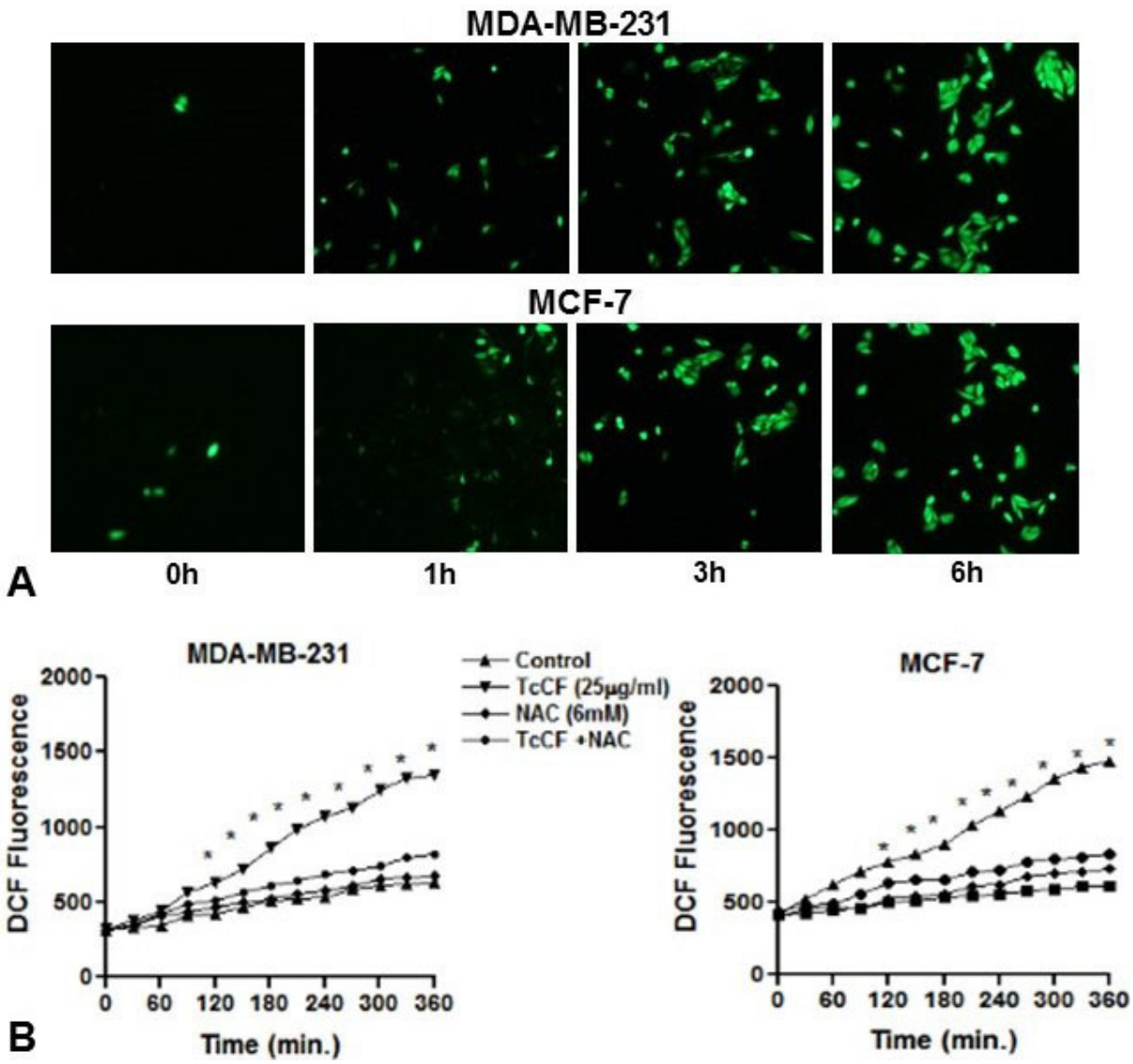

Figure 7. Effect of TcCF on intracellular ROS generation in human breast cancer cells. (A) Cells were stained with DCFDA dye for 1h and then treated with TcCF (25 µg/mL) for 1, 3 and 6h. Increase in DCF fluorescence was observed after treatment for 3 and 6h through fluorescence microscopy. (B) TcCF treatment significantly increased DCF fluorescence in both cells as early as 2h after treatment as measured by slow kinetics through flourimetry.

cleaved fragment of caspase-3 which is considered to be the hallmarks of apoptosis. Therefore, these results confirmed that TcCF treatment induced apoptosis in MDA-MB-231 and MCF-7 cells through modulating the expression of pro- and anti-apoptotic proteins.

5. DISCUSSION

Pre-clinical studies have shown that both novel drugs from synthetic and natural origin share similar platform in terms of their anti-cancer properties. However, agents from natural resources have mostly outweighed the synthetic molecules in toxicity assessment and symptom relief. Therefore, use of natural agents represents a rational and appealing strategy to treat cancer patients either alone or in combination with standard drugs. In this context more research has been focused on the evaluation of anti-cancer property of medicinal plant which has been used since ancient times in traditional medicine. The purpose of this study was to evaluate the effect of the chloroform fraction of *T. cardifolia,* an important Ayurvedic herb on breast cancer and determine its potential to be an anti-cancer agent. We also attempted to delineate its possible mode of action to inhibit breast cancer cell growth and proliferation. We found that TcCF exhibited anti-cancer property against breast cancer cells MDA-MB-231 and MCF-7.

A recent study has shown that ethanolic extract of *T. cordifolia* decreased the percentage of cancer

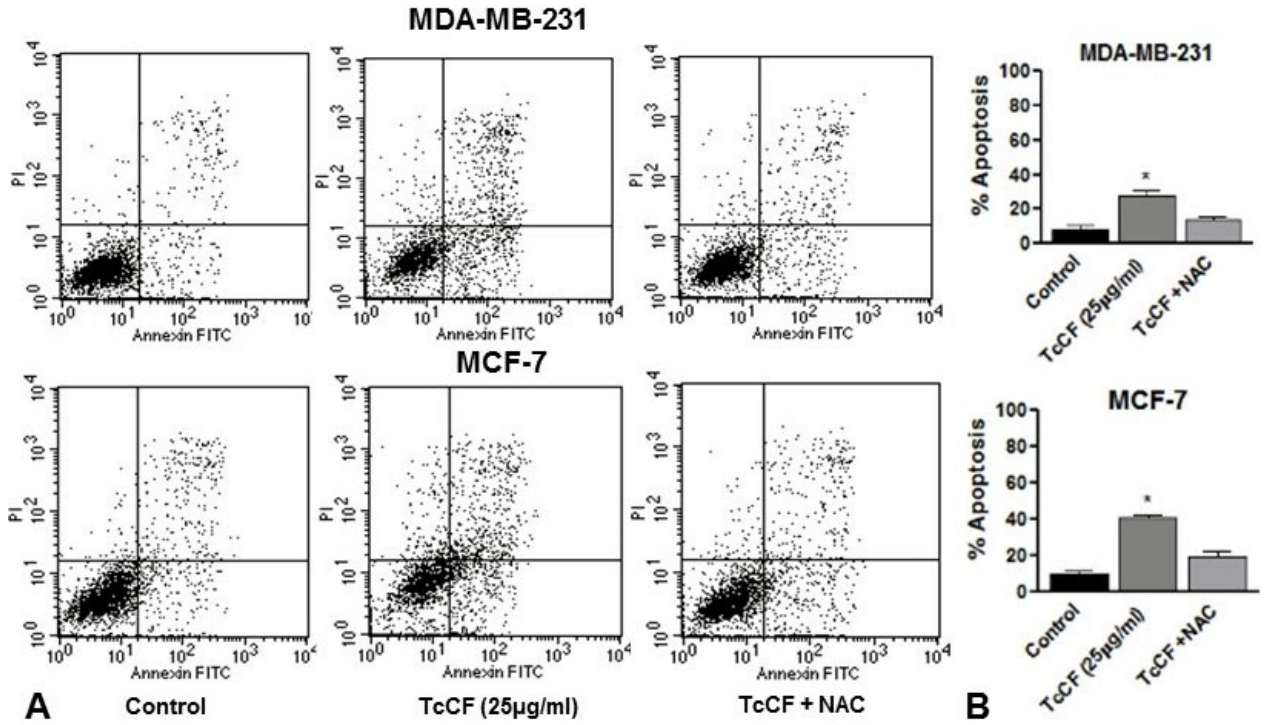

Figure 8. TcCF induces apoptosis in human breast cancer cells. (A) Cells were treated with TcCF (25 µg/mL) along with NAC (10mM) and subjected to Annexin V-FITC/PI staining. Percentage of live, necrotic, early and late apoptosis were analysed through flow cytometry and represented as quadrants. (B) Bar graphs represent percentage of apoptotic cells (early and late). Data are represented as mean±SEM, p≤0.5.

stem-like cells isolated from MDA-MB-435S breast cancer cells (22). Since the colony formation assay defines the indefinite proliferative ability of self-renewing cancer stem cell sub-population. Our study showed that treatment of breast cancer cells with TcCF showed significant inhibition of number of colonies formed as compared to untreated controls. Therefore, our results also suggested that TcCF may also be efficient in inhibiting the small population of cancer stem cells which are considered to be the cause of disease relapse in breast cancer (5). Apoptosis is being continuously viewed as the most relevant strategy to restrict the growth of cancer cells by killing them. Therefore, agents that induce apoptosis in cancer cells have gained considerable attention in the development of anti-cancer therapies (23, 24). In our study, we also found features of apoptotic cell death induced by TcCF. These results were well in accordance with previous studies where other natural compounds have been shown to exert similar morphological changes to indicate cancer cell death (25).

The role of ROS generation in mediating apoptosis in various cancer cells is well established. ROS generation imposes serious insults and dysfunctions in cancer cells, including oxidative stress, mitochondrial dysfunction, DNA damage, cell cycle arrest leading to apoptosis (6, 26-28). We observed that treated MDA-MB-231 and MCF-7 cells showed a significant increase in intracellular levels of ROS as early as 2h after treatment which was completely abrogated in NAC pre-treated group. This indicated that ROS generation is an early event in the molecular cascade of TcCF induced cell death in breast cancer cells. Many natural agents have been shown to use ROS generation as either their primary or supporting event to kill cancer cells (6, 29). Our results indicated that NAC pre-treatment in breast cancer cells partially decreased the percentage of apoptotic cells in both the cell lines suggesting involvement of other key events in addition to ROS generation in these cells. Therefore, it would be interesting to find out other downstream events associated with TcCF induced ROS generation in these cells so that we could identify its cellular targets and other molecular mechanisms of cell death.

Apoptosis is a genetically programmed regulatory process with interplay between pro and anti-apoptotic factors (29-32). Chemotherapeutic agents have been known to disturb the balance between these factors in favor of apoptosis (33). Moreover there are many natural agents which have been previously reported to induce apoptosis in cancer cells, including breast cancer by altering the expression of one or more of these factors (5, 6). Our results also showed decreased expression of anti-apoptotic genes Bcl-2 and c-myc and increased expression of pro-apoptotic genes p21, p53, Bax and Bad in both the cell lines after treatment with TcCF for 24h. Apoptosis process involves cleavage of

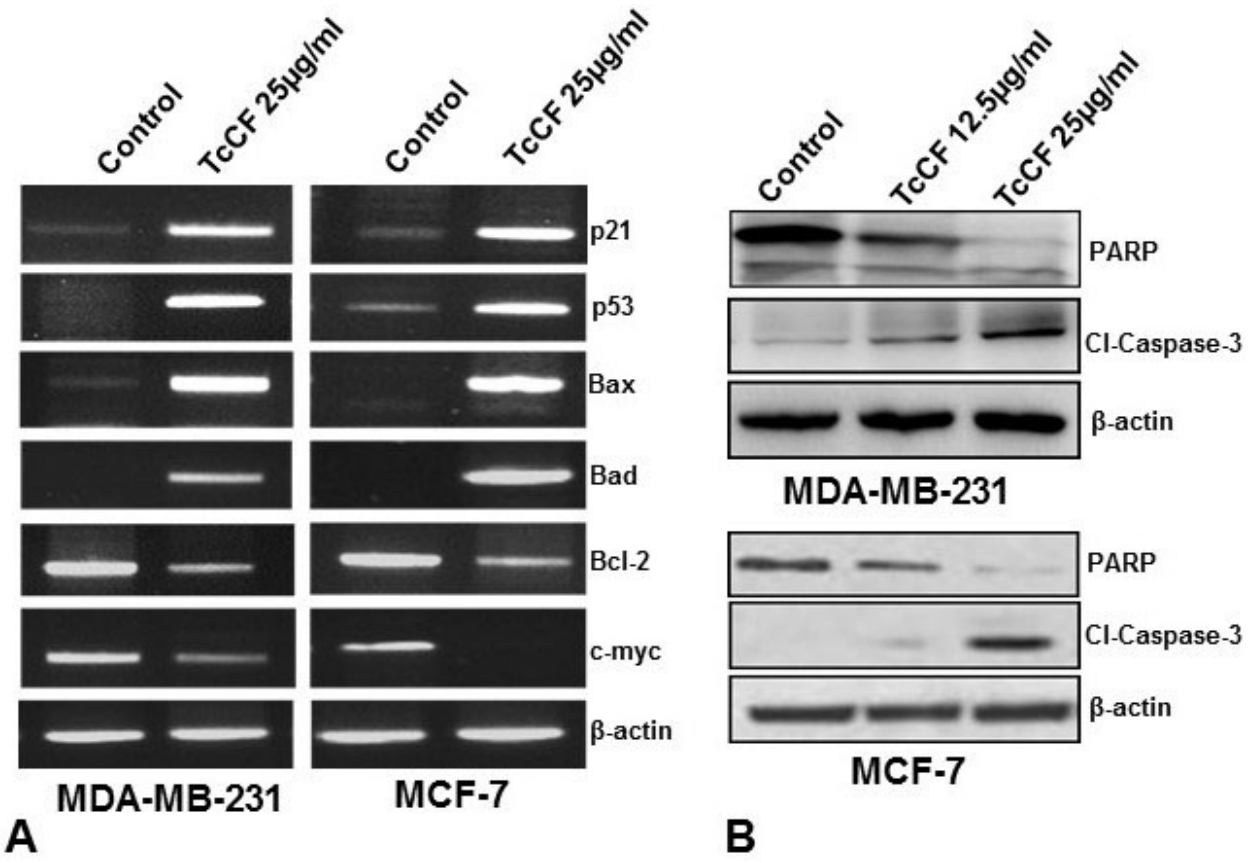

Figure 9. TcCF alters expression of pro and anti-apoptotic markers in breast cancer cells. (A) Cells were treated with TcCF (25 µg/mL) for 24h and subjected for RNA isolation and cDNA sysnthesis. Semi quantitative PCR was performed to analyze the expression of pro- and anti apoptotic genes. (B) Western blot analysis was done to evaluate the expression of total PARP and Cleaved Caspase-3 after TcCF (25 µg/mL) treatment for 24h.

hallmark proteins, namely Caspases and PARP and their protein expressions are determined to confirm the occurrence of apoptotic cancer cell death (6). Additively, in our results also we observed decrease in protein expression of total PARP and increase in expression of cleaved caspase-3. Therefore, our results suggested that TcCF induced apoptosis in breast cancer cells and in the process, altered the expression of anti- and pro-apoptotic factors.

Phenols and flavonoids are among the active ingredients responsible for pharmacological properties of plant products. Several studies demonstrated a significant role of polyphenols in growth inhibition of breast, colon, prostate, ovary, endometrium and lung cancer cells (34, 35). Furthermore, many polyphenols tend to selectively act as pro-oxidant in cancer cells thereby increasing intracellular ROS levels as observed in our study. Previous studies have shown active components like Tinosporide, Furanolactone diterpenes and Cordifolioside been isolated from chloroform fraction of *T. cordifolia* (36-39). However, in our HPLC results we found that TcCF contained a rich source of quercetin followed by rutin along with some other metabolites. Quercetin has been previously described for its antitumor effects on breast cancer cells in vitro (40). It was observed that quercetin treatment induce proliferation inhibition, cell cycle arrest and apoptosis in breast cancer cells (40). However, we did not observe any effect of TcCF on cell cycle distribution in breast cancer cells (data not shown) suggesting that other compounds of TcCF might have more influencing effect on breast cancer cells in our case. On a similar note rutin has also been reported to exert anti-tumor effect against many cancer types including breast, colon and hematological cancers (41, 42). Therefore, our experimental findings suggested that the anti-cancer effects of TcCF is accredited to the presence of compounds like quercetin and rutin as well as presence of other bioactive ingredients present in TcCF.

Conclusively, our study captivates the anti-proliferative and pro-apoptotic activity of TcCF against breast cancer cells *in vitro*. We also observed that TcCF induced intracellular ROS levels as its preliminary event which can be attributed to the presence of compounds like quercetin and rutin. Hence this study summarizes the pharmacological property of TcCF as anti-cancer agent. However, other metabolites which were found

to be present in TcCF may also have pharmacological properties. Therefore, isolation and identification of active principles from TcCF is ongoing and demands further research attention in terms of their pharmacodynamics and pharmacokinetic profiles, pre-clinical evaluation and identification downstream molecular targets in cancer cells.

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