



## EXPRESSION OF THE APOPTOSIS RELATED GENES AND TELOMERASE ACTIVITY IN *TINOSPORA CORDIFOLIA* TREATED MCF-7 CELLS

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**Abstract:** The present study was taken up to explore the anti-proliferative activity of aqueous and hydro-alcoholic extracts of *Tinospora cordifolia* in MCF-7 human breast cancer cells each at the dose rate of 600 µg/ml. Their influence on the induction of apoptosis was detected by determining the expression of apoptosis related genes Bcl-2, Bax and Fas keeping β actin as an internal control and the expression of the tumour marker enzyme telomerase by using RT-PCR. There was an expression of the internal control β actin, and the apoptosis related genes Bax and Fas after treatment with both the extracts of the plant similar to that noticed in the positive control. Whereas in the negative control group there was expression of the anti-apoptotic gene Bcl-2 and the internal control β actin. Telomerase RNA, hTERT mRNA was expressed in the negative control but not in the treated groups as evidenced by RTPCR analysis. The internal control, GAPDH was expressed in both the groups. These results suggest that aqueous and hydro-alcoholic extracts of *Tinospora cordifolia* could induce apoptosis in MCF-7 cell line and act as effective natural anticancer agents.

**Key words:** *Tinospora cordifolia*; Apoptotic genes; MCF-7 cell line; Telomerase activity; hTERT

### INTRODUCTION

Cancer is one of the most life threatening diseases and possess many health hazard in both developed and developing countries. Cancer claims the lives of more than six million people each year in the world. Current treatment approaches have yielded significant progress in the fight against cancer, but the incidence of developing certain types of cancer continues to rise. This is especially true in the African American communities. African Americans are about 33% more likely to die of cancer than are whites and more than twice likely to die of cancer as are Asian-Islander, American-Indians, and Hispanics. This increase coupled with the harsh side effects of some of the cancer chemotherapies have led to the search for more natural biological products, especially those derived from plant products, currently known as herbal medicine. (Izevbigie E, B. 2003). Ethno historical accounts shows that medicinal plants have been used as a remedy for various human ailments, the reason of using these plants is that they contain certain types of chemical constituent which is having greater therapeutic value that produces a definite pharmacological actions on with lesser side effects. (Shobia, N *et al.*, 2011). *Tinospora cordifolia* (TC) (Menispermaceae) also known as Giloy or Guduchi, is an important medicinal plant cultivated throughout the Indian subcontinent. Through centuries, it has been extensively used in various ayurvedic preparations for the treatment of various ailments (Kritikar 1933 and Bhat 2013). *T. cordifolia* has been extensively screened for many pharmacological activities. It had been shown to possess hypolipidaemic (Prince *et al.*, 1999), antioxidative and hepatoprotective (Bishayi *et al.*, 2002), immunostimulant (Nair *et al.*, 2004), antiangiogenic (Leyon and Kuttan, 2004), antiallergic (Badar *et al.*, 2005), anti-inflammatory and analgesic activity (Thejomoorthy and Raju, 2007) and causes retardation in the tumour development (Jageta and Rao, 2006).

Hence with an attempt to focus on the *in vitro* antiproliferative effect of *Tinospora cordifolia* on MCF-7 cells this study was undertaken.

### MATERIALS AND METHODS

Aqueous (T<sub>AE</sub>) and hydro-alcoholic extracts (T<sub>HAE</sub>) of stem part of *T. cordifolia* were obtained from M/s. Natural Remedies Pvt. Ltd., Bangalore. Human breast carcinoma cell line MCF-7 was obtained from the National Centre for Cell Sciences (Pune, India) and doxorubicin (positive control) from M/s Dabur Pharma Ltd. (Himachal Pradesh, India). Cells were grown in Minimum Essential Medium (MEM: Gibco) with 10% foetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco BRL) at standard culture conditions. Other standard chemicals with analytical grade were used throughout the study.

The study was conducted in four groups of culture with six replications for each group for the MCF-7 cell line.

| Group | Treatment                                       | Dose rate            |
|-------|---|----------------------|
| I     | Negative control (NC)                           | Only Medium          |
| II    | Positive control (PC)                           | Doxorubicin (2µg/ml) |
| III   | <i>Tinospora cordifolia</i> (T <sub>AE</sub> )  | 600 µg/ml            |
| IV    | <i>Tinospora cordifolia</i> (T <sub>HAE</sub> ) | 600 µg/ml            |

### Determination of the expression of apoptosis related genes and tumour marker (telomerase activity) enzyme

**Procedure:** The mRNA expression levels of widely established apoptosis-related genes (Bcl-2, Bax and Fas) and internal control β actin to find the treatment effect on apoptosis and the expression of telomerase RNA, hTERT mRNA keeping GAPDH as an internal control to find the treatment effect on reducing telomerase activity were carried out using semi quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) as described by (Kahta *et al.*, 2006) and (Kosciolek *et al.*, 2003), respectively.

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The primers are as follows;

| S.No | Gene           | Primers                        |                               |
|------|----------------|--------------------------------|-------------------------------|
|      |                | Forward                        | Reverse                       |
| 1    | $\beta$ -actin | 5'-ggcatcctcaccctgaagta-3'     | 5'-ggggtgttgagggtcctcaaa-3'   |
| 2    | Bcl-2          | 5'-gagaaggtgagaagccctga-3'     | 5'-gccaggatgaataaacct-3'      |
| 3    | Bax            | 5'-ttgcttcagggttccatcc-3'      | 5'gcatcctcctgatgattccat-3'    |
| 4    | Fas            | 5'-ttgcttcagggttccatcc-3'      | 5'gcatcctcctgatgattccat-3'    |
| 5    | Telomerase RNA | 5'ctggaggagggtggggccattt-3'    | 5'cgacgggcccagcagctgacat-3'   |
| 6    | hTERT mRNA     | 5'gccagaacgttccgccagagaaaa -3' | 5'aatcatccaccaaacgcaggagc -3' |
| 7    | GAPDH          | 5'-cagccgagccacatcg -3'        | 5'-tgaggctgttgcatactctc -3'   |

### RNA extraction and cDNA synthesis

The cells were cultured in T-25 flasks and treated with the plant extracts and incubated for 2 hours. After incubation, the total cellular RNA was isolated using Tri reagent (MRC, Ohio) according to the manufacturer's protocol. Subsequently, 10 $\mu$ l was reverse transcribed into cDNA by using the cDNA synthesis kit (First strand cDNA synthesis kit- Fermentas, USA) using the random hexamer primer. The cDNA synthesized was used as the template for PCR amplification. PCR was carried out in a final concentration of 25 $\mu$ l containing 12.5 $\mu$ l of Mastermix (Fermentas, USA), 2 $\mu$ l of cDNA, 1 $\mu$ l of each forward and reverse primer (10 pmol) and 8.5 $\mu$ l DEPC treated water.

### PCR amplification for expression of the apoptotic genes

The PCR protocol for determining the expression of apoptotic genes consisted of initial cycles of denaturation at 94°C for 2 minutes, 94°C for 30 seconds, annealing at 60°C for 30 seconds, 72°C for 1 min and extension at 72°C for 7 min. The PCR cycle conditions include 28 ( $\beta$  actin), 33 (Fas) and 38 (Bax and Bcl-2). The PCR products were electrophoresed on a 2 % agarose gel and visualized with ethidium bromide staining. The expected sizes of the PCR products for  $\beta$  actin, Bcl-2, Bax, Fas were 203, 179, 342, 269, base pairs (bp), respectively.

### PCR amplification for expression of telomerase (Tumor marker enzyme)

The thermal cycling conditions for telomerase RNA includes 94°C at 5 minutes, followed by 25 cycles of 94°C at 20 seconds, 50°C at 45 seconds and 72°C at 30 seconds and final extension at 72°C for 7 minutes. hTERT includes 94°C at 5 minutes, followed by 30 cycles of 94°C at 20 seconds, 48°C at 45 seconds and 72°C at 30 seconds and final extension at 72°C for 7 minutes. GAPDH includes 94°C at 5 minutes, followed by 25 cycles of 94°C at 1 minute, 58°C at 30 seconds and 72° at 45 seconds and final extension at 72° for 7 minutes. The PCR products were electrophoresed on a 2% agarose gel and visualized with ethidium bromide staining. The expected sizes of the PCR products for GAPDH, telomerase RNA and hTERT mRNA were 460, 179 and 649 bp respectively.

### Nucleotide sequencing

The PCR products of the hTERT gene were purified using Purelink (PCR purification kit, Invitrogen, USA) and the purified PCR products were subjected to automatic sequencer (ABI, Switzerland). The nucleotide sequence data was then used for blast analysis.

## RESULTS AND DISCUSSION

### Effect of *Tinospora cordifolia* on the expression of the apoptosis related genes

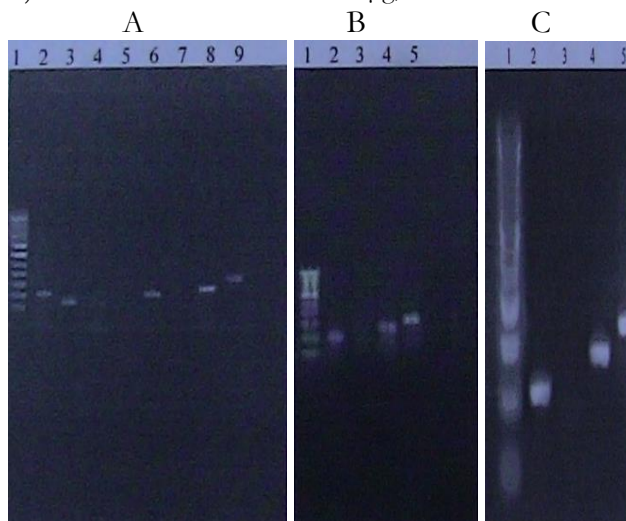
The effect of aqueous and hydro alcoholic extracts of *T. cordifolia* in high doses (600  $\mu$ g/ml) to detect the expression of apoptosis related genes Bcl-2, Bax and Fas keeping  $\beta$  actin as an internal control on MCF-7 cell line is depicted in Fig. 1.

**Figure 1:** The effect of aqueous and hydro-alcoholic extracts of *T. cordifolia* in high doses (600  $\mu$ g/ml) on the expression level of apoptosis related genes Bcl-2, Bax, Fas, keeping  $\beta$ -actin as an internal control on MCF-7 cells.

A) DNA extracted from vehicle (lane 2-5) and doxorubicin (lane 6-9) treated cells.

B) DNA extracted from T<sub>AE</sub> 600  $\mu$ g/ml treated cells.

C) DNA extracted from T<sub>HAE</sub> 600  $\mu$ g/ml treated cells.



\* Lane : 1 - Marker  
 Lane : 2 & 6 -  $\beta$  actin (203 bp)  
 Lane : 3 & 7 - Bcl2 (179 bp)  
 Lane : 4 & 8 - Fas (269 bp)  
 Lane : 5 & 9 - Bax (342 bp)

\* AE - Aqueous extracts, HAE - Hydro-alcoholic extracts.

The expression of apoptotic genes and  $\beta$  actin were noticed in all the treatment groups when compared to the negative control (vehicle) where there was expression of anti-apoptotic gene and the internal control. Bcl-2, Bax and Fas play an important role in apoptosis. Bcl-2 has an antiapoptotic effect whereas Bax and Fas are apoptotic in nature (Lai and Thomas, 1999). The increased ratio of Bax/Bcl-2 might contribute to the induction of apoptosis in resveratrol (Kim *et al.*, 2004) and *Antrodia camphorata* (Yang *et al.*, 2006) treated MCF-7 cells. Treatment with *Siegesbeckia glabrescens* decreased the level of Bcl-2 mRNA expression and increased the level of Bax expression in MCF -7 cells

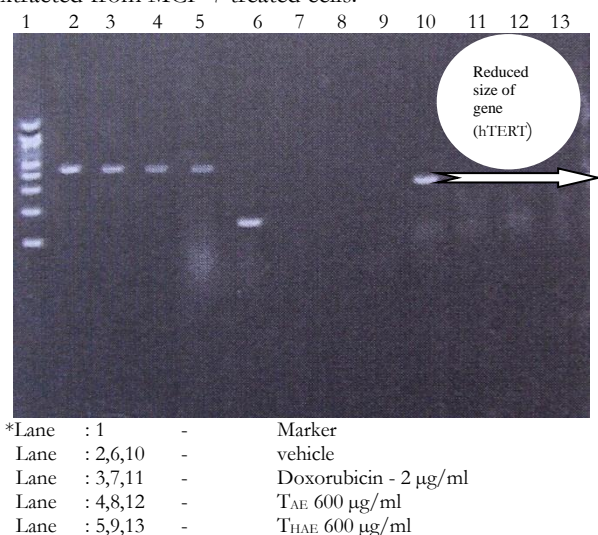
(Jun *et al.*, 2006). Our studies also showed the similar expression of Bcl-2 gene in the negative control and Bax and Fas in the treated groups.

Since only qualitative assay of expression was done in our studies, it requires further probe using quantitative tests like real time PCR to provide unequivocal results.

### Effect of *T. cordifolia* on tumour marker enzyme (Telomerase)

The effect of aqueous and hydro alcoholic extracts of *T. cordifolia* in high doses (600 µg/ml) to detect the expression level of telomerase RNA, hTERT mRNA keeping GAPDH as an internal control on MCF-7 cell line is depicted in Figure 2.

**Fig. 2:** The effect of aqueous and hydro-alcoholic extracts of *T. cordifolia* in high doses (600 µg/ml), on the expression of telomerase RNA (lane 6-9), hTERT mRNA (lane 10-13) keeping GAPDH as an internal control (lane 2-5) on cDNA extracted from MCF-7 treated cells.



\*AE - Aqueous extracts, HAE - Hydro-alcoholic extracts

Telomerase is a ribonucleoprotein polymerase that maintains telomere ends by the addition of the telomere repeat TTAGGG. The enzyme consists of two molecules each of hTERT which is a protein and an RNA component which serves as a template for the telomere repeat and dyskerin. Increase in telomerase activity and hTERT mRNA expression are features of the early stages of the development of squamous cell carcinoma of the lung, with strong telomerase activity and hTERT mRNA expression being prominent during the later stages (Shibuya *et al.*, 2001). In the present findings, negative control (Vehicle) showed the expression of GAPDH, telomerase RNA and hTERT mRNA, whereas treatment with the aqueous and hydro-alcoholic extracts of *T. cordifolia* and the positive control showed only the expression of GAPDH. Some of the proven anticancer compounds like adriamycin and 5-fluorouracil reduced telomerase activity and human telomerase mRNA expression in MCF-7 cells (Ishikawa *et al.*, 1999). In our study too, decrease in telomerase activity is found as is evident by the absence of hTERT amplification. However, in the negative control, the gene amplified

corresponds to a much smaller size (less than 649 bp). This may be attributed to the following reasons.

- (i) Alternative spliced variants encoding different isoforms (Yokoyama *et al.*, 2001).
- (ii) The amplified gene was further sequenced and the blast analysis of the sequenced product revealed to be similar to a U5 snRNP (small nuclear Ribonucleo protein) which is responsible for maintaining cell viability in *Saccharomyces cerevisiae* (Dix *et al.*, 1998). It is possible that the plant extract could induce cell death through a different pathway by involving U5 snRNP. The blast analysis also revealed that hTERT primers did not show homology with the U5 snRNP. Hence the mechanism by which U5 snRNP was amplified is uncertain.

However the results of our study point that telomerase activity could be a useful marker in *in vitro* assessment of tumour cell chemosensitivity. Hence, to confirm identity of the expressed products, it was subjected to nucleotide sequencing. Blast analysis of the sequenced product was found to be homologous to U5 snRNP present in Homo sapiens chromosome. However the blast analysis of hTERT primers with the U5 snRNP did not show homology.

Thus the study revealed that *Tinospora cordifolia* could be an effective inhibitor of breast cancer as evidenced by the expression of apoptotic genes and inhibition of the telomerase activity (tumour marker cells. enzyme) in the cancer cells *In Vitro*. Thus *Tinospora cordifolia* may be considered as a natural anticancer agent for human breast cancer and contribute to clarify the complex mechanisms involved in its anticancer properties. Hence it will be worthy to undertake further exploration of its anticancer effects to realize its full potential as a cancer chemotherapeutic agent.

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