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Original Article

# Antiproliferative activity and apoptosis-inducing mechanism of *Curcuma longa* (Turmimax®) on HeLa cell lines

## Atividade antiproliferativa e mecanismo indutor de apoptose de *Curcuma longa* (Turmimax®) em HeLa

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**Abstract**

Curcumin, the primary polyphenol found in turmeric, is derived from the *Curcuma longa* plant. Since curcumin is nontoxic and has a wide range of medicinal qualities, including anti-oxidant, analgesic, anti-inflammatory, and antibacterial action, it has been widely employed in Ayurveda medicine for ages. Curcumin has recently been discovered to have anti-cancer properties through its impact on numerous biological pathways involved in carcinogenesis, metastasis, tumorigenesis, cell cycle regulation, mutagenesis, and oncogene expression. In this study, we determined the Antiproliferative activity and apoptosis-inducing mechanism of *C. longa* (Turmimax®) on human cancer cells. The cytotoxic effect was evaluated against HeLa cell lines using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Flow cytometric analysis was performed to detect apoptotic cell death. Turmimax® exhibits promising properties as a potential anti-cancer therapeutic agent in human cervical adenocarcinomas and possibly other cancer types, with an $IC_{50}$ value of 87.89 µg/mL. In HeLa cells treated with Turmimax®, cell cycle arrest was seen in the G0/G1 and S phases. By inducing apoptosis and increasing the number of apoptotic cells in a dose-dependent manner, the experimental data suggest that Turmimax® has considerable promise in cancer prevention and treatment.

**Keywords:** antiproliferative, *Curcuma longa*, HeLa cell lines, apoptosis.

**Resumo**

A curcumina, o polifenol primário encontrado no açafrão, é derivada da planta *Curcuma longa.* Como a curcumina não é tóxica e possui ampla gama de qualidades medicinais, incluindo ação antioxidante, analgésica, anti-inflamatória e antibacteriana, ela tem sido muito empregada na medicina ayurvédica há séculos. Descobriu-se recentemente que a curcumina possui propriedades anticancerígenas por meio de seu impacto em várias vias biológicas envolvidas na carcinogênese, metástase, tumorigênese, regulação do ciclo celular, mutagênese e expressão de oncogenes. Neste estudo, determinamos a atividade antiproliferativa e o mecanismo de indução de apoptose de *C. longa* (Turmimax®) em células cancerígenas humanas. O efeito citotóxico foi avaliado em linhagens de células HeLa usando o ensaio MTT – brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio. Uma análise de citometria de fluxo foi realizada para detectar a morte celular apoptótica. Turmimax® exibe propriedades promissoras como um potencial agente terapêutico anticancerígeno em adenocarcinomas cervicais humanos e possivelmente em outros tipos de câncer, com um valor de IC50 de 87,89 µg/mL. Nas células HeLa tratadas com Turmimax®, a parada do ciclo celular foi observada nas fases G0/G1 e S. Ao induzir a apoptose e aumentar o número de células apoptóticas de maneira dependente da dose, os dados experimentais sugerem que Turmimax® tem uma indicação considerável na prevenção e tratamento do câncer.

**Palavras-chave:** antiproliferativo, *Curcuma longa*, linhas de células HeLa, apoptose.

## 1. Introduction

Cancer is a major cause of mortality in people around the world, among other diseases. Because they are either extremely toxic, ineffective at treating cancer, or extremely expensive, the majority of drugs currently used to treat cancer have limited potential. There is a demand for treatments without these drawbacks. Therefore, a crucial and difficult issue in cancer treatment is the development and synthesis of novel, effective, and less toxic anticancer medicines. Numerous plant-based products have been reported for advancements in the treatment of cancer, and using plant extracts as medicine is undoubtedly an efficient strategy. In comparison to synthetic medicines, plant-based products are affordable, safe, and naturally multi-targeting (Kintzios, 2006). Plants have long been

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used to treat cancer, and there is still a lot of interest in nature as a source of possible chemotherapeutic drugs. Current research and development targeted at finding new anti-proliferative agents from natural products has been accelerated by the growth of science and technology in the identification of anticancer drugs. Oxidative stress and persistent inflammation are the starting points for cancer. These newly formed cells are promoted by inflammation, which may send signals that encourage cell growth and stop apoptosis. It has been investigated how inflammation affects the development of tumor and subsequent malignancy. The inflammatory reaction produces cytokines, which function as growth and/or angiogenic agents, promoting the proliferation and development of altered cells (Srivastava and Srivastava, 2015).

According to studies, curcumin produced from the rhizomes of *C. longa* exhibits anti-inflammatory, anti-mutagenic, antibacterial, anticarcinogenic, and antioxidant effects (Ali et al., 2017). Despite years of research, Curcumin's efficacy as a medicine has been compromised by its poor solubility (Zhang et al., 2013). As per statistics, recently developed medications often have poor water solubility, which limits how much of the drug may be absorbed after oral administration (Srinivasan, 1972). In order to improve selectivity against cancer cell lines, it is necessary to explore potential solutions or alternatives that modify the natural compound structure. Curcumin inhibits cancer cell invasion, angiogenesis, metastasis, cell proliferation, and inflammation. It has also been proven to cause apoptosis and make tumor cells more sensitive to cancer treatments (Aggarwal et al., 2003; Lin et al., 2009). As a potent antioxidant, curcumin reduces lipid peroxidation in rat liver microsomes, erythrocyte membranes, and brain homogenates. Inflammation, heart disease, and cancer are all caused by lipid peroxidation (Araujo and Leon, 2001; Reddy and Lokesh, 1994). Due to its multi-targeting properties against a range of various cancers, such as leukemia, gastrointestinal tumors, genitourinary cancers, breast cancer, etc., it has broad therapeutic promise (Barthelemy et al., 1998; Bartine and Tanaoui-Elaraki, 1997; Kawamori et al., 1999). Turmimax® is a unique, proprietary substance that dissolves quickly and retains solubility over time, making it 35x times more bioavailable than ordinary curcumin. It contains 95% curcuminoids and 100% water dispersibility. This study evaluates Turmimax® cytotoxicity toward HeLa cell lines.

## 2. Materials and Methods

### *2.1. Preparation of sample*

Turmimax® is manufactured and registered by Star Hi Herbs Pvt Ltd Jigani, Bangalore, Karnataka, India.

### *2.2. Reagents and cell culture*

HeLa cell lines were obtained from the NCCS, Pune, India. The drugs and chemicals were purchased as follows; MTT reagent (5mg/mL; Cat. No. 4060, Himedia), DMSO (Dimethyl sulfoxide), DMEM (Dulbecco's Modified Eagle Medium; Cat No. #AL127S, Himedia) medium, PBS (Phosphate-buffered saline) (Cat No. TL1006, Himedia), 5 µL of Annexin V FITC (Cat No: 51-65874X, BD Biosciences), and Propidium Iodide (PI; Cat No. 51-66211E, BD Biosciences) and camptothecin (Cat. No. C9911, Sigma). HeLa cells were grown in 10 mL of DMEM supplemented with 10% FBS and 2mM L-glutamine. Medium was replenished every three days.

### *2.3. MTT assay*

The cytotoxicity of Turmimax® was evaluated using MTT assay. HeLa cells were seeded at a density of 20,000 cells per well in a 24-well culture plate (Corning, USA). Cells were treated with Turmimax® concentrations of 6.25, 12.5, 25, 50 and 100 µg/mL for 24 hours. Prior to MTT assay, the media was replaced and MTT reagent (5 mg/mL; Cat. No. 4060, HiMedia) was added to each well at a final concentration of 0.5 mg/mL. Wells with DMEM only served as the controls. The plate was incubated at 37 °C for 3 hours. To solubilize the MTT formazan crystals, the MTT reagent medium was replaced with DMSO and gently rocked on a gyrator. The absorbance was read at a wavelength of 570 nm. Control wells were used to eliminate absorbance from DMEM. Percentage cytotoxicity and viability were calculated with the following Equations 1 and 2 respectively:

$$Cytotoxicity\% = 1 - \frac{Mean\ absorbance\ of\ toxicant}{Mean\ absorbance\ of\ control} \times 100 \quad (1)$$

$$Viability\% = 100 - Cytotoxicity\% \quad (2)$$

### *2.4. Flow cytometry*

In a 24-well culture plate (Biolite, Thermo), cells were seeded at a density of $3x10^5$ cells/2 mL and incubated overnight at 37 °C. In fresh 2 mL of DMEM medium, each well was treated for 24 hours with concentrations of the apoptotic standard and camptothecin as per the manufacturer's instructions, and the $IC_{50}$ concentration of Turmimax®. Cells were harvested after being rinsed with PBS (Cat No. TL1006, Himedia). The cells were centrifuged at 300 x g for 5 minutes at room temperature, and the supernatant was carefully decanted. Before staining with 5 µL of Annexin V FITC, the pellet was washed twice with PBS (Cat No: 51-65874X, BD Biosciences). Each round bottom tube was vortexed and incubated in the dark for 15 minutes at room temperature. Post-incubation, PI (Cat No. BD Biosciences, 51-66211E) and 400 µL of 1 x Annexin V binding buffer was pipetted into each tube. Using a BD FACS Calibur (BD Biosciences), a 10 000-event cell population was analyzed for apoptotic activity.

### *2.5. Cell cycle arrest*

Cells were cultured for 24 hours at 37 °C at a density of $2 x 10^5$ cells/2 mL. Cells were replenished with new media and treated with Turmimax® and camptothecin. Cells were harvested and centrifuged at 300×g for 5 minutes. Cells were rinsed with PBS before being fixed for 30 minutes in 1 mL of cold 70% ethanol (Cat. No. #GRM8525, Himedia) on ice. Cells were centrifuged and PBS washed twice. After washing, cells were stained with 400 µL PI/RNAse staining

solution (Cat No: 550825, Sigma) and incubated at room temperature for 20 minutes. Cell cycle arrest was detected using the BD FACS Calibur flow cytometer.

### *2.6. Statistical analysis*

Statistical significances of difference throughout this study were calculated using a Student's t-test and by one-way variance analysis. P<0.05 was considered statistically significant.

*p*-values (Statistically significant if<0.05) were calculated using the Graph Pad Prism V.5. software.

## 3. Results

### *3.1. Cytotoxicity effects of Turmimax on HeLa cell line*

The effect of Turmimax® was studied as a dose-response experiment. Turmimax® cytotoxicity in HeLa cells was assessed using the MTT assay at concentrations of 6.25, 12.5, 25, 50, and 100 µg/mL after a 24-hour exposure period. The proliferation of HeLa cells was significantly inhibited by Turmimax® in a concentration-dependent manner during 24 hours ($P < 0.01$). Cell proliferation was shown to decrease with increasing concentrations of Turmimax® at both microscopic observations (Figure 1) and MTT absorbance rates (Figure 2). MTT analyses have shown that Turmimax® may possess potentially significant cytotoxic effects on HeLa cells, with an $IC_{50}$ value of approximately 87.89 µg/mL (Table 1). $IC_{50}$ was determined by probit analysis using the Pharm PCS (Pharmacologic Calculation System) statistical package (Springer-Verlag, USA). There were significant differences in $IC_{50}$ Turmimax® ($P < 0.05$).

### *3.2. Turmimax® apoptosis induction in HeLa cells*

The effect of Turmimax® on cell apoptosis was investigated. HeLa cells were treated with Turmimax®

**Table 1.** Cytotoxic effects of Turmimax® on HeLa cancer cell lines.

| Sl. No. | Drug Concentration (µg/ mL) | % of Cell Viability | $IC_{50}$ Value |
|---|---|---|---|
| 1 | Cell Control | 100 | |
| 2 | Std Control | 49.446 | |
| 3 | 6.25 | 80.600 | |
| 4 | 12.5 | 76.594 | 87.89 µg/mL |
| 5 | 25 | 70.426 | |
| 6 | 50 | 63.995 | |
| 7 | 100 | 45.861 | |

$IC_{50}$: Drug concentration effective in inhibiting 50% of the cell viability measured by the MTT assay after 24-hour exposure, and is expressed as mean ± SD by at least three independent experiments.

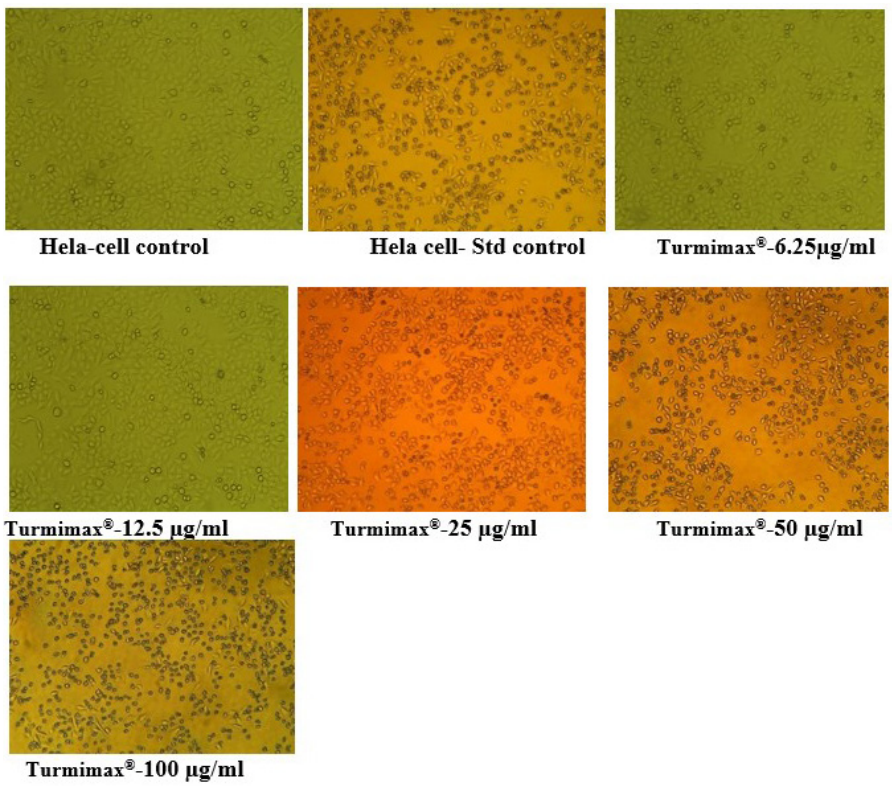


**Figure 1.** HeLa cells treated to various concentrations of Turmimax® over 24 hours. Cells exhibited morphological changes after treatment. Images were captured at a 10x magnification using an inverted phase contrast microscope.

for 24 h. According to the results of the HeLa proliferation assay, the results demonstrated that Turmimax® promoted cell apoptosis. We used the Annexin V-FITC/PI assay to determine whether the cells were in apoptosis or necrosis, and the results revealed by flow cytometry showed that the percentage of apoptosis in cervical cancer cells increased significantly after treatment with the established $IC_{50}$ value of Turmimax® 87.89 µg/mL, demonstrating Turmimax® capacity to induce apoptosis in HeLa cells. As shown in Figure 3, when compared to the apoptotic standard camptothecin, when compared to camptothecin, Turmimax® had a significantly higher percentage of apoptotic cells (83.93% and 44.19%). There is a clear differential between apoptotic activities in comparison to the untreated (control) cells with the largest percentage of viable cells. The observed population of necrotic (dead) cells per treatment was essentially the same, with the control treatment clearly devoid of any dead cells. This demonstrates clearly that the turmeric rhizome extract can induce anticancer effects in HeLa cells via the apoptotic mechanism.

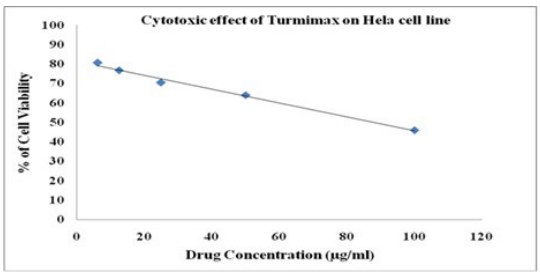


**Figure 2.** MTT assay showed considerable cytotoxic effects on HeLa cells with a maximal inhibitory concentration.

*3.3. G0/G1 and S phase cell cycle arrest in HeLa cells by Turmimax®*

To assess Turmimax® cell cycle arrest activity, HeLa cells were once again treated at the IC50 dose. When compared to the control (untreated), the percentage of cells stopped at the G0/G1 and G2/M phases decreased in the Turmimax®-treated group, while the sub G0/G1 and S phases increased (Figure 4). However, both of these phases had the highest cell cycle arrested populations during the treatment. Camptothecin treatment at 10 µM demonstrated a substantial reduction in cell cycle arrest in the G0/G1 phase population and an increase in the observed percentage in the G2/M phase. Turmimax® interacts with HeLa cells in a clearly promising manner for inducing apoptosis by stopping the cell cycle.

## 4. Discussion

Apoptosis is a vital physiological process that is necessary for the development and homeostasis of healthy tissues. Cancer, on the other hand, upsets the balance between cell division and death (Hanahan and Weinberg, 2000). As a result, it is critical to focus on apoptosis when treating cancer. Phosphatidylserine (PS) translocate to the extracellular side of the membrane in apoptotic cells. Because Annexin V is a phospholipid-binding protein, it can be used to detect the translocation of PS to the outside of the membrane, revealing early-stage apoptosis (Plati et al., 2011).

The anti-tumoricidal and antimetastatic effects of curcumin have been reported in the treatment of oral, colon, breast, prostate, oesophageal, lung, and colon cancers (Allegra et al., 2017). In this investigation, we examined Turmimax® turmeric rhizome extract's anticancer

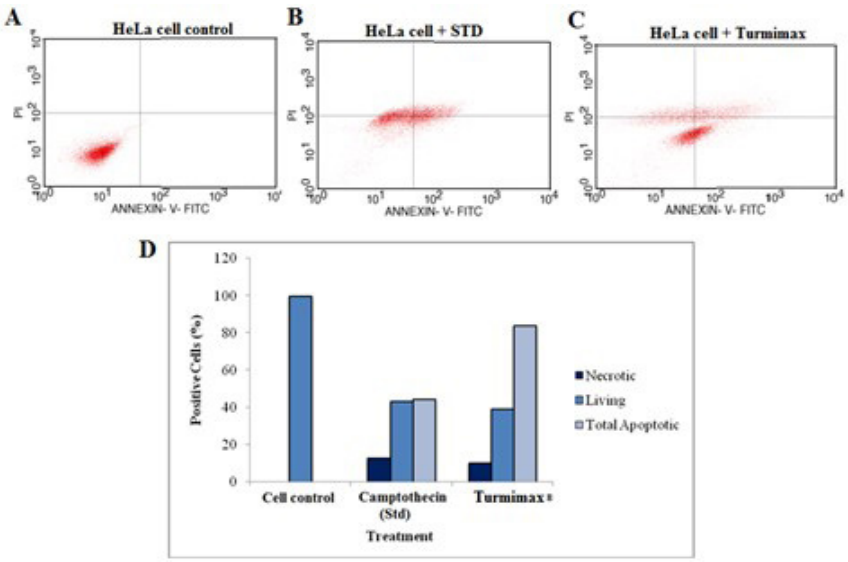


**Figure 3.** Apoptosis induction in Turmimax® treated HeLa cells. (A) Control (untreated) HeLa cells. Cells were treated with (B) camptothecin (apoptotic standard) and (C) 87.89 µg/mL of Turmimax® for 48h; (D) Percentage positive necrotic (PI) and apoptotic (Annexin V-FITC) cell populations in control, camptothecin and Turmimax® treatments.

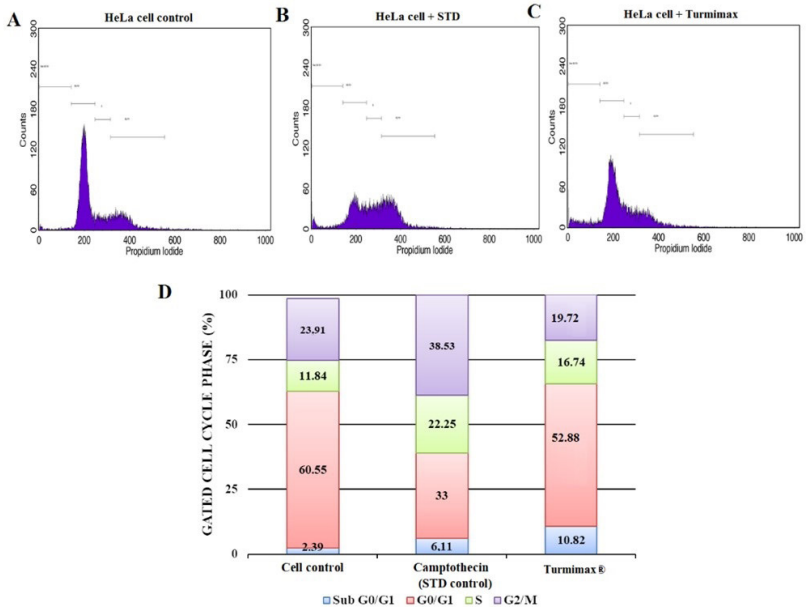


**Figure 4.** Cell Cycle Analysis of Turmimax® treated HeLa cells. (A) Cell cycle phase identification using BD FACS Caliber scatterplots for PI positive populations in control, (B) Cell cycle phase identification using BD FACS Caliber scatterplots for PI positive populations in 10 µM of camptothecin treatment (C) Cell cycle phase identification using BD FACS Caliber scatterplots for PI positive populations in 83.89 µg/mL of Turmimax® treatment; (D) PI histogram of the gated cell singlets distinguished cells at the Sub G0/G1, G0/G1, S, and G2/M cell cycle phases.

properties in HeLa cells (cervical adenocarcinoma). Turmimax® induces cell arrest at the G0/G1 and S phases. Wherein earlier studies found that arrest at certain points led to apoptosis and, in some situations, DNA synthesis suppression in cell lines (Karunagaran et al., 2005). Cells that are stopped in the G0/G1 phase do not differentiate into new cells that inhibit cell growth or induce apoptosis; however, with prolonged treatment, these cells might relocate to the following cell cycle phase where micronuclei cause apoptosis to occur formation. In phase I/II clinical trials, oral dosages of curcumin are administered as the primary therapeutic agent or as a chemo sensitizer to lessen the harmful and cytotoxic effects of chemotherapeutic drugs (Soleimani et al., 2018).

Cancer chemotherapy is critical in the treatment of various malignancies because it targets cancer cells while not harming healthy host cells. Oncologists are primarily interested in cancer-fighting compounds that are selective and induce cellular apoptosis or autophagy, which most secondary plant metabolites do. Several studies have found that the mechanism of action of these anticancer drugs causes apoptosis (Scaria et al., 2020).

Turmimax® has intriguing capabilities as a possible anticancer treatment agent in HeLa cells and maybe other cancer types. This is when all the study's findings for apoptosis induction and cell cycle arrest are taken into account and compared to currently published curcumin data. Additionally, extended *in vitro* treatment times may reveal the precise processes controlled to activate programmed cell death for a more specialized medication design.

## 5. Conclusion

Our present findings indicate that Turmimax® is a promising anti-tumor agent in human cervical adenocarcinomas. The antiproliferative effect strengthens with an increase in the concentration of the Turmimax®. The induction of apoptosis by Turmimax® appears to be dependent on the formation of reactive metabolites. Therefore, it is important to promote the development of therapies that involve Turmimax® in the treatment of cancer. This finding will greatly benefit the clinical use of Turmimax® and suggests that it is a potent anti-tumor drug in the Pharmaceutical industry.

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