

EVALUATION OF THE ANTICANCER PROPERTIES OF TURMESAC® IN HUMAN CERVICAL ADENOCARCINOMA

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ABSTRACT

Objective: In the current study, an extract of turmeric rhizome (Turmesac®) was evaluated for possible anticancer activity in human cervical adenocarcinoma (HeLa) cells.

Methods: Turmesac®'s ability to elicit cytotoxicity in cancer cells was evaluated by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (MTT) assay, where the IC₅₀ value was determined. Apoptosis induction and cell cycle arrest were analyzed by flow cytometry post-Turmesac® IC₅₀ value treatment for 24 h.

Results: The determined IC₅₀ value of Turmesac® in HeLa cells was 115.12 µg/ml. This concentration was able to induce apoptosis 2 times greater than the apoptotic standard, camptothecin, treated cells. Cell cycle arrest was observed at the G₀/G₁ and S phases in Turmesac® treated HeLa cells.

Conclusion: Turmesac® shows the potential of being a promising anticancer agent that may be incorporated into chemotherapies, but further study is required to elucidate the exact mechanisms involved with longer treatment duration, as would be the case in clinical trial phases.

Keywords: Apoptosis, Cell cycle arrest, Cytotoxicity, HeLa cells, Turmesac®

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INTRODUCTION

Turmeric (*Curcuma longa* [Linn.]) is a member of the ginger (Zingiberaceae) family [1-3]. It is a herbaceous, aromatic, and perennial plant species that is cultivated in subtropical and tropical regions around the world, including Bangladesh, India, China, and South America [4-6]. The largest world supplier of turmeric is India. This short-stemmed species grows to a height of 100 cm. The plant has oblong leaves and funnel-shaped yellow flowers while the rhizome may be oblong, ovate, cylindrical, or pyriform often with short branches. The rhizome is boiled, dried, and ground for the creation of the spice powder that is included as a principal ingredient in curry powder or as a coloring agent in western sauces, mustards, and pickles. Turmeric has been used for centuries as a traditional/ayurvedic medicine for the treatment of numerous ailments and disease. It is commonly used in foods, therapeutics (personal and health care), and as a dietary supplement and has been characterized as a pharmaceutical crop [7] that is safe for consumption by the food and drug administration and WHO.

The highly researched component of turmeric, curcumin, is a natural, yellow polyphenolic that comprises 75% of its constitution [8]. Curcumin was first chemically characterized in 1910. Based on published literature, curcumin has many biological targets, including signaling pathways, cytokines, transcription and growth factors, and oncogenic molecules. Conventionally, curcumin was known as a blood purifier, in addition to this characteristic, it exhibits anti-inflammatory, anti-proliferative, anti-venom, antioxidant, anti-microbial, anti-malarial, anti-aging, anti-angiogenic, wound healing, and anti-depressant properties [9]. In addition, the current research has been focused on natural, targeted alternative medicine to be inclusive in cancer therapy, and treatment with curcumin being of great focus [10]. Cancer is the result of genetic alterations within a cell that leads to a drastic change in its phenotype that allows for continuous cell longevity and excessive proliferation of

said phenotype within the body [11]. This cell proliferation is an abnormal behavior since these cells do not follow the normal cell cycle checkpoints with the possibility of metastases events [12]. It has been shown that countries with high turmeric consumption have a lower cancer incidence rate which highly suggests to the possibility of possessing chemopreventative properties. Due to the multi-targeting action of curcumin and its metabolites, studies have shown that it has significant anti-tumoricidal activity in a wide range of normal and cancer cell lines, animal models, and clinical trials [13,14]. By inhibition of cell proliferation, cell cycle arrest, and apoptosis induction [3] previous studies have shown that curcumin interacts with the apoptotic proteins of the Bcl₂ family, NF-κB pathway, and cyclin kinases. In this study, turmeric rhizome (Turmesac®) extract was evaluated for eliciting anticancer effects in human cervical adenocarcinoma (HeLa) (cervical adenocarcinoma) cells.

METHODS

Turmeric rhizome (Sangli Turmeric) was collected from Maharashtra, March 2019, India, and then authenticated by a botanist, R&D division, Star Hi Herbs Pvt. Ltd, Jigani, Bengaluru, Karnataka, India. The turmeric rhizomes were carefully cleaned, dried, powdered, and stored in an airtight container until the extraction procedure.

Preparation of the Turmesac®

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

Cell culture and treatment

The HeLa cell line (NCCS, Pune) was grown in high glucose Dulbecco's Modified Eagle Medium (DMEM) medium (#AL111, HiMedia) supplemented with 10% fetal bovine serum (FBS; #RM10432, HiMedia) in a 5% CO₂ humidified incubator at 37°C (Heal Force, China), where applicable HeLa cells were treated with specific concentrations of Turmesac® and 10 µM camptothecin (Cat. No. C9911, Sigma) for 24 h.

MTT assay

HeLa cells were seeded in a 96-well plate (Corning, USA) at a density of 20,000 cells per well. Cells were treated with concentrations of Turmesac® ranging from 0 to 100 µg/ml for a duration of 24 h. The media were replaced before the preparation of MTT assay where MTT reagent (5 mg/ml; Cat. No. 4060, HiMedia) was added to each well at a final concentration of 0.5 mg/ml. The plate was incubated for 3 h at 37°C. The MTT reagent medium was replaced by dimethyl sulfoxide to solubilize the MTT formazan crystals by gentle rocking on a gyrator. Absorbance was read at a wavelength at 570 nm. The IC₅₀ value for the Turmesac® compound was determined by linear regression using Microsoft Excel 2010 software.

Flow cytometry

Cells were seeded at a density of 3×10^5 cells/2 ml in a 6-well culture plate (BioLite, Thermo) and incubated overnight at 37°C. Each well was treated with the appropriate concentration of the apoptotic standard, camptothecin, or Turmesac® in fresh 2 ml DMEM medium for 24 h. Post-treatment cells were washed with phosphate-buffered saline (PBS) (Cat No. TL1006, HiMedia) and harvested. Cells were centrifuged for 5 min at $300 \times g$ at room temperature and the supernatant was carefully decanted. The pellet was washed twice with PBS before staining with 5 µl of Annexin V FITC (Cat No: 51-65874X, BD Biosciences). Each was vortexed and incubated in the dark at room temperature for 15 min. Cells were stained with 5 µl propidium iodide (PI; Cat No. 51-66211E,

BD Biosciences) and 400 µl of $1 \times$ Annexin V binding buffer. Cells were analyzed for apoptotic activity in a BD FACSCalibur (BD Biosciences).

Cell cycle arrest

Cells were cultured at a density of 2×10^5 cells/2 ml and incubated at 37°C for 24 h. Cells were replenished with fresh media and treated with Turmesac® or camptothecin. Cells were harvested and centrifuged for 5 min at $300 \times g$. Cells were rinsed with PBS and fixed in 1 ml of cold 70% ethanol on ice for 30 min. Cells were centrifuged and washed twice with PBS. After the wash step, cells were stained with 400 µl PI/RNase staining solution (Cat No: 550825, Sigma) and incubated for 20 min at room temperature. Using the BD FACSCalibur flow cytometer, cells were analyzed for cell cycle arrested.

RESULTS**Determination of IC₅₀ of Turmesac®**

Cytotoxicity of Turmesac® in HeLa cells was evaluated through MTT assay activity at concentrations of 6.25, 12.5, 25, 50 and 100 µg/ml after 24 h exposure period. Cell proliferation was shown to decrease with increasing concentrations of Turmesac® at both microscopic observations (Fig. 1a) and MTT absorbance rates (Fig. 1b). MTT analyses have shown that Turmesac® may possess potentially significant cytotoxic effects on HeLa cells bearing an IC₅₀ value of approximately 115.12 µg/ml.

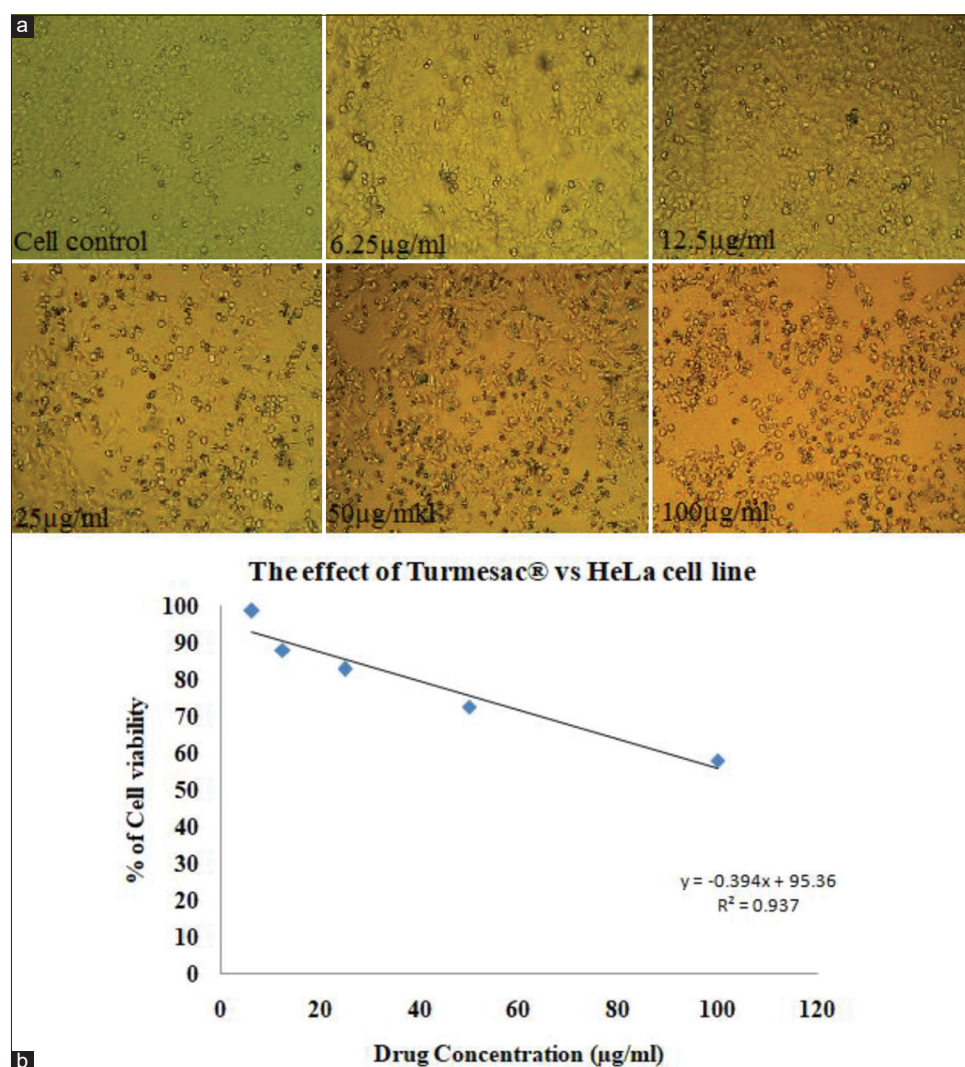


Fig. 1: Morphological changes in human cervical adenocarcinoma cells treated to different concentrations of Turmesac® for 24 h. Images were taken using on inverted phase-contrast microscope at $\times 10$

Turmesac® apoptosis induction in HeLa cells

Turmesac® apoptosis induction activity was evaluated using the IC₅₀ value of 115.12 µg/ml on HeLa cells. The ability of Turmesac® to induce apoptosis in cervical adenocarcinoma cells was in comparison to the apoptotic standard, camptothecin. There is a clear distinction of apoptotic activity in comparison to the untreated (control) cells (Fig. 2) with the highest percentage of viable cells, while Turmesac® had a significantly larger apoptotic cell population to camptothecin, 83.93 % to 44.19 %, respectively. The observed necrotic (dead) cell population per treatment was relatively the same with a clear absence of dead cells in the control treatment. This clearly indicates that the turmeric

rhizome extract can elicit anticancer effects through the apoptotic pathway in HeLa cells.

G0/G1 and S phase cell cycle arrest in HeLa cells by Turmesac®

HeLa cell was once again treated with the IC₅₀ concentration to evaluate Turmesac® cell cycle arrest activity. In comparison to the control (untreated), there was a decrease in the percentage of cells arrested at the G0/G1 and G2/M phases but an increase in the sub G0/G1 and S phases (Fig. 3) in the Turmesac® treatment. Camptothecin treatment at 10 µM showed a drastic reduction in cell cycle arrest in the G0/G1 phase population and increased observed percentage in the G2/M

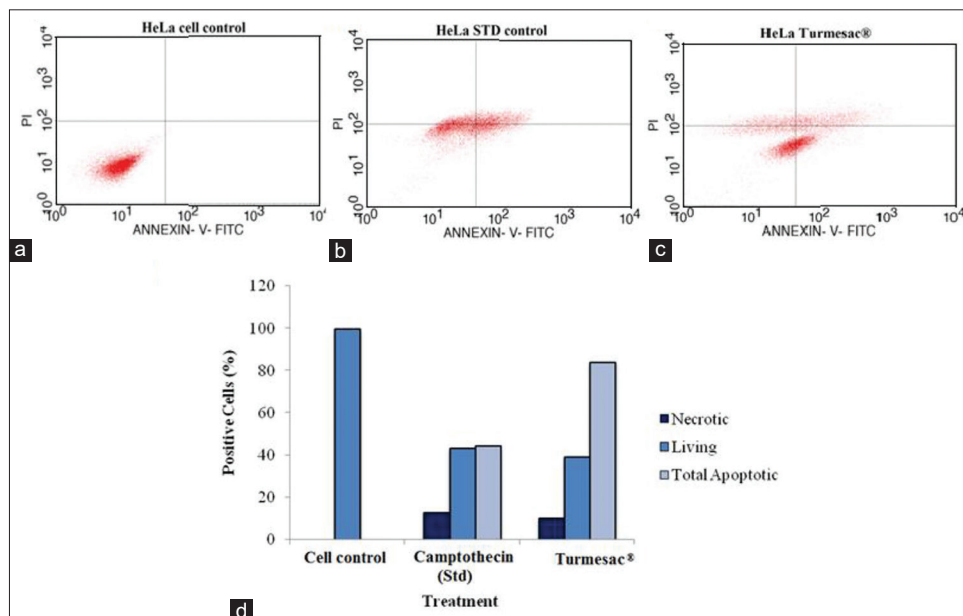


Fig. 2: Apoptosis induction of Turmesac® in human cervical adenocarcinoma cells. Cells were treated with 115.12 µg/ml of Turmesac® and camptothecin (apoptotic standard) for 48 h. Flow cytometric analysis for necrotic (PI) and apoptotic (Annexin V-FITC) positive cell populations (n=3)

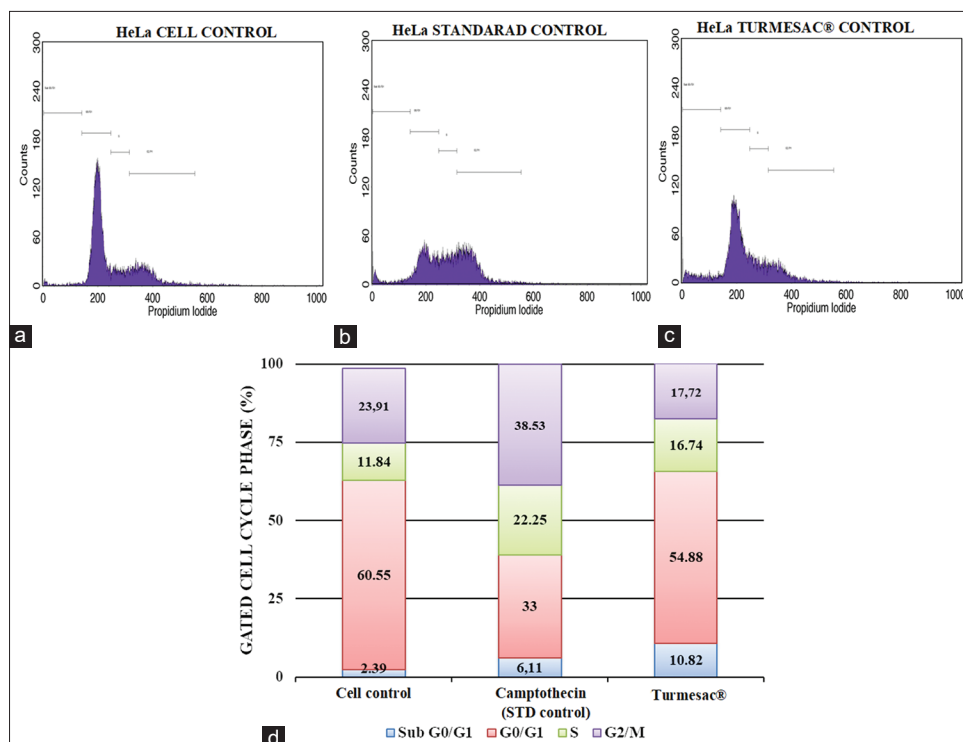


Fig. 3: Cell cycle analysis of Turmesac® against human cervical adenocarcinoma cells using BD FACSCaliber. PI histogram of the gated Cell singlets distinguishes cells at the Sub G0/G1, G0/G1, S, and G2/M cycle phases (n=3)

phase; however, both these phases had the highest cell cycle arrested populations within the treatment. Turmesac® clearly shows promising interaction with HeLa cells to induce apoptosis through cell cycle arrest.

DISCUSSION

Turmeric has been used for centuries as a traditional medicine in India and China to treat numerous ailments, diseases, and general health care [1]. Over the past four decades, the major constituent, curcumin, and its metabolites have been a favored research target in designing therapeutic medicines due to the numerous biological targets it interacts with [9]. The recent research focus is on the efficacy of curcumin as a natural anticancer agent to be incorporated into chemotherapies for delivery enhancement of other anticancer drugs or as a designed anticancer drug itself (Gupta et al. 2017). Curcumin has been known to exhibit anti-tumoricidal and anti-metastatic properties in colon, breast, prostate, esophagus, lung, and oral cancers [4]. In this study, we focused on evaluating the anticancer effects of the turmeric rhizome extract, Turmesac®, in HeLa cells (cervical adenocarcinoma).

The extent of the effects curcumin may have is concentration, time, and cell type dependent. Curcumin cytotoxicity is also dependent on these factors. In HeLa cells, Turmesac® showed cytotoxic effects at a concentration of 115.12 µg/ml. We have previously shown that Turmesac® had cytotoxic effects at IC₅₀ values of 163.19 µg/ml in MCF-7 cell lines but none in HUH-7 cell line [15]. This cytotoxic effect inhibited HeLa cell proliferation which was also seen in brain, prostate, leukemic, gastric, hepatic, breast, oral epithelial, ovarian, pancreatic, bladder, colon, and other cervical cell lines (Hamidpour et al., 2018). With the inhibition of cell proliferation through the various signaling pathways leads to apoptosis induction of these cells.

Turmesac® has shown promising apoptosis induction at the IC₅₀ value in HeLa cells. In porcine ovarian cells, curcumin was able to induce apoptosis at concentrations of 0.1, 10, and 100 µg/ml. At the 100 µg/ml treatment, there was a 2 times greater effect at inducing programmed cell death [3] similarly seen in this study with the HeLa cells. According to Allegra et al. 2017, curcumin is able to mediate its cytotoxic effect through the mitochondrial apoptotic pathway with caspase-8 activation, BID cleavage, cytochrome c release, and activation of caspase-3. This coupled by the upregulation of the pro-apoptotic protein, Bax, and downregulation of anti-apoptotic protein, Bcl-XL. To further facilitate programmed cell death, curcumin upregulates pro-apoptotic proteins BIM and PUMA (p53 independent or dependent pathways) and downregulation of Bcl-2, NF-κB, cyclin D1, and cyclooxygenase-2 considered being carcinogenesis-related genetic products. These carcinogenic gene products are also involved in the manipulation of the cell cycle, ensuring cell longevity.

Turmesac® was able to induce cell arrest at the G0/G1 and S phases, wherein previous studies, arrestment at these points resulted in inhibition of DNA synthesis and in some cases apoptosis in cell lines [13]. Halting cells at the G0/G1 phase prevent cellular differentiation that results in apoptosis induction or inhibition of cell proliferation; however, with longer treatment duration, these cells may move to the next cell cycle phase for apoptosis induction due to micronuclei formation. Curcumin has been previously shown to induce cell cycle arrest at a concentration range of 10–20 µM in different cancer cell lines (A431, A375, HCT-116, colon cancer, and MCF-7) [4]. Curcumin is currently in phase I/II clinical trials where oral doses are given as the principal therapeutic agent or a chemosensitizer that reduces the adverse and cytotoxic effects of chemotherapeutic agents [3]. In the entirety of all the results in the study for apoptosis induction and cell cycle arrest that parallels currently published data on curcumin, Turmesac® shows promising properties as being a potential anticancer

therapeutic agent in HeLas and possibly other cancer types. Further investigation into the *in vivo* effects of Turmesac® is necessary for future possible incorporation into clinical trials as well longer *in vitro* treatment durations may elucidate the exact mechanisms manipulated for activation of programmed cell death for a more targeted drug design.

CONCLUSION

Turmesac® at an IC₅₀ value of 115.12 µg/ml shows promising properties as being a potential anticancer therapeutic agent in HeLas and possibly other cancer types. Further investigation into the *in vivo* effects of Turmesac® is necessary for future possible incorporation into clinical trials as well as longer *in vitro* treatment durations may elucidate the exact mechanisms manipulated for the activation of programmed cell death for a more targeted drug design.

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AUTHORS' CONTRIBUTIONS

All the authors have contributed for preparation and editing of the manuscript.

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Self.

CONFLICTS OF INTEREST

Nil.

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