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Curcuma caesia rhizome extract shows selective cytotoxicity and antiproliferative effect against lung and breast cancer cell lines

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ABSTRACT

Background: Black turmeric (*Curcuma caesia* Roxb.) exhibits various pharmacological properties, including anticancer, antifungal, and antibacterial activities. Objective: This study evaluates the cytotoxic and antiproliferative potential of crude methanolic rhizome extracts of black turmeric in selected cell lines. Materials and methods: The crude methanolic extract was prepared by the Soxhlet extraction method. Short-term cytotoxicity was determined by Trypan blue exclusion method in murine cancer cells (DLA and EAC), whereas *in vitro* cytotoxicity was determined by MTT assay in human cancer cell lines (Breast and lung cancer cells). The antiproliferative effect and induction of apoptotic machinery were measured by clonogenic assay, and DNA laddering assay respectively. Results and conclusion: The Trypan blue exclusion assay demonstrated IC_{50} values of 207.69 ± 18.74 µg/ml and 122.35 ± 1.29 µg/ml in Ehrlich Ascites Carcinoma (EAC) and Dalton's Lymphoma Ascites (DLA) cells respectively. Cytotoxicity assessment of the methanolic extract in A549 and MDA-MB-231 cell lines yielded IC_{50} values of 100.14±0.56 µg/ml and 165.43±1.76 µg/ml respectively, with negligible toxicity to normal cell line. The clonogenic assay revealed significant inhibition of colony formation with the crude extract suppressing colony formation by 80.2% in A549 cells and complete inhibition in MDA-MB-231 cells at IC_{50} concentration. DNA fragmentation analysis of the treated cell lines for apoptosis showed no visible DNA shearing in agarose gel electrophoresis. Conclusion: The crude methanolic extract of *C. caesia* showed a significant cytotoxic effect against murine and human cancer cells. It significantly reduced colony formation, particularly in MDA-MB-231 cells. The mechanism of action needs to be elucidated.

Keywords: Black turmeric, Trypan blue assay, MTT assay, Clonogenic assay, DNA fragmentation assay.

INTRODUCTION

Cancer arises from genetic aberrations and disruptions in normal cellular regulatory mechanisms [1]. According to the 2024 American Cancer Society report, cancer cases continue to rise, contributing to 9.7 million global deaths. Conventional treatments are expensive, often cause adverse health effects, and lead to drug resistance, highlighting the urgent need for cost-effective and more efficacious therapeutic alternatives. Plants have long been valued for their medicinal properties, with approximately 30 plant-derived compounds currently in clinical trials for cancer treatment and over 3,000 plant species documented for their anticancer potential [2,3]. However, many traditionally recognized plant species with anticancer properties remain unexplored, necessitating further research to develop novel therapeutics against cancers.

Curcuma caesia Roxb., commonly known as black turmeric, belongs to the Zingiberaceae family. Its bluish-black rhizome possesses numerous medicinal properties and has been traditionally used to treat ailments such as piles, leprosy, bronchitis, asthma, wounds, diarrhoea, epilepsy, fever, menstrual disorders, and infertility. Reports highlight its antifungal [4], anti-ulcer [5], antioxidant [6], antibacterial [7], and bronchodilatory properties [8].

The anticancer properties of *C. caesia* extracts have been reported against different cell lines. Ethanol extract exhibited direct cytotoxicity against EAC cells, whereas the methanol extracts reduced tumor volume and prolonged lifespan in mice [9,10]. The hexane extract demonstrated significant anti-proliferative activity in HepG2 cell lines (IC_{50}: 0.98µg/mL) and proved induction of cell arrest at

G2/M phase along with cellular apoptosis [11]. Hexane extract showed anti-proliferative effect against Hep2 (IC_{50}: 7.8 µg/ml), HepG2 (IC_{50}: 0.98 µg/ml), HT29 (IC_{50}: 7.81µg/ml), and Vero cells (IC_{50}: 50µg/ml). These extracts upregulated pro-apoptotic proteins, resulting in induced cell death [12]. The aqueous ethanol extract exhibited no cytotoxic activity against MCF-7 and 4T1 breast cancer cell lines, whereas the ethanolic extract displayed cytotoxic activity in MCF-7 and 4T1 cells [13].

This study aims to evaluate the anticancer and antiproliferative effects of methanolic rhizome extracts of *C. caesia* in breast and lung cancer cell lines and also EAC and DLA cells.

MATERIALS AND METHODS

Materials

Reagents and chemicals used in this study included Trypan blue (Spectrum Pvt. Ltd., India), Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), antimycotic-antifungal solution (Thermo Fisher Scientific), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5 fluorouracil (5-FU), and trypsin (HiMedia, Mumbai, India), ethanol, methanol (Merck, Mumbai, India), and TRIzol reagent (Ambion). All reagents were of analytical grade.

Methods

Preparation of extract

Rhizomes of *Curcuma caesia* were sourced from the Aromatic and Medicinal Plants Research Station, Odakkali, Kerala. They were dried, powdered and Soxhlet extracted [14] using methanol at 60°C for 8 hours. The methanol was evaporated to obtain crude extract (figure 1) which was stored at -20°C and dissolved in ethanol (10 mg/ml) before syringe filtration.

Short-term cytotoxicity assay

Murine tumour cells, Dalton's Lymphoma Ascites (DLA) and Ehrlich's Ascites Carcinoma cells (EAC) were collected from the peritoneal cavity of female Swiss albino mice maintained at the Amala Cancer Research Centre, Thrissur, Kerala. DLA and EAC cells were collected, washed with phosphate-buffered saline (PBS), and prepared as a 1×10^6 cells/ml suspension. Various extract concentrations (50-200 µg/ml) were incubated with 100 µl of stock cell suspension at 37°C for 3 hours. Cells were stained with Trypan blue, and live/dead cells were counted using a hemocytometer. Cytotoxicity (%) was calculated as: % Cytotoxicity = (No. of dead cells/Total no. of cells) × 100 [15].

Cytotoxicity of rhizome extract on cell lines

Breast cancer (MDA-MB-231), lung cancer (A549) and normal (HEK-293) cell lines were obtained from the National Centre for Cell Sciences, Pune and were maintained in 10% DMEM under 5% CO_2 at 37°C. A total of 5,000 cells/well were seeded in 96-well plates and incubated for 24 hours at 37°C at 5% CO2. Extract at various concentrations (50-250 µg/mL) was added, and cells were incubated for 48 hours. 5-Fluorouracil served as a positive control, while ethanol served as a negative control. After 48 hours, cell viability was assessed using the MTT assay [16]. MTT (1 mg/ml in PBS) was added to the wells and incubated for 3 hours at 37°C in the dark. Formazan crystals formed were solubilized with lysis buffer (DMSO: Isopropanol, 1:1) and incubated at room temperature for 15 minutes. The absorbance was measured at 570 nm. Cell viability (%) and inhibition (%) were calculated as:

(i) Cell Viability (%) = (OD treatment/OD control) × 100

(ii) Inhibition (%) = 100 - Cell viability (%)

Clonogenic assay

A549 and MDA-MB-231 cells (1,000-2,000 cells/well) seeded in 6 well plates were incubated for 24 h and then treated with IC_{10}, IC_{25}, and IC_{50} concentrations of *Curcuma caesia* rhizome extract. The control treatments included untreated cells, ethanol-treated cells, and positive control (IC_{50} of 5-Fluorouracil) [17]. After 48 h, the medium in each well was replaced with fresh 10% DMEM, and the cells were incubated for a week. The colonies were fixed with methanol, stained with 1% crystal violet and counted. Plating efficiency and survival rates were calculated as follows.

(i) Plating Efficiency (PE) = (Colonies observed) / (Number of cells seeded)

(ii) Survival Fraction= Number of colonies counted / Number of cells seeded x (PE /100)

Estimation of apoptosis

DNA laddering assay was performed using the modified protocol of Chen *et al*. [18] and Aravind *et al*. [19]. A549 and MD-MB 231 cells (500,000 cells per well) were treated with IC_{50}, IC_{25}, and IC_{10} concentrations of *Curcuma caesia* rhizome extract for 48 h. Controls included untreated, ethanol-treated and 5 FU (IC_{50}) treated cells. DNA was extracted, washed with 70% ethanol, air-dried, and resuspended in TE buffer. DNA was subjected to agarose gel electrophoresis (1.8% gel, 100V, 2 h) using TBE buffer. DNA bands were visualized using a gel documentation system (BIO-RAD).

RESULTS

Cytotoxic activity

The cytotoxicity of the crude methanolic extract of *C. caesia* (CCM) was assessed against DLA and EAC cells using a short-term trypan blue assay. CCM showed a dose-dependent cytotoxicity in DLA cells, with an IC_{50} value of 122.35±1.29 µg/ml. In EAC cells, the IC_{50} value was 207.69±18.74 µg/ml. The dose response curve is shown in Figure 2.

The CCM demonstrated moderate cytotoxicity against A549 and MDA-MB-231 cell lines in a dose dependent manner. In A549 cells, viability decreased to 50.75% at 100 µg/mL, whereas in MDA-MB-231 cells, the viability dropped to 46.43% at 150 µg/mL (figure 3). The IC_{50} was 100.14±0.56 µg/ml for A549 and 165.43±1.76 µg/ml for MDA-MB-231 cells. CCM did not exhibit any toxicity to HEK-293 cells up to the highest treated concentration (250 µg/ml). The ethanol treated cells (vehicle control) showed a viability of 83.5% in A549 and 85% in MDA-MB-231. The lowest cell viability was observed in positive control (5FU). In the presence of 5FU, the cell viability was reduced to 48.3% in A549 and 41.5% in MDA-MB-231 at its respective IC_{50} values (10.32±0.69 µM for A549 cells [20] and 11.40 µM for MDA-MB-231) [21].

Morphological changes in A549 and MDA-MB-231 cells on treatment with the crude rhizome extract were observed under a microscope as shown in figure 4 and 5 respectively. The number of cells decreased with an increase in the concentration of the methanolic *C. caesia* rhizome extract. Morphological changes such as vacuole formation, rounding and floating of the cells indicative of cytotoxicity were observed in the treatment with the rhizome extract in a dose-dependent way. In ethanol (vehicle) treated cells (both A549 cells and MDA-MB-231 cells) the morphology was similar to their respective control cells. In 5-FU treated cells, the viability was less, and the morphology was changed, they became rounded in shape.

Effect on reproductive death of cancer cells

CCM significantly inhibited colony formation in A549 and MDA-MB-231 cell lines (figure 6 and 8). The survival fraction for each cell line is plotted in figure7 and 9. At IC_{50}, the extract inhibited colony formation by 80.2% in A549 cells and 100% in MDA-MB-231 cells.

Estimation of Apoptosis

DNA was isolated from the cells treated with CCM and analysed on an agarose gel (figure 10). No visible DNA fragmentation or shearing was observed on 1.8% agarose gel.

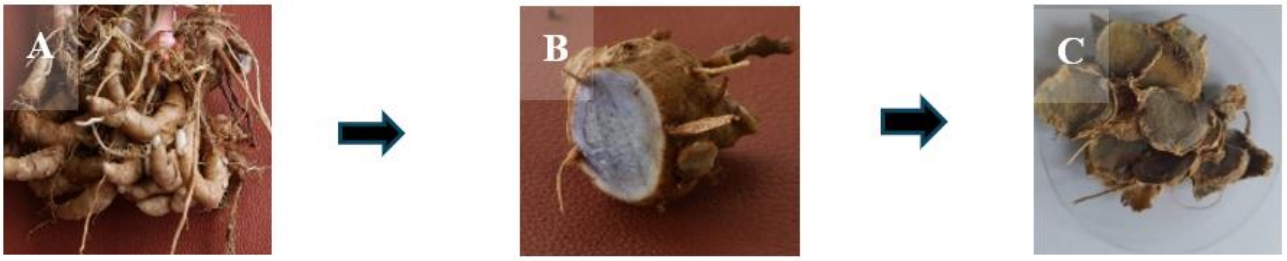

Figure 1: Extraction of *C. caesia* rhizome in methanol (A) *C. caesia* rhizome (B) Cross section of rhizome (C) Dried Rhizome

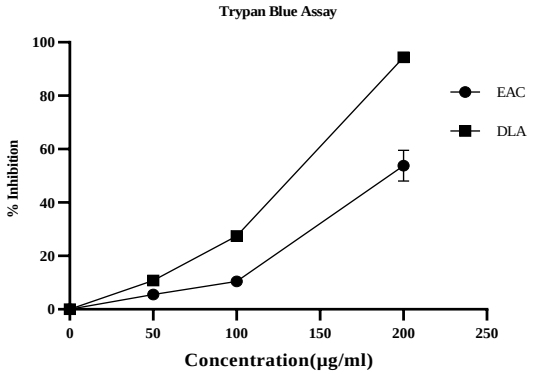

Figure 2: *In vitro* cytotoxicity of crude methanolic extract of black turmeric on DLA cells, and EAC cells. Values are expressed as mean ± SD at two consecutive experiments

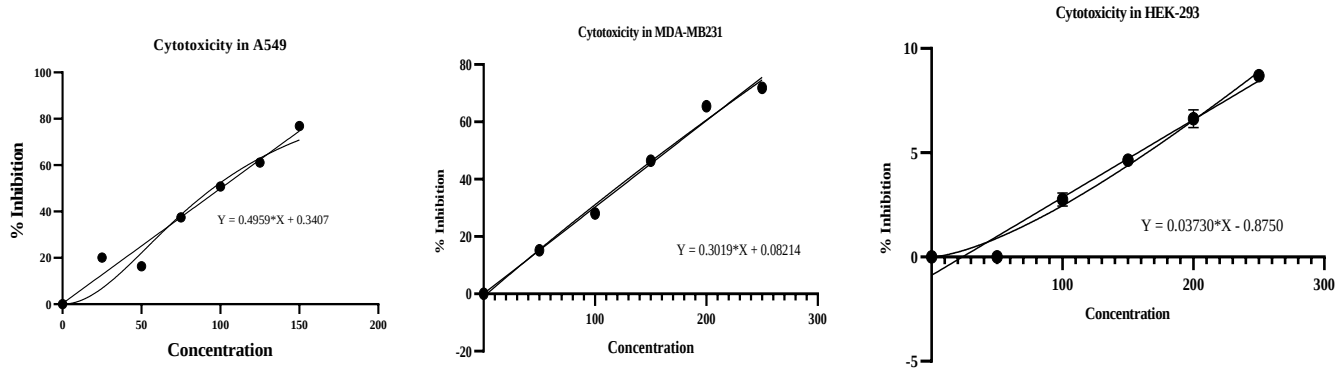

Figure 3: *In vitro* cytotoxicity of crude methanolic extract of black turmeric on A549, MDA-MBM-231, and HEK-293 cells. Values are expressed as mean ± SD of at two consecutive experiments

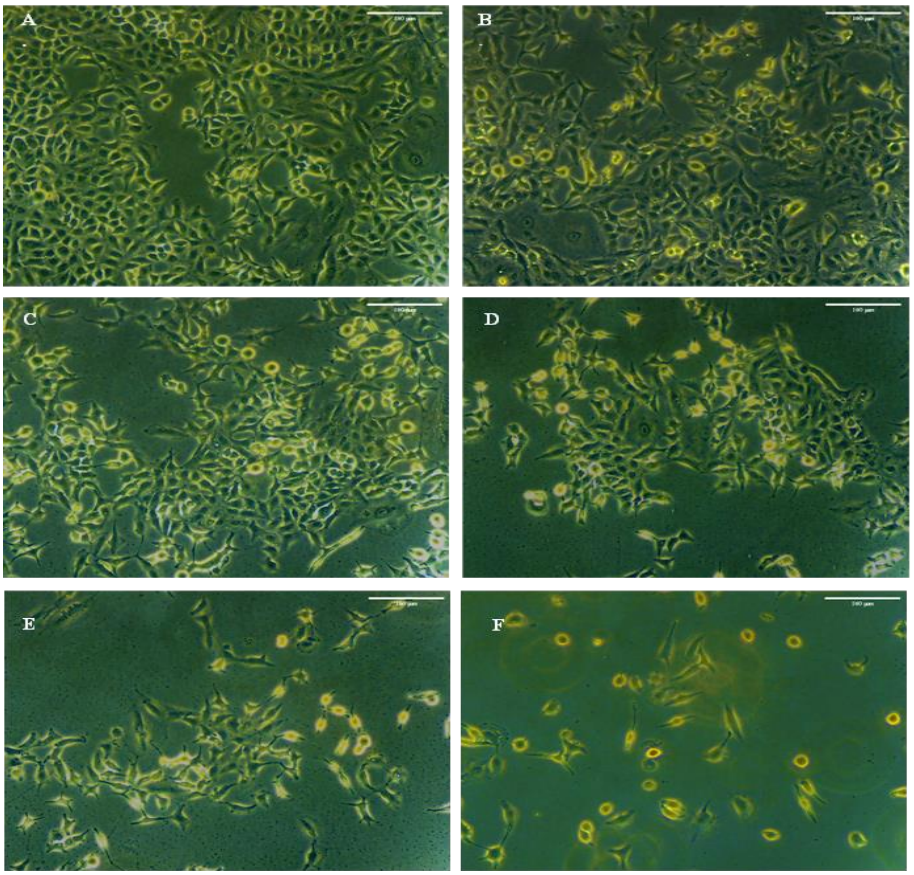

Figure 4: Morphological changes of A549 cells when treated with methanolic extract of *C. caesia* rhizome (CCM). (A) Untreated (B) Ethanol treated (C) CCM IC_{10} (D) CCM IC_{25} (E) CCM IC_{50} (D) 5FU IC_{50}. Scalebar=100µm

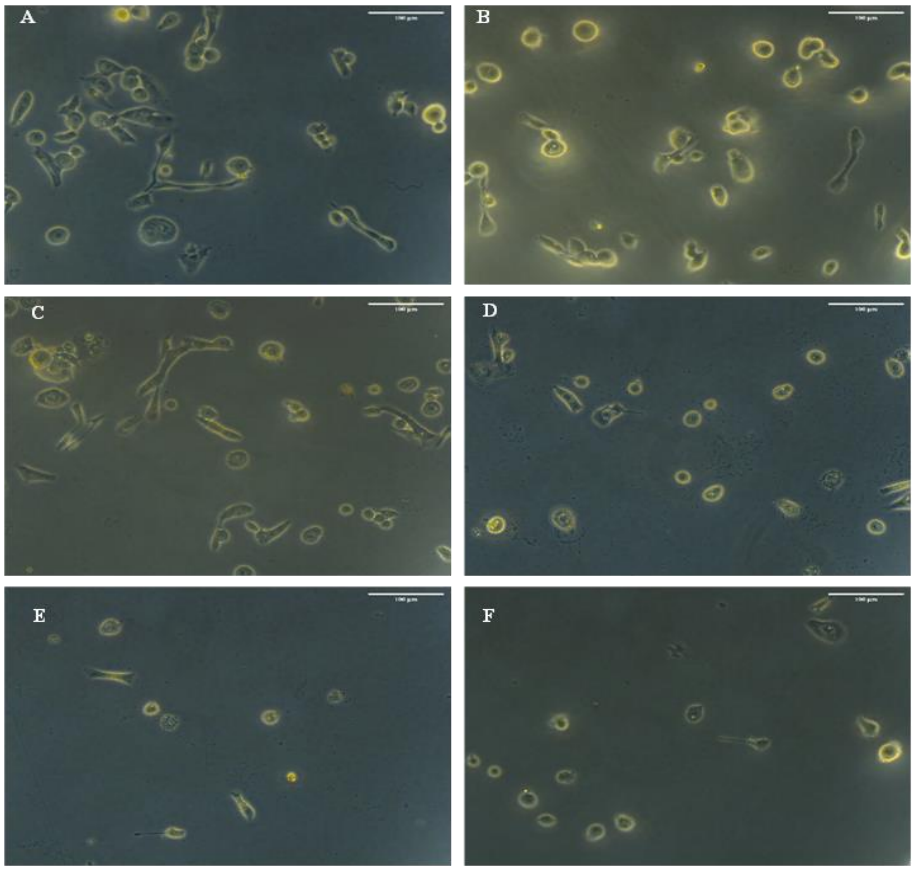

Figure 5: Morphological changes of MDA-MB-231 cells when treated with methanolic extract of *C. caesia* rhizome (CCM). (A) Untreated (B) Ethanol treated (C) CCM IC_{10} (D) CCM IC_{25} (E) CCM IC_{50} (D) 5FU (IC_{50}). Scalebar=100µ

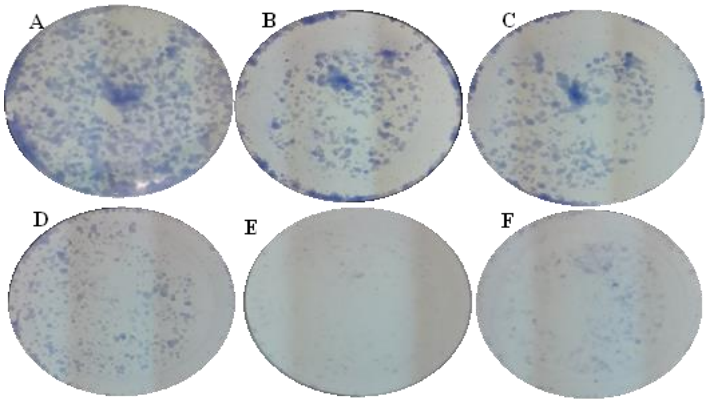

Figure 6: Colony formation by MDA-MB-231 cells with a plating efficiency of 10.4 % treated with the CCM. (A) Control (B) Ethanol (C) CCM IC_{10} (D) CCM IC_{25} (E) CCM IC_{50} (F)5FU

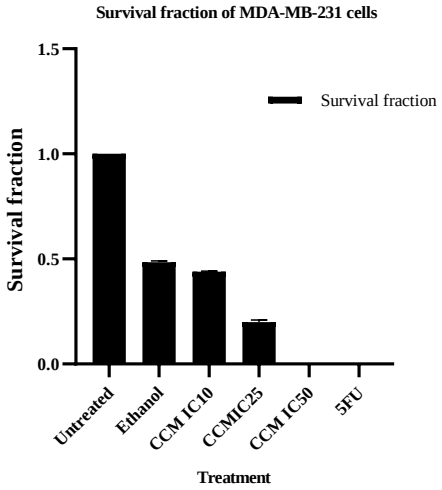

Figure 7: Survival fraction of MDA-MB-231 cell line treated with the methanolic rhizome extract in clonogenic assay

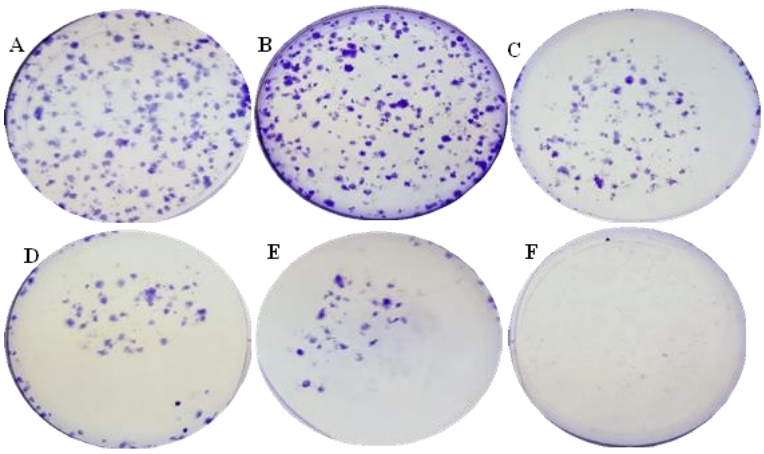

Figure 8: Colony formation by A549 cells with a plating efficiency of 21.2% when treated with the CCM. (A) Control (B) Ethanol (C) CCM IC_{10} (D) CCM IC_{25} (E) CCM IC_{50} (F) 5-FU

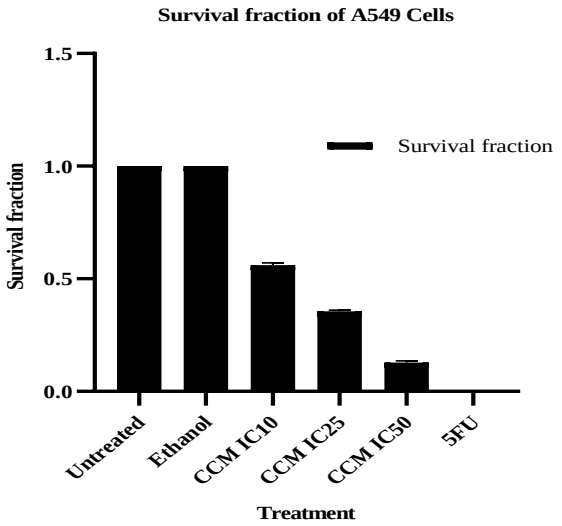

Figure 9: Survival fraction of A549 cell line treated with the methanolic rhizome extract in clonogenic assay

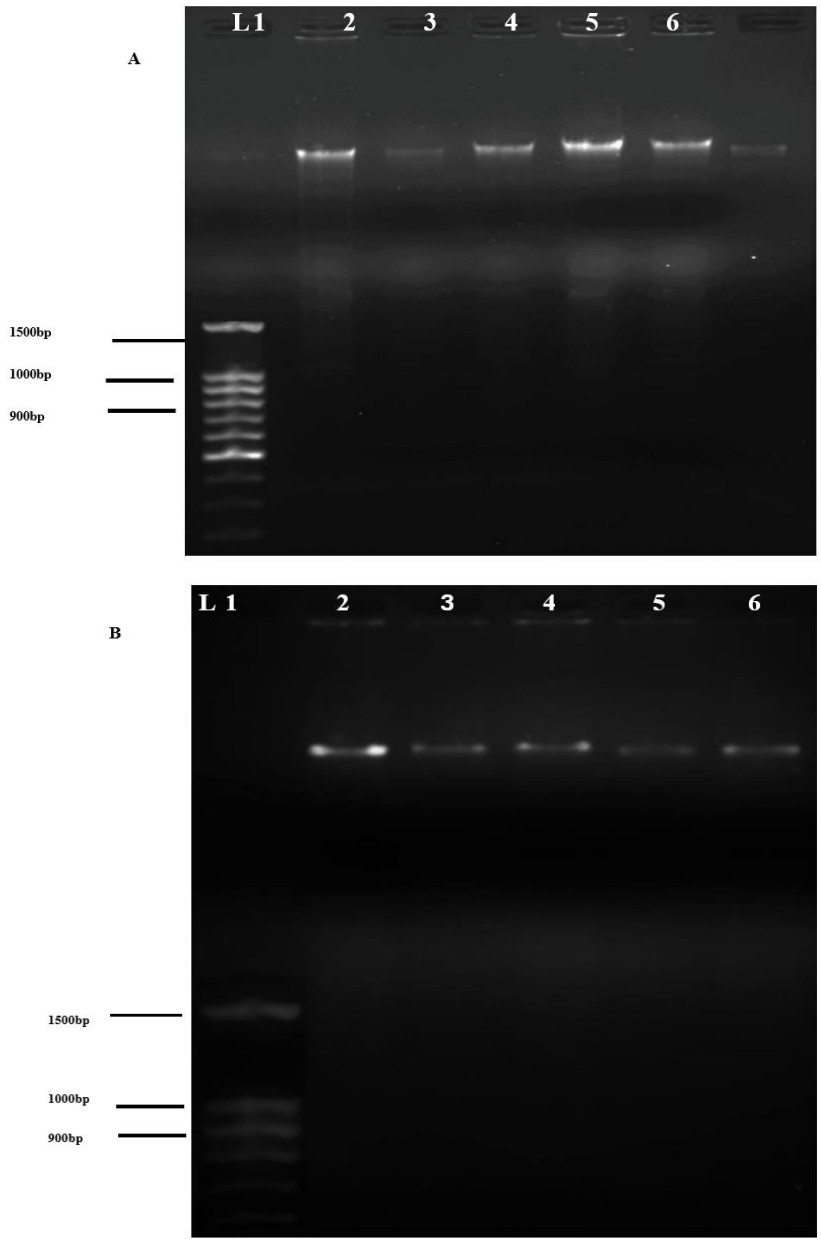

Figure 10: Profile of genomic DNA isolated from (A) A549 and (B) MDA-MB-231 cells treated with CCM on 1.8% gel. Lane 1- Ladder; Lane 2-Untreated: Lane3- CCM (IC_{50} treated); Lane 4-CCM (IC_{25} treated); Lane 5 -CCM (IC_{10} treated); Lane 6-5FU treated

DISCUSSION

Curcuma caesia rhizome has been traditionally used for treating various ailments, including cancer [22-24]. Several phytochemicals have been identified in *C. ceasia* with known pharmaceutical properties. Key components such as isoborneol, alloaromadendrene, trans sesquisabinene hydrate, α-santalol, ar-turmerone, Megastigma-3,7(E),9-triene, Retinal,9-cis, Androstenediol, (+)-2-Bornanone, 2-Pentaone, Isoborneol, 4-hydroxy-4-methyl acid, exhibit anti-inflammatory, anticancer, antibacterial, analgesic, antifungal, antipyretic, antibronchitic and antioxidant properties [25-29].

Earlier studies have reported that *C.caesia* acts against murine cancer cells [9,10]. In this study, showed the inhibitory effect of CCM against both DLA and EAC cells. It showed IC_{50} values of 207.69 ± 18.74 µg/mL and 122.35 ± 1.29 µg/mL in Ehrlich Ascites Carcinoma (EAC) and Dalton's Lymphoma Ascites (DLA) cells respectively. Earlier reports showed that ethanol extract exhibited direct cytotoxicity against EAC cells, whereas the methanol extracts reduced tumor volume and prolonged lifespan in mice [9,10].

Various studies highlight the cytotoxic and anticancer potential of *C. caesia*. Campos *et al*., [30] demonstrated a potential anticancer activity of its phenolic compounds against oropharynx cancer cells, with an IC_{50} of 30.13µg TE PC/ ml in FaDu and 13.36µg TE PC/ml in HaCat cells. Mangla *et al*. [31] demonstrated its antioxidant activity, while Borah *et al*., [32] reported anti-inflammatory and cytotoxic effects of the essential oil from leaves. Shaikh *et al*., [33] found that an aqueous extract exhibited moderate to weak cytotoxic activity against MCF-7, HCT-116, and PA-1 cancer cell lines compared to the standard drug 5-FU. Mukunthan *et al*. [34] observed high cytotoxic potential (IC_{50}: 0.98 µg/ml) of a hexane extract against liver cancer cell lines, inducing apoptosis and cell cycle arrest. Similarly, inhibitory effects on hepatocellular carcinoma in BALB/c mice were reported by Hadem *et al*. [35]. In the present study, the crude methanolic extract demonstrated moderate cytotoxicity against lung (A549) and breast (MDA-MB-231) cancer cell lines, IC_{50} values of 100.14±0.56 µg/ml and 165.43±1.76 µg/ml, after 48 hours of treatment, which are aligned with the previous reports.

The extract selectively targeted cancer cells while sparing normal HEK cells, a crucial feature of potential anticancer agents [36]. Comparing IC_{50} values, the extract was more effective against lung cancer (A549) than breast cancer (MDA-MB-231), though less effective than 5-FU.

Cancer progression is characterized by hallmark features such as unlimited replicative potential, invasion, and metastasis [37]. A single metastatic cancer cell can initiate secondary tumour growth and recurrence. Effective anti-cancer drugs must inhibit the formation of cancer cell colonies. The clonogenic assay assesses the reproductive potential of individual cells [38]. In this study, methanolic extract from *C. caesia* rhizomes significantly inhibited colony formation by 80.2% in A549 and complete inhibition in MDA-MB-231 cells. 5FU completely inhibited colony formation in MDA-MB-231 cells. While multiple Curcuma species exhibit antiproliferative effects, studies on *C. caesia* remain limited. Shaikh *et al*. [33] reported its antiproliferative activity against various cancer cell lines, and Al-Amin *et al*. [39] demonstrated the inhibitory effects of curcuzederone, a bioactive compound from *C. caesia* rhizome on MDA-MB-231 metastasis.

The DNA laddering assay was conducted to assess apoptosis as the mechanism of cell death. Apoptosis involves intrinsic, extrinsic, and caspase-dependent/independent pathways [37,40]. Apoptotic cells exhibit DNA fragmentation, forming a characteristic 'ladder' pattern on agarose gel electrophoresis [41]. However, in this study, no laddering pattern was observed, suggesting an alternative mode of cell death.

This study highlights that the methanolic extract of *Curcuma caesia* rhizome is selectively cytotoxic and anti-proliferative to lung (A549) and breast cancer (MDA-MB-231) cell lines without causing any cytotoxicity to normal cell line (HEK-293) at the tested concentrations. Many plant-derived anti-cancer compounds are reported to induce apoptosis [42-45]. However, the crude methanolic extract of *C.caesia* did not induce significant DNA fragmentation, indicating an alternative cell death pathway other than apoptosis. Non-apoptotic mechanisms such as autophagy, necrosis, mitotic catastrophe, or senescence may contribute to its cytotoxic effects [46].

CONCLUSION

The present study demonstrates that the crude methanolic extract of *Curcuma caesia* rhizome exhibits selective cytotoxicity and anti-proliferative activity against lung (A549) and breast (MDA-MB-231) cancer cell lines while sparing normal HEK-293 cells. The extract effectively inhibited cancer cell viability in a dose-dependent manner and significantly reduced colony formation, particularly in MDA-MB-231 cells. However, the absence of DNA fragmentation suggests that its cytotoxic mechanism may involve alternative pathways beyond apoptosis, such as autophagy, necrosis, or mitotic catastrophe. These findings highlight the potential of *C. caesia* as a natural source of anticancer compounds, warranting active compound isolation to elucidate its precise mode of action and therapeutic potential.

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Conflict of interest

The authors declared no conflict of interest.

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Supplementary part

Table 1: Concentration and percentage inhibition of the extract in each cell

Concentration	Inhibition	
	EAC	DLA
0	0	0
50	5.55±0.323	10.803±0.669
100	10.47±0.315	27.4922±1.177
200	53.78±4.052	94.41555±0.076
IC_{50}	207.69±18.74	122.35±1.289

Table 2: Concentration and percentage inhibition of the extract in each cell lines. IC_{50} of the extract in A549 and MDA-MB-231 are also given. Values are expressed as mean ± SD of at least two consecutive experiments

Cytotoxicity in A549		**Cytotoxicity in MDA-MB- 231**		**Cytotoxicity in HEK-293**	
Concentration (µg/ml)	% inhibition	Concentration (µg/ml)	% inhibition	Concentration (µg/ml)	% inhibition
0	0	0	0	0	0
25	20.085±.459	50	15.195±0.785	50	0
50	16.38±.891	100	28.025±0.445	100	2.9725±0.308
75	37.49±1.598	150	46.43±1.075	150	4.805±0.233
100	50.75±.665	200	65.42±1.838	200	6.935±0.431
125	61.14±.806	250	71.85±.481	250	8.885±0261
150	76.88±.184				
IC_{50}	100.139±.562	IC_{50}	165.43±1.76		

Table 3: TBE buffer composition: TBE Buffer (5x) for 1 litre

Component	**Mass**
Tris base	54 g
Boric acid	27.5 g
0.5M EDTA	20 ml