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Evaluation of *in vitro* anticancer activity of rhizome of *Curcuma longa* against human breast cancer and Vero cell lines

[1] Rumana Ahmad, [1] AN Srivastava, [2] Mohsin Ali Khan
[1] Dept. of Pathology, Era's Lucknow Medical College & Hospital, Sarfarazganj, Hardoi Road, Lucknow, India.
[2] Chairman Research, Era's Lucknow Medical College & Hospital, Sarfarazganj, Hardoi Road.

Abstract
Curcuma longa Linn (CL), also known as commonly known as 'Haldi' in Hindi is an Indian spice and medicinal plant belonging to Zingiberaceae family and is extensively used in Ayurveda, Unani and Siddha medicine and as home remedy for various diseases. The active constituent of turmeric is curcumin, a polyphenol, responsible for the yellow color and volatile oils like tumerone, atiantone and zingiberone. In the current study, *in vitro* cytotoxic activity of 50% ethanolic extract of CL rhizomes was evaluated against human breast cancer cell line MDA-MB-231 and normal Vero epithelial cell line. The anticancer activity of CL ethanolic extract was determined using MTT assay and cytometer based analysis. The percentage viability of the cell lines was determined by using Trypan blue dye exclusion method. HPLC profiling was also carried out for identification of the active components, which demonstrated the presence of curcumin in the ethanolic extract. Ethanolic extract of CL showed significant anticancer activity on MDA-MB-231 human breast cancer cell line. IC_{50} values of CL ethanolic extract with respect to MDA-MB-231 was found to be 49 ± 2.08 µg/ml in 0.25% DMSO and 40±1.03 µg/ml in 0.5% DMSO. The ethanolic extract would be studied further for isolation and characterization of active components for lead optimization studies. Future studies would entail evaluation and comparison of anti-cancer potential of pure curcumin with that of the whole CL extract.

Keywords: Anticancer, Cytotoxicity, MTT assay, *in-vitro*, MDA-MB-231, Vero, Breast cancer, *Curcuma long*.

1. Introduction

In spite of the recent domination of the synthetic chemistry as a method to discover and produce drugs, the potential of bioactive plants or their extracts to provide new and novel products for disease treatment and prevention is still enormous. The continuing search for new anticancer compounds in plant medicines and traditional foods is a realistic and promising strategy for its prevention [1, 2]. Numerous groups with antitumor properties are plant derived natural products including alkaloids, phenylpropanoids, and terpenoids [3, 4].

Among various diseases attributed to mortality in humans all over the world, cancer is a leading cause. Most drugs currently available for the treatment of cancer have limited potential, because they are highly toxic, inefficient in treating cancer, or highly expensive. Treatments without these disadvantages are needed. Hence, the identification and synthesis of novel, efficient and less toxic anticancer agents remains an important and challenging task for the cancer treatment. Use of plant extracts as medicine for cancer treatment is certainly the effective method and dozens of plant based products have been reported for cancer treatment progress. Plant based products have the natural multi-targeting ability and are inexpensive and safe as compared to synthetic agents [3]. Among them, plant based products such as curcumin occupy significant role against cancer.

Curcuma longa Linn. (CL) is a plant native to India which is remarkable for being the source of a culinary spice known as 'Turmeric' and its medicinal properties [5-8]. The plant is a member of the Zingiberaceae family. In its usual form it is a dry yellow powder that is oil-soluble. Curcumin, one of the most studied chemopreventive agents, is a naturally occurring compound extracted from CL. It is widely used as coloring and flavoring agent in food [5]. Curcumin is known for its antitumor, antioxidant, anti-amyloid and anti-inflammatory properties [5-8]. Anti-inflammatory properties may be due to inhibition of eicosanoid biosynthesis [9]. It acts as a free radical scavenger and antioxidant, inhibiting lipid peroxidation and oxidative DNA damage. Curcuminoids induce glutathione S-transferase and are potent inhibitors of cytochrome P450.

Curcumin exhibits wide therapeutic potential due to the multi-targeting nature against variety of different cancers including leukemia, gastrointestinal cancers, genitourinary cancers, breast cancer etc. Curcumin has been shown to suppress transformation, proliferation, and metastasis of tumors. It also inhibits proliferation of cancer cells by arresting them in various phases of the cell cycle and by inducing apoptosis. It is obvious that curcumin's multitargeting ability may be the key to its therapeutic potential against cancer.

In the present study, dose-dependent cytotoxic activity of ethanolic extract of CL against human breast cancer cell line MDA-MB-231 and normal epithelial cell line Vero by Trypan blue and MTT method. The study revealed that ethanolic extract of induced apoptosis in cancer cells without having any such effect on normal cells. As per National Cancer Institute (NCI, USA) for anti-cancer screening on a panel of human cell lines, an IC_{50} value of less than 100 µg/ml for medicinal plant extracts would be considered as potential anticancer agents and recommended for further isolation and characterization of bio-active molecules. The data suggest that CL ethanolic extract exhibited potent cytotoxic activity with an IC_{50} value less than 100 µg/ml. Therefore, ethanolic

extract of CL may be considered for further isolation and characterization of bio-active molecules other than curcumin.

2. Materials and methods

2.1 Reagents

0.4% Trypan blue, PBS (pH=7.2, 1X), 0.25% Trypsin-EDTA (1X), DMEM/F-12 (1X) (Dulbecco's Minimum Essential Medium) and Antibiotic (100X) were obtained from Gibco, Life Technologies; whereas FBS and MTT were from Himedia. Doxorubicin hydrochloride solution was purchased Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl Sulfoxide (DMSO) was purchased from Calbiochem. All other chemicals used in the study were of reagent grade. Pure curcumin was purchased from Natural Remedies Ltd. Veerasandra, Bangalore-100. All reagents used in HPLC analysis were of HPLC grade. All other chemicals used in the study were of reagent grade.

2.2 Collection of plant material

The fresh rhizomes of CL were purchased from a local market in Lucknow, India. The rhizomes were chopped into small pieces and sun dried for a week and then dried at 50 °C in hot air oven for 6 hours. Dried rhizomes were then blended into fine powder by means of a grinder.

2.3 Sample Preparation

For experimental purpose, 25g of CL powder was extracted with 50% ethanol (1:8). After 24 h, the upper layer of solvent was collected in a beaker and the procedure was repeated thrice in the interval of 24 h continuously till the color of solvent disappeared. All extracts were pooled together and filtered using Whatman No.1 filter paper (125 mm). The filtered extracts were concentrated at 100 ^{o}C in water bath. The semi-solid paste formed was transferred to a Petri plate and kept in hot air oven till it attained a powdered form. The total weight of powder was measured and stored in air tight container for further use. For biological studies, 20 mg of ethanolic extract was dissolved in 10% and 50% DMSO (Calbiochem) respectively at a concentration of 20 mg/ml. The extracts were passed through 0.22 µm sterile Millipore syringe filter units (Fisher Scientific) prior to being used in cell culture studies.

2.4 Biological evaluation

2.4.1 Cell lines

MDA-MB-231 (human breast carcinoma, ER^-, tumorigenic and invasive cell line) and Vero (ATCC- CCL-81 normal kidney epithelial cell line) were obtained from the National Centre for Cell Science (NCCS), Pune, India, and as such, were maintained by sub-culturing and passaging as monolayers in 25 and 75 cm^2 cell culture flasks (Nest, Tarsons) at 37 °C in Tissue and Cell Culture Lab, Era's Medical College, Lucknow, in an incubator gassed with an atmosphere of 5% CO_2 at 95% humidity, in advanced Dulbecco's Minimum Essential Medium (DMEM) containing phenol red as a pH indicator and supplemented with 5% FBS. The medium, prior to being used in cell culture experiments was vacuum filtered using a Corning filtration system. The medium requires an atmosphere of 5% CO_2 to produce HCO_3 buffering capacity to maintain pH at 7.4 for normal cell growth.

2.4.2 Cell Culture

For experiments, cells were trypsinized and cultured in 6-well plates (0.5 x 10^5 cells/well) initially for 24 h, so as to allow the cells to attach. After 24h of incubation, the cells were exposed to 50 and 100 µg/ml of ethanolic extracts of CL (in 50% DMSO) for the next 48h. Suitable untreated controls (containing 50% DMSO as vehicle) were also concomitantly employed. Each dose was tested in at least 3 replicate wells. Results were interpreted as cell viability *versus* time period graph.

2.4.3 Morphological Study

For morphological analysis, cells in 6-well plate were observed under phase contrast microscope & photographed (Nikon Eclipse Ti, Japan).

2.5 Cytotoxicity assays

2.5.1 Trypan blue dye exclusion assay

A cell suspension was made at a suitable dilution (1.0 x10^5 cells/ml) in PBS. 50 µl of cell suspension was taken and mixed with an equal volume of 0.4% trypan blue. The solution was mixed thoroughly and allowed to stand for 5 min at room temperature. 50 µl of the solution was transferred to a hemocytometer and viable cells were counted as clear cells and dead cells as blue ones. The number of live cells per ml was calculated using the following formula:

% viability = (live cell count/total cell count)*100.

2.6 (Methyl tetrazolium-MTT assay)

a. Determination of optimal cell number for assay

In order to determine optimal cell number required for the assay, serial dilutions of MDA-MB-231 (2,000, 4,000, 6,000, 8,000, 12,000, 14,000, 16,000 and 18,000 cells/100 l) were made in cell culture media and seeded in 96 well microtiter tissue culture plates (Linbro, MP Biomedicals). Cells were cultured, in a humidified 5% CO_2 incubator at 37 °C for 24 h. At the end of the incubation period, 20 µl of MTT solution (Stock concentration, 5.0 mg/ml in PBS) was added to each well and incubated for 4 h under the same conditions. Thereafter, medium containing MTT was gently replaced by 200 µl DMSO to dissolve formazan crystals and the absorbance values were read in an ELISA plate reader (Biorad PW41) at 550 nm with a reference wavelength of 630 nm. A graph was plotted with the number of cells in X-axis and absorbance at 570/630 nm in Y-axis. Optimal cell density of cell line corresponding to absorbance values of 0.9 to 1.0 in the assay was selected for MDA MB 231 to facilitate measurement of both stimulation and inhibition of cell proliferation within the linear range.

b. Evaluation of cytotoxicity and cell viability

Briefly, MDA-MB-231 cells were trypsinized and resuspended in the culture medium to get a defined cell number for MDA MB 231 (16,000/100 µl) in a 96-well microtiter tissue culture plate and cultured in a humidified 5% CO_2 incubator at 37 °C for 24 h. Defined concentrations of the extracts in 10% and 50% DMSO were freshly prepared in culture media by serial dilution to get final concentration of 20, 40, 60 and 100 µg/ml (for CL ethanolic extract). Serial dilution was carried out in cell culture media in such a way that the final concentration of DMSO in the well did not exceed 0.5% (v/v). Three control wells containing medium

alone to serve as blanks were also included. After 24 h of incubation, cells were treated with the above-mentioned concentrations of CL extract in triplicates for 48h. Doxorubicin hydrochloride, an anticancer drug was used as a positive control. Equal volume of DMSO was used as a vehicle control. At the end of treatment, 20 µl of MTT (stock made in PSS at 5.0 mg/ml) reagent was added to each well and incubated for further 4h. Thereafter, the culture medium was removed and formazan crystals were dissolved in 200µl of DMSO. The plates were read in an ELISA plate reader (Biorad PW41) at 550 nm with a reference wavelength of 630 nm. Percentage cell viability (Y-axis) was calculated from absorbance and plotted against concentration in µg/ml (X-axis).

% Cell survival was calculated as = $\{(A_T-A_B)/ (A_C-A_B)\}$ x100 where,

A_T= Absorbance of treatment well
A_B= Absorbance of blank
A_c=Absorbance of control well
% cell inhibition= 100-Cell Survival
IC_{50} values of the extracts were obtained from the graph as concentration which decreased cell by viability 50%.

2.7 Comparison of Cytotoxic Activity of Extract

The question whether CL extract-mediated induction of apoptosis was selective to cancer cells, and not to normal cells was addressed by evaluating the effects of the ethanolic extract of CL on normal epithelial cells (Vero) at the concentration that was cytotoxic to human breast cancer cells. Briefly, Vero cells were seeded (14,000/100 µl) in 96-well microtiter tissue culture well plates initially for 24 h and then treated with CL ethanolic extract (50 µg/ml), and doxorubicin (0.50 µM) for the next 48 h. At the end of the treatment, cells were subjected to MTT assay. Percentage cell viability (Y-axis) was calculated from absorbance and plotted against concentration in µg/ml (X-axis).

2.8 HPLC Profiling of Ethanolic Extract

a. Preparation of standard for HPLC analysis

Stock solution of curcumin standard was prepared in ethanol at 1.0 mg/ml. One ml of the stock solution was transferred to a 10-ml volumetric flask and volume was adjusted with ethanol.

b. Sample preparation

20 mg of ethanolic extact was dissolved in 20 ml ethanol. 1 ml of the sample was then diluted to 5 ml with ethanol HPLC grade) and filtered through 0.2 µm membrane filter before injection. Injection size for standard and samples was 10 µl each.

c. Procedure

HPLC was performed on a Waters Alliance 515 HPLC system, equipped with two Waters 515 pumps, a Waters Pump Control Module, degasser, injector and a Waters 2998 photodiode array detector (Waters, Milford, MA, USA). For separations, an ODS-2 Hypersil C18 Reverse Phase column (250x4.6 mm, 5 µm particle size, maintained at 25°C) was used. The mobile phase consisted of water (Solvent A) and acetonitrile (Solvent B), which was applied in the following gradient elution for 55 min: 95% A, 5%B for 0-5 min, 85% A, 15% B for 5 min, 60% A, 40% B for 40 min, 10% A, 90% B for 10 min, 50% A, 50% B in 2 min, 95% A, 5% B for 3 min. Flow rate and sample volume were set at 1.0 ml/min and 10 µl, respectively. The detection wavelength was set at 420 nm.

2.9 Data interpretation and Statistical Analysis

Absorbance values that were lower than the control wells indicated a reduction in the rate of cell proliferation. Conversely, a higher absorbance value indicated an increase in cell proliferation. Rarely, an increase in proliferation might be offset by cell death; evidence of cell death was inferred from morphological analysis.

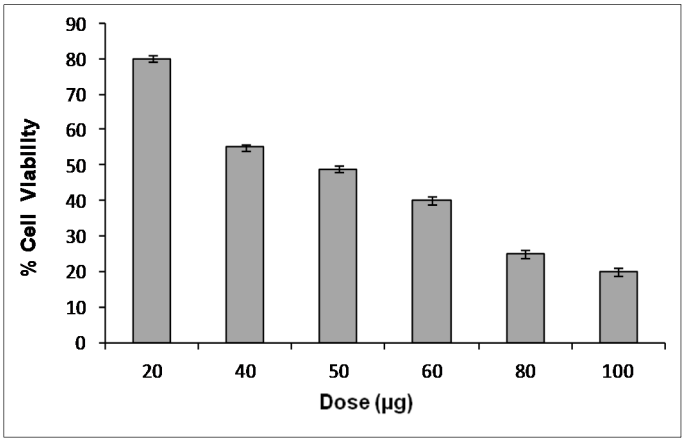
Results were expressed as mean ± SD of experiments done in triplicates.

3. Results

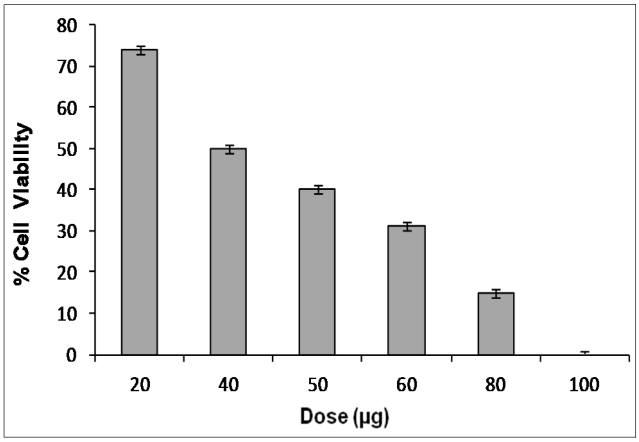
3.1 Ethanolic extract of CL shows cytotoxicity against human breast cancers cells with less effect on normal cells

Cytotoxic effect of CL on human cancer cell line MDA-MB-231 was studied by MTT assay (Fig. 1). Optimal cell densities corresponding to absorbance values of 0.9 to 1.0 in MTT assay were selected to facilitate measurement of inhibition of cell proliferation within the linear range. The optimal cell number to be seeded for a cytotoxicity assay for MDA-MB-231, was determined from the plot and was found to be 16,000 cells/100l respectively. In order to evaluate the cytotoxic effects of the ethanolic extract of CL, MDA cells were treated with specified concentrations of the extract for 48 h. Doxorubicin was used as a positive control. The ethanolic extract of CL showed cytotoxic and dose dependent inhibitory effects on human breast cancer cells MDA-MB-231. IC_{50} values of CL ethanolic extract with respect to MDA-MB-231 were found to be 49± 2.08 µg/ml in 0.25% DMSO and 40 ± 1.03 µg/ml in 0.5% DMSO.

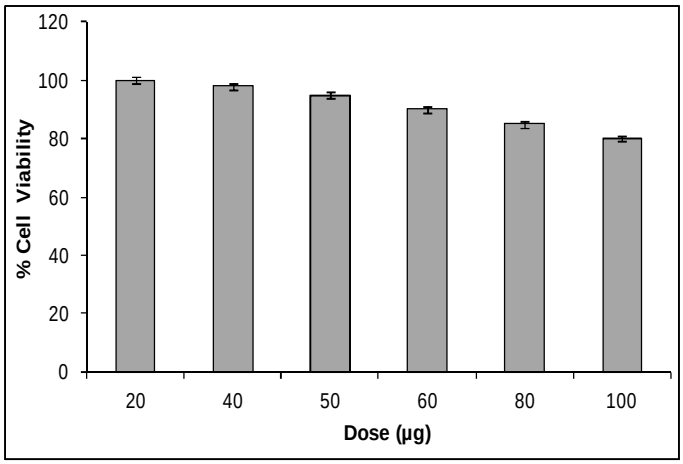
Fig 2 depicts the morphological analysis of untreated versus treated cells with respect to CL ethanolic extract at 50 and 100 µg/ml respectively. It is evident from the figures that CL showed cytotoxic effect on human breast cancer cells MDA-MB-231. The positive control, doxorubicin imparted cytotoxic and dose dependent inhibition of cell proliferation and the IC_{50} value of doxorubicin on MDA-MB-231 cells was found to be 0.50 ± 0.03 µM (Fig. 2j). The next question was to determine whether CL extract-mediated cytotoxicity was selective to cancer cells, and not to normal cells. This would be a highly desirable trait for a potential therapeutic anti-cancer agent. This question was addressed by determining the cytotoxic effect of bio-active CL methanolic extract on Vero normal kidney epithelial cell line. Results indicated that methanolic extract of CL possesses cytotoxic activity with an IC_{50} value of >100± µg/ml. However, the results revealed that the methanolic extract of CL failed to induce cytotoxicity in normal cells at the concentration that was cytotoxic to human breast cancer cells (Fig 3a, b).



(a)



(b)



(c)

Fig 1: Dose dependent effect of CL ethanolic extract in (a) 0.25 % (b) and 0.5 % DMSO on viability of MDA cells *in vitro* using MTT assay. (c) Dose dependent effect of CL ethanolic extract in 0.5 % DMSO on viability of Vero cells *in vitro* using MTT assay**.**

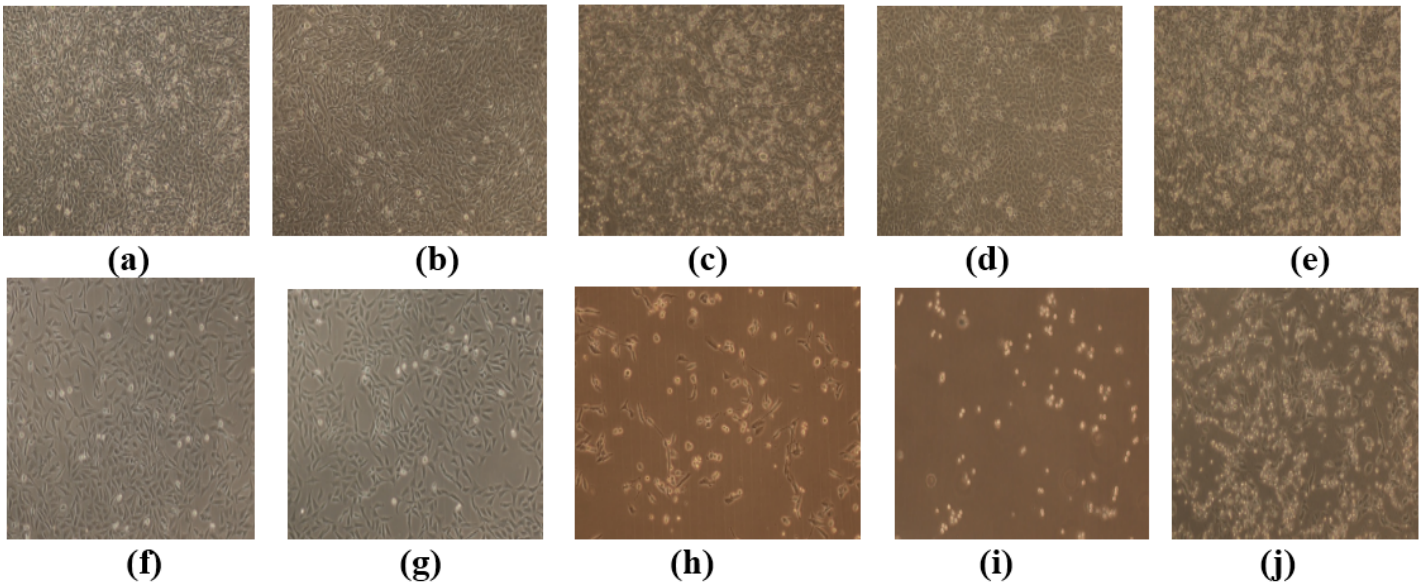


Fig 2: Controls showing untreated MDA human breast cancer cells in presence of (a, c) 0.25% DMSO and (b, d, e) 0.5% DMSO after 48 h (Magnification 10X); Cytotoxic activity of CL ethanolic extract at (f) 50 µg/ml in 0.25% DMSO (g) 50 µg/ml in 0.5% DMSO (h) 100 µg/ml in 0.25% DMSO (i) 100 µg/ml in 0.5% DMSO and (j) 0.5µM doxorubicin chloride in 0.5% DMSO after 48 h (Magnification 10X)

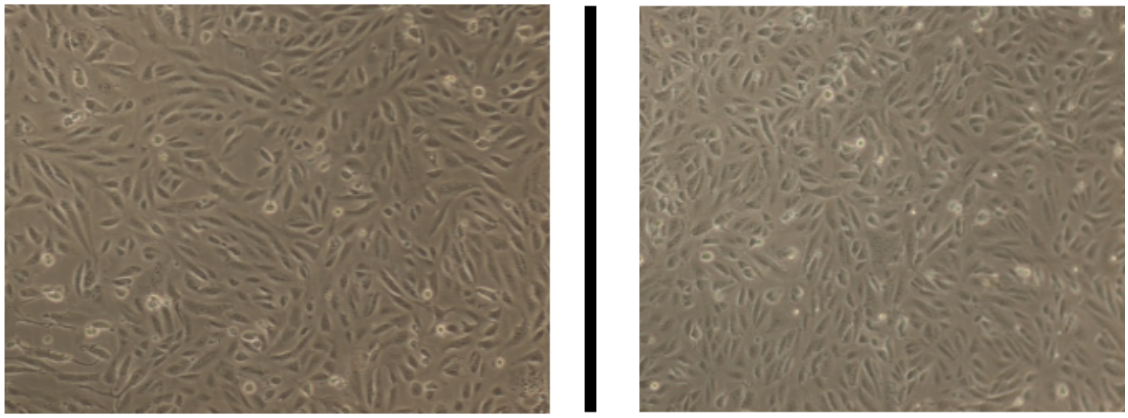


Fig 3(a): Control showing untreated normal epithelial cells (Vero) in presence of 0.5% DMSO and (b) in presence of CL ethanolic extract at 50 µg/ml in 0.5% DMSO after 48 h (Magnification 10X).

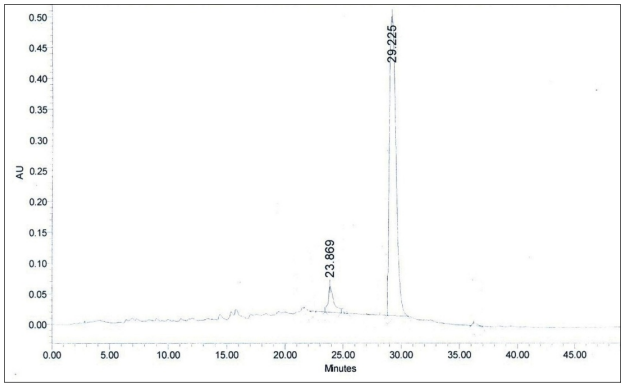


Fig 4(a): HPLC profile of Pure Curcumin Working Standard. Curcumin, (R_t = 29.225 min)

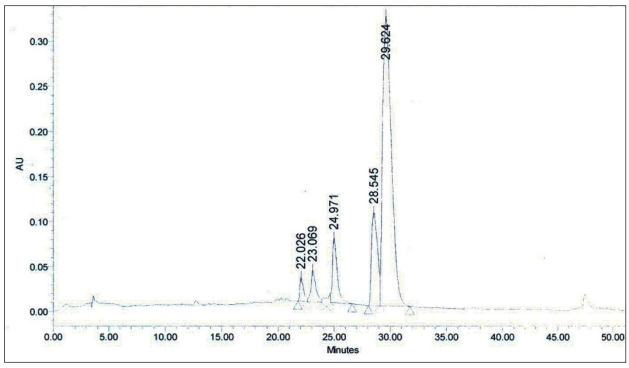


Fig 4(b): HPLC profile of ethanolic extract of CL. Curcumin, (R_t = 29.624 min)

4. Discussion

In the last few decades, extensive work has been done to establish the pharmacological actions of turmeric and its extracts [10-12]. The ethanolic extract of turmeric has been studied using a number of cell lines viz. Colon 502713, Colo-205, Hep-2, A-549, OVCAR-5, PC-5, leukemia, murine mammary adenocarcinoma (AMN-3) and rat embryonic fibroblast transformed (REF-3) cell lines [13-15]. Curcumin I, curcumin II (monodemethoxycurcumin) and curcumin III (bisdemethoxycurcumin) from CL have been assayed for their cytotoxicity, antioxidant and anti-inflammatory activities. These compounds showed activity against leukemia, colon, CNS, melanoma, renal, and breast cancer cell lines [16].

The ability of curcumin to induce apoptosis in cancer cells without cytotoxic effects on healthy cells contributes to the understanding of the anti-cancer potential of curcumin. Curcumin efficiently induces apoptosis in various cell lines like HL-60, K562, MCF-7 and HeLa [17]. Curcumin also leads to apoptosis in scleroderma lung fibroblasts (SLF) without affecting normal lung fibroblasts (NLF) [18]. This effect seems to be due to the weak level of protein kinase (PK) C3 in SLF, generating low levels of glutathione S-transferase (GST). Studies reporting anticancer activity of curcumin and ethanolic extract of turmeric *in vivo* (mice) and *in vitro* (human liver carcinoma cell line) have revealed that both curcumin and the crude ethanolic extract have great potential in the prevention and cure of cancer [10].

In the present study, *in vitro* anticancer activity of CL has been evaluated against human breast carcinoma cell line MDA. The results were found to be in agreement with previous studies done on curcuma extract by other authors. In the present study, ethanolic extract of CL showed significant cytotoxicity on breast cancer cells, but little to no activity on Vero cells. The anti-proliferative effect increased with increase in the concentration of the extract. The treated cells displayed an altered morphology under inverted microscope. Curcumin caused MDA cells to develop characteristic features of cell shrinking, rounding and partial detachment, thus demonstrating the lobulated appearance of apoptotic cells (Fig. 2 h, i).

Qualitative phytochemical characterization of extract constituents was carried out using HPLC. HPLC analysis revealed the presence of the active component curcumin in the ethanolic extract. The analytical HPLC method used in the study provided a good baseline resolution of peaks of curcuminoids present in CL extract with reference to curcumin standard (R_t = 29.6 min). The retention times (R_t) obtained from HPLC analysis of CL extract and curcumin standard were quite similar, viz. 29.2 and 29.6 respectively (Fig. 8a, b). Curcumin was also found to be the major component in HPLC tested samples of CL in a number of studies [19, 20].

Curcumin, a naturally occurring polyphenolic compound in rhizomes of curcuma longa, is known to have a wide range of therapeutic and pharmacological properties. Curcumin also has potent anti-cancer properties as demonstrated in a plethora of human cancer cell line and animal carcinogenesis models. Although it is a considerably promising compound,

its poor water solubility and fast degradation profile make it compromise over its bioavailability way below the threshold level on administration. Over a period of time, a lot of emphasis has been given to improve the biodistribution of native curcumin, but it is only recently that the application of the field of nanotherapeutics has significantly improved its therapeutic efficacy. This is through the development of nanorange formulations of curcumin, popularly known as the "nanocurcumin." These attempts have given a strong platform to reap all the biological benefits from this phytodrug, which was not significantly plausible earlier [21, 22, 23]. Future studies would entail synthesis of nanocurcumin and comparison of its therapeutic efficacy and cytotoxicity in a panel of human cell lines as well as in preclinical animal models of cancer with free or native curcumin. Also, it would be interesting and essential to establish whether curcumin will be more effective in humans if consumed as an individual agent or as part of turmeric. Bioavailability and preventive efficacy as well as therapeutic activity of curcumin may also form an interesting field of research for future human clinical studies. It would be interesting to determine whether or not other chemical constituents present in CL rhizome extract augment the anticancer effect of curcumin.

5. Conclusion

This study showed that 50% ethanolic extract of stem of CL is cytotoxic to the human breast cancer cell line MDA-MB-231 and contains curcumin as the main anticancer agent. Also, the ethanolic extract did not seem to have any significant *in vitro* effect on normal kidney epithelial cell line Vero.

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