

Apoptosis-induced *in vitro* anticancer activity of methanolic extract of leaves and rhizomes of *Curcuma amada* Roxb. against breast cancer cells

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Abstract

Objective: Cancer is one of the most dreadful diseases which is globally distributed among the world's population. Cancer occurs due to excessive free radical damage, which ultimately causes damage to the genetic material DNA, protein, and lipids. This DNA damage leads to mutations that cause normal cells to transform into a cancer cell. Breast cancer is the major health problem among women in both developing and developed countries. Although several treatment strategies have been employed, there are still chances of recurrence of cancer. Hence, in this study, the mechanism of apoptosis inducing the effect of methanolic extract of leaves and rhizomes of *Curcuma amada* has been analyzed. **Materials and Methods:** Immunocytochemical analysis was used to detect the expression of apoptosis-associated proteins Bax, Bcl-2, and p53. Various phytochemicals present in the leaves, and the rhizomes were analyzed using various chromatographic and spectral studies. **Results:** The results revealed that the extracts upregulated the expression level of pro-apoptotic proteins p53 and Bax in cancer cells compared to the non-tumorigenic cells, whereas there was a downregulation in the expression of anti-apoptotic protein Bcl-2 in the extract treated cancerous cells. **Conclusion:** The result of this study indicated that the extracts induced apoptosis in breast cancer cells which is mediated through apoptosis.

Key words: Apoptosis, breast cancer, cancer, cell lines, *Curcuma amada*, immunocytochemical

INTRODUCTION

Medicinal plants are invaluable for the survival of both human and animal population globally. They are a rich source of phytochemicals, which can be used to treat various ailments of humankind.^[1,2] In India, natural products derived from medicinal plants have been used traditionally for the treatment of various disorders including cancer.^[3] However, the use of these medicinal plants needs proper validation and documentation for utilizing it as a drug for treating various pathophysiological conditions.^[4-6] About 6000 medicinal plants have been estimated to be present in India, of which, only 3000 plants have been proved to have medicinal value and still many remains unexplored.^[7]

Cancer is one of the most dreadful diseases which is globally distributed among the world's population. Cancer occurs due to excessive

free radical damage, which ultimately causes damage to the genetic material DNA, protein, and lipids. This DNA damage leads to mutations that cause normal cells to transform into a cancer cell.^[8] Failure of apoptosis and increased rate of cell survival occurs due to DNA damage that results in cancer development. Apoptosis is the major form of programmed cell death, which takes place in all the cells to maintain homeostasis and cellular integrity. Cancer treatment therapies target this apoptotic pathway by increasing apoptosis in cells and thus preventing cancer.^[9,10]

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Curcuma amada Roxb is a well-known rhizomatous herb which is commonly known as mango ginger that belongs to the family Zingiberaceae (Ginger family) that is widely cultivated in various parts of South India. *C. amada* rhizomes have been used for culinary purposes and pickle preparations in South India. This plant has been proved to have many biological activities such as antioxidant,^[11] anti-inflammatory, antihelminthic,^[12] hypoglycemic, antihyperglycemic,^[13] cytotoxic, platelet aggregation inhibitory, and antimicrobial activities.^[14] Our previous studies with this plant showed that both the leaves and the rhizomes possess free radical scavenging, cytotoxicity, antioxidant, and anticancer activity against breast cancer cell lines.^[15-18] Hence, this study was formulated to explore the mechanism of anticancer action.

MATERIALS AND METHODS

Plant Material

C. amada Roxb rhizomes were procured from Arya Vaidya Pharmacy, Centre for Indian Medicinal Plant Heritage, Kanjikode, Kerala and were grown as pot culture in our university herbal garden and were identified by Botanical Survey of India, Southern Circle. Both fresh leaves and rhizomes were collected for the study. Previous studies conducted by us showed that the methanol extract of the leaves and rhizomes were rich with antioxidants.

Extract Preparation

The fresh leaves and rhizomes collected were rinsed with tap water, blotted dry using a filter paper and used for extract preparation. The components present in the leaves and rhizomes were extracted using methanol. The methanol extract prepared after evaporation of methanol was dissolved in dimethylsulfoxide (0.2 mg/ml - inhibitory concentration 50% dose).

Culturing of Cell Lines

All the cell lines MCF-7, MDA-MB 231, and HBL-100 were purchased from National Centre for Cell Science, Pune, India. The cell count was done and the cell viability was tested by Trypan blue using hemocytometer. The cells were grown in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% non-essential amino acids in tissue culture flasks and incubated in a CO₂ incubator in a 5% CO₂ and 95% humidity atmosphere. Once the cells attained confluent growth, the cells were trypsinized using trypsin-ethylenediaminetetraacetic acid (PAA) and the required number of cells 10⁶ and 10³ cells/mL were seeded into 6-well, respectively, for carrying out various assays. Cell

viability and cytotoxicity assays were carried out in 96-well plates, and the staining was performed in 6-well plates. In each well of the 6-well plates, a clean, dry, sterile cover slip was placed before the cells were seeded, followed by incubation in a CO₂ incubator in a 5% CO₂ and 95% humidity atmosphere (Innova CO-170, United States).

Treatment Groups

MCF-7, MDA-MB 231, and HBL-100 were treated with the leaf and rhizome extract (0.2 mg/ml) for 24, 18, and 12 h, respectively, which was the optimal treatment time of the extracts in each of the cell lines. The effect induced was also compared to the standard drugs used, *viz.*, Tamoxifen for estrogen receptor positive MCF-7 and HBL-100 cells and etoposide for triple negative MDA-MB 231 cells. The following treatment groups are set up of the study. Negative control: Cells alone. Positive control: Cells + tamoxifen/etoposide. Test groups: Cells+ methanol extract of *C. amada* leaves; cells + methanol extract of *C. amada* leaves + tamoxifen/etoposide; cells + methanol extract of *C. amada* rhizomes; cells + methanol extract of *C. amada* rhizomes + tamoxifen/etoposide.

Immunocytochemical Analysis

Sterile coverslips were placed inside 6-well plates and 0.1% gelatin was added and left for 30 min. The gelatin was then discarded, and cells were seeded onto the plates. The treatments were given according to the optimized time for each cell line. The treated cells were then fixed with 4% paraformaldehyde (in phosphate-buffered saline [PBS]) for 30 min. Then, the cells were washed 3 times with PBT for 10 min each (optional: Skip for membrane staining, essential for nucleus staining). The cells were then treated with 0.5% Triton-X-100 (in PBS-PBT) for 5-60 min (often 15 min). Then, the cells were washed three times with PBT for 10 min each. Then, 200 µl of 10% normal goat serum (in PBT) was added and incubated for 30 min followed by incubation with 200 µl of primary antibody (From Dako-Catalogue Nos.: IS61630-P53, IS61430-Bcl-2, A3533-Bax) (1:100 to 1:10,000 dilution in PBS + 1% bovine serum albumin [BSA]) at 4°C overnight. The cells were washed 6 times with PBT for 10 min each followed by incubation with 200 µl of fluorescein isothiocyanate-labeled secondary antibody (From Dako-Catalogue No.: F0232 - 1:100 to 1:500, in PBS + 1% BSA) at 37°C for 1 h. The cells were washed 3 times with PBT for 10 min each and incubated with 200 µl of DAPI for 10-15 min, and then washed the cells 6 times with PBT for 10 min each. 5 µl of the antifading solution was placed onto clean glass slide, and the cover slip with the cells was mounted over it and the edges were sealed using manicure and the expression of the proteins in the cells were observed under ×400 magnification using a fluorescent microscope (Moticam, Hong Kong).

Phytochemical Analysis

Preliminary phytochemical screening

The methanolic extract of the leaves and the rhizomes of *C. amada* were screened for the presence of phytochemicals according to the method of Khandelwal (2002).

High-performance thin layer chromatography (HPTLC) analysis

The methanol residue (100 mg) of the leaves and the rhizomes of *C. amada* were dissolved in methanol (1 ml) and centrifuged at 3000 rpm for 3 min. The supernatant was collected and used for HPTLC analysis. The test sample (2 µl) was loaded as an 8 mm band in the 4 × 10 silica gel G60F254 plate using a Hamilton syringe in CAMAG LINOMAT 5 instrument. After saturation with the solvent vapor, the TLC plate loaded with the test sample and the reference was kept in a TLC twin trough developing chamber with the respective mobile phase (mentioned below) and developed up to 90 mm.

The developed plates were dried in hot air oven to evaporate the solvents from the plates. The plates were kept in a photo-documentation chamber (CAMAG REPROSTAR 3), and the images were captured in white light, ultraviolet (UV) 254 nm, and UV 366 nm. After derivatization with the appropriate reagents (as given below), the plates were photo-documented at daylight, UV 254 nm, and UV 366 nm for alkaloids and flavonoids; daylight and UV 366 nm for phenolics, saponins, steroids, and terpenoids; daylight alone for tannins. The peak table, peak display, and peak densitogram of alkaloids, phenolics, flavonoids, saponins, steroids, tannins, and terpenoids were noted [Table 1 and Figure 3].

Infrared (IR) spectral analysis

IR spectral analysis was carried out in the methanolic extract of the leaves and the rhizomes of *C. amada* using a scanning Michelson interferometer and Fourier transformation (Shimadzu, Japan). A residue of the methanol extract was placed in the beam. The intensities that are reduced in the

interferogram were subjected to Fourier transform. This Fourier transform is the IR absorption spectrum of the sample.

Gas chromatography–mass spectrometry (GC-MS) analysis

The methanolic extract of the leaves and the rhizomes of *C. amada* were analyzed using a Shimadzu GC apparatus (Model: GC 8000 series and MS was MD 800) using a DB-S capillary column (30 m) equipped with quadrupole MS detector (EI, 70 eV) with helium as a carrier gas at a flow rate of 1 ml/min. The compounds were identified by comparison with the standards and also matched with the in-built libraries.

RESULTS

Immunocytochemistry is a common laboratory technique that uses antibodies to detect specific antigens in the cell via specific epitopes. Immunocytochemistry allows researchers to evaluate whether or not cells in a particular sample express the antigen in question. When an immunopositive signal is found this technique allows the researchers to determine which sub-cellular compartments are expressing the antigen.

p53 is a tumor suppressor gene which prevents the formation of cancer. The mutations in p53 gene lead to the formation of cancer by modulating the complex network of molecular events. In a cell, p53 protein binds to DNA, which in turn stimulates another gene to produce a protein p21 which interacts with a cell cycle regulatory protein Cdk2. When p21 complexes with Cdk2, the cell cannot pass to the next stage of the cell division. Mutant p53 can no longer bind DNA in an effective way and as a consequence, the p21 protein is not available to stop the cell division. Thus, cells divide uncontrollably and form tumors.

Bax (Bcl-2 associated X protein) is a protein of the Bcl-2 gene family. Bax is a pro-apoptotic member of the Bcl-2 gene family. It promotes apoptosis by competing with Bcl-2. In healthy mammalian cells, the majority of Bax is found

Table 1: HPTLC analysis of the methanol extract of rhizomes and leaves of *Curcuma amada* (Rf values shown)

Profile	Number of bands		Rf values	
	Leaves	Rhizomes	Leaves	Rhizomes
Alkaloids	3	2	0.05, 0.23, 0.28	0.07, 0.23
Flavonoids	2	1	0.46, 0.54	0.95
Phenolics	5	1	0.09, 0.13, 0.17, 0.60, 0.70	0.70
Saponins	6	3	0.29, 0.48, 0.63, 0.31, 0.42, 0.49, 0.56, 0.66, 0.94	0.29, 0.48, 0.63
Steroids	6	10	0.06, 0.45, 0.49, 0.72, 0.80, 0.89	0.04, 0.09, 0.16, 0.22, 0.28, 0.34, 0.38, 0.46, 0.52, 0.79
Tannins	5	4	0.03, 0.08, 0.45, 0.86, 0.92	0.40, 0.55, 0.80, 0.94
Terpenoids	3	6	0.64, 0.69, 0.82	0.15, 0.37, 0.45, 0.50, 0.59, 0.64

HPTLC: High-performance thin layer chromatography

in the cytosol but on initiation of apoptotic signaling, Bax undergoes a conformation shift and inserts into mitochondrial outer membrane. The expression of Bax is upregulated by the tumor suppressor protein p53. p53 is a transcriptional factor, which, when activated as part of cell's response to stress, regulates many downstream target genes including Bax.

Bcl-2 is a human proto-oncogene whose product is an integral membrane protein Bcl-2 located in the membranes of endoplasmic reticulum, nuclear envelope, and the outer membrane of the mitochondria. Bcl-2 protein suppresses apoptosis by preventing the activation of the caspases either by preventing the release of cytochrome C from the mitochondria and/or by binding to the apoptosis-activating factor.

The results revealed that the expression level of the tumor suppressor protein p53 increased in both the extract and drug-treated groups and combinations in all the three cell lines HBL-100, MCF-7, and MDA-MB 231. The expression level of Bax, a pro-apoptotic protein, was found to be increased in extracts, and drug treated groups compared to the control in the breast cancer cell lines MCF-7 and MDA-MB 231. There is no change in the expression level of Bax protein in non-cancerous breast cell line HBL-100. The expression level of anti-apoptotic protein Bcl-2 was found to be decreased on treatment with the methanolic extract of leaves and rhizomes of *C. amada* in both the breast cancer cell lines MCF-7 and MDA-MB 231 as shown in Figures 1 and 2. However, in non-tumorigenic HBL-100 cells, there is only slight increase in the expression of Bax.

These findings revealed that the leaf and the rhizome extracts induced apoptosis in tumor cells which are mediated through p53 which may be the reason for the cell death. The extracts also increased the expression levels of pro-apoptotic protein Bax and decreased the level of the anti-apoptotic protein

Bcl-2 as shown by decrease in the green fluorescence, and thus prevent the formation of cancer.

The results of the phytochemical analysis revealed that the steroids and terpenoids were the major components present in the methanolic extract of leaves and rhizomes. Spectral analysis revealed the presence of carbonyl and hydroxyl group in both leaves and rhizomes.

DISCUSSION

Our studies are in line with the below scientific investigations. In oxygen-glucose deprivation induced human neuroblastoma SH-SY5Y cells, immunocytochemical analysis showed that apoptosis inducing factor was translocated from mitochondria to the nucleus. This was accompanied by increased levels of Bax and decreased levels of Bcl-2.^[19]

The expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors was identified in varicocele-induced testicular dysfunction in an experimental rat model using immunohistochemical staining.^[20] Most frequently described markers of apoptosis such as p53, Bcl-2, survivin, Fas, and TRAIL-R1 receptors and their ligands were identified in colorectal cancer patients using immunohistochemical staining.^[21]

A decrease in the expression level of anti-apoptotic protein Bax has been detected in streptozotocin-induced diabetic rats by immunohistochemical studies (Bhan *et al.*, 2013). The treatment with rhein, a primary anthraquinone found in the roots of Chinese herb, rheibarb for 48 h to human gastric cancer cells SGC-7901 induced apoptosis by increasing the level of pro-apoptotic protein Bax and decreasing the level of anti-apoptotic proteins Bcl-2, Bcl-XL, and procaspase 3.^[22] The pre-treatment of Genistein followed by irradiation increased the expression of DNA repair protein Rad 51, which

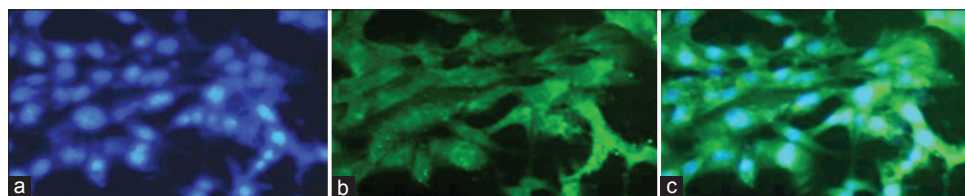


Figure 1: Expression of apoptotic proteins in HBL-100 (representative photograph), (a) 4',6-diamidino-2-phenylindole, (b) fluorescein isothiocyanate, (c) merge

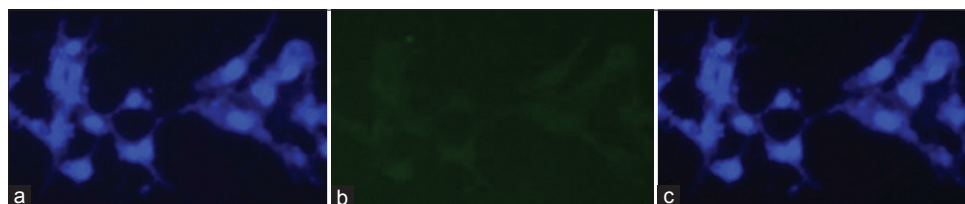


Figure 2: No expression of apoptotic proteins in MCF-7 (representative photograph), (a) 4',6-diamidino-2-phenylindole, (b) fluorescein isothiocyanate, (c) merge

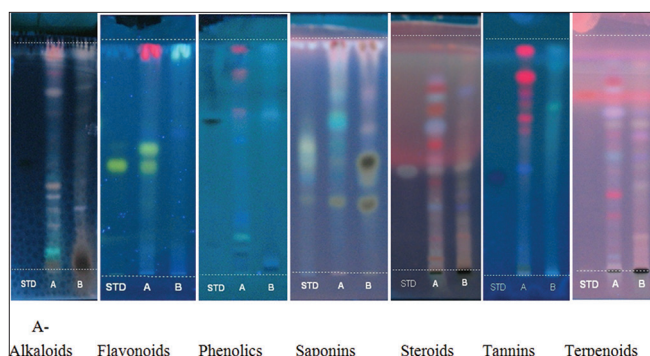


Figure 3: High-performance thin layer chromatography analysis of the methanol extract of rhizomes and leaves of *Curcuma amada*, (A) Leaves, (B) rhizomes

was detected by immunocytochemical analysis in MCF-7 and MDA-MB 231 breast cancer cells.^[23] A decrease in the level of anti-apoptotic protein Bcl-2 and Bcl-XL and increase in the level of tumor suppressor protein p53 was observed in breast cancer cell line MCF-7 on exposure to benzyl isothiocyanate, a constituent found in edible cruciferous vegetables.^[24]

The methanolic extract of the stem and the leaves of *Kedrostis foetidissima* increased the level of tumor suppressor protein p53 in breast cancer cell lines MCF-7 and YMB-1.^[25] The administration of *Cerastes cerastes* and *Vipera lebetina* snake venoms upregulated the pro-apoptotic genes p53 and Bax and downregulated the anti-apoptotic gene Bcl-2.^[26]

Elkady *et al.*^[27] found that the administration of the ethanolic extract of *Zingiber officinale* caused the upregulation of Bax and downregulation of Bcl-2 proteins. An upregulation of Bcl-2 and p53 genes were observed in MCF-7 breast cancer cells after exposure to the phenolic extract of *Cichorium endivia* L. roots.^[28]

In tune with the above results, in this study, we have demonstrated that pro-apoptotic p53 and Bax protein expression is significantly enhanced, and anti-apoptotic Bcl-2 protein expression is reduced. Therefore, the results show the potential of the methanolic extract of *C. amada* leaves and rhizomes as an anticancer agent to inhibit cell growth and trigger apoptosis.

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