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*Full Length Research Paper*

# Phytochemical Analysis and Cytotoxicity Studies of *Curcuma amada rhizomes* in BHK-21 Cells

Ankita Joshi[1], R.S. Chauhan[2]*
[1]PDF, Institute of Biotechnology, G B Pant University of Agriculture & Technology, Patwadangar-263128, Nainital, Uttarakhand, India, joshi.ankita11@gmail.com; 9897570194.
[2]Director, Institute of Biotechnology, G B Pant University of Agriculture & Technology, Patwadangar-263128, Nainital, Uttarakhand, India.
*Corresponding Authors: Email: rs_chauhan@rediffmail.com; Tel.: 9412288343.

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**Abstract.** *Curcuma amada* is medicinal plant which is used in several traditional medicines to cure various diseases. *Curcuma amada* has been shown to possess anti-inflammatory, antioxidant and antitumor properties. In this investigation, it had been planned to study its anticancer properties in BHK-21 cells. Acetone, Methanol, Ethanol and aqueous extracts of the rhizomes of *Curcuma amada* were screened for their anticancer properties. The cells were seeded with all the extracts separately and then allowed to grow for 24hrs; the cell growth was inhibited within 24hrs. The cytopathology observed were included rounding and clumping of cells, detachment of cells, flagging of cells and apoptosis. Methanolic and ethanolic extracts showed better response than that of its aqueous and acetone extract. The concentration of 10 mg/ml of ethanolic extract inhibited the cancerous cell growth.

**Key words:** BHK-21 cells, *Curcuma amada*, Phytochemical, Cytotoxicity

## 1. INTRODUCTION

Medicinal plants are a rich source of numerous pharmacologically active molecules. India is a continent with wide field of diversity. This diversity includes both flora as well as fauna. This variation is due to the varied climatic condition, vegetation, topography etc. resulting in enriched heterogeneity. As a result, many such herbs are present with increased medicinal value that is left unnoticed. These herbs may possess medicinal values, domestic values and therapeutic values (Sofowara, 1993). *Curcuma amada* belonging to Zingiberaceae family is known as mango ginger in English; Manghainchi and Kathumachal in Malayalam and Suraniyika in Sanskrit (Wealth of India, 1952; Warrier et al., 1994; Kirtikar and Basu, 1984). The rhizome, the portion of the plant used medicinally, is usually boiled, cleaned, and dried, yielding a yellow powder. Dried *Curcuma amada* is the source of the spice turmeric, the ingredient that gives curry powder its characteristic yellow color. *Curcuma amada* is used extensively in foods for both, its flavor and color, as well as having a long tradition of use in the Chinese and Ayurvedic systems of medicine, particularly as an anti-inflammatory and for the treatment of flatulence, jaundice, menstrual difficulties, hematuria, hemorrhage, and colic. *Curcuma amada* can also be applied topically in poultices to relieve pain and inflammation (Govindarajan, 1980).

Various researches had focused on turmeric's antioxidant, hepatoprotective, anti-inflammatory, anticarcinogenic, and antimicrobial properties, in addition to its use in cardiovascular disease and gastrointestinal disorders (Leung, 1980). The rhizome of the plant *Curcuma amada* has been used for centuries in traditional medicine and is known to have cancer preventive or therapeutic capabilities (Anand et al., 2008). It has been shown to suppress multiple signaling pathways and inhibit cell proliferation, invasion, metastasis, and angiogenesis (Kunnumakkara et al., 2008; Duvoix et al., 2005). Its safety combined with its low cost, and multiple targeting potential makes *Curcuma amada* an ideal agent to be explored for prevention and treatment of various cancers and fits very well as a candidate for chemoprevention by edible phytochemicals (Aggarwal, 2008; Gupta, 2007). This study was a step towards evaluation of the plant against cancer. Methanolic, ethanolic, acetone and aqueous extract of the rhizome of *Curcuma amada* were screened for their anticancer properties. The result of the phytochemical examination of the flowers of this plant is described in this communication.

## 2. MATERIALS AND METHODS

Plants have been selected from high altitude area (1600m from sea level) from the polyhouse nursery of Institute of Biotechnology, Patwadangar (Nainital),Uttarakhand. Rhizome of *Curcuma amada*

were collected and washed with tap water thrice. Washing was again repeated five times by using distilled water. Then the rhizome of *Curcuma amada* were air dried and thereafter kept in incubator at $37^0$C for 24 hrs. The dried material was then crushed in mechanical grinder in order to make fine powder which was stored at room temperature for further use.

## 3. EXTRACT PREPARATION

### 3.1. Aqueous Extract

The aqueous extract was prepared according to the standard method with slight modifications. Now, 5g of rhizome powder was mixed in 120 ml of water and was kept in incubator shaker at 36˚C and 100 rpm. The extract so obtained was evaporated to drying through heating in a china dish. Dry extract was then scrapped off, weighed and reconstituted in normal saline (Marks et al., 2008).

### 3.2. Solvent Extraction

Ethanol, Acetone and methanol extracts were prepared in Soxhlet's apparatus. Soxhlet's extraction was carried out at room temperature. Dried rhizome powder of *Curcuma amada* weighed accurately 5gm and taken in thimble and subjected to extraction in a Soxhlet's apparatus at room temperature using ethanol (150ml), acetone (170ml) and methanol (165ml) (Govindachari et al., 1999). The extract obtained was first filtered through Whatman No. 1 filter paper and solvent was then removed under reduced pressure in a vacuumed rotary evaporator and dried. The dried extract was stored in airtight containers for further studies.

For the present study, BHK-21 cells were cultured in 24 well sterile polystyrene plates using GMEM media supplemented with 5% fetal bovine serum as per standard procedure. The cells were seeded into 24 well sterile polystyrene plates and were incubated for 24 hours at 37ºC. Thereafter, the medium was removed and 0.5ml of each dilution (10mg,1mg,10µgm) of each extracts added to the assigned wells, Control were also kept (medium without test sample) and triplicate sets of each dilution were maintained. Finally the cells were incubated for 24hours at 37ºC and thereafter, examined under inverted microscope for their morphological studies.

### 3.3. Confirmatory Tests

#### 3.3.1. MTT Assay

The MTT Assay is a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) into a dark formazan product that is insoluble in water. The amount of formazan produced is directly proportional to the cell number in a range of cell lines. It was performed as, prepared an MTT stock solution of 5 mg ml- 1 in phosphate-buffered saline (PBS), pH 7.5, and filter through a 0.22-µ filter to sterilize and the small amount of insoluble residue was removed. Add10 µl of MTT (5mg $ml^{-1}$), after 24 h of incubation and the cells were further incubated in incubator at 37°C for3 h. Then 100 µl 0.04 M HCl in propan-2-ol to eachwell were added and mixed thoroughly to dissolve insoluble blue formazan crystals. The Plates were read on a micro-ELISA reader using a test wavelength of 570nm (Mosmann, 1983).

#### 3.3.2. Neutral Red Assay

Neutral red (3-amino-7-dimethyl-2-methylphenazine hydrochloride) is a water soluble, weakly basic, supravital dye that accumulates in lysosomes of viable cells. The neutral red (NR) assay is an invitro cell viability test that was developed and extensively studied for in vitro cytotoxicity determination. After incubation of cells with extracts, 0.33% of NR (NR in PBS) was added in each well and incubated for 1 h at 37ºC. Dye-containing medium was removed and the well was washed twice with 150µl/well warmed PBS. The cells were then lysed with 125 µl of 50% of v/v mixture of ethanol and 0.1Mmonobasic sodium phosphate to solubilise the neutral red. The plate was then incubated for 15 min and take O.D at 550 nm (Flick and Gifford, 1984).

#### 3.3.3. Cytotoxicity % = A-B/A X 100

A = O.D of untreated well;
B = O.D of wells treated with plant extract.

### 3.4. Phytochemical Analysis

In recent years, secondary plant metabolites (phytochemicals), previously with unknown pharmacological activities, have been extensively investigated as a source of medicinal agents. Thus, it is anticipated that phytochemicals with adequate antibacterial efficacy will be used for the treatment of various infections. These are the qualitative tests performed to analyze the presence or absence of various phytochemicals such as alkaloids, tannins,

flavonoids etc. in plant extract (Wagner and Bladt, 1996).

## 4. RESULTS

Phytochemical analysis of *Curcuma amada* methanolic extract, showed positive result for alkaloids, tannins, phenolic compounds, phytosterols, terpenoids, saponins and flavonoids. Aqueous extract of *Curcuma amada* showed the presence of reducing sugar, Amino acid, steroids, cardiac glycosides, saponins and alkaloids. Ethanolic extract of *Curcuma amada* showed the presence of reducing sugar, amino acid, steroids, cardiac glycosides, anthraquinone glycosides, saponins and alkaloids. Acetone extract of *Curcuma amada* showed the presence of phenolic compounds, phytosterols, terpenoids, saponins and amino acid.

The cells were observed after 24 hours to record the changes in morphology. The induction of apoptosis resulted in cell shrinkage, eventually leading to the formation of apoptotic bodies. The cells also got detached from the substratum shown by flagging of the cells and also they formed clumps after detachment from the substratum. The morphological changes revealed that the ethanolic and methanolic extract of *Curcuma amada* rhizome were better than the aqueous and acetone extract of rhizome. The shapes of cells were changed. Elongated cell turn to round cell and clumping was observed. The cytotoxicity study also clearly indicated, that the ethanolic and methanolic extracts of *Curcuma amada* rhizome killed more cells then that of aqueous and acetone extracts.

**Table 1**: Percent cell scytotoxicity due to the extract of *Curcuma amada* rhizome measured by MTT Assay (mean±SE).

| Sl.No. | Extracts | 10mg/ml | 1mg/ml | 10µgm/ml |
|---|---|---|---|---|
| 1. | Aqueous extract | 69.84±3.51 | 67.99±3.63 | 64.65±3.43 |
| 2. | Acetone extract | 41.22±3.18 | 40.91±3.42 | 38.47±3.63 |
| 3. | Ethanolic extract | 84.32±3.21 | 82.54±3.09 | 80.06±3.11 |
| 4. | Methanolic extract | 74.29±3.07 | 71.64±3.57 | 70.41±3.27 |

**Table 2:** Percent cell cytotoxicity due to the extract of *Curcuma amada* rhizome measured by NR Assay (mean±SE)

| Sl.No. | Extracts | 10mg/ml | 1mg/ml | 10µgm/ml |
|---|---|---|---|---|
| 1. | Aqueous extract | 69.97±2.47 | 68.92±2.51 | 65.39±2.37 |
| 2. | Acetone extract | 43.27±2.11 | 41.83±2.18 | 39.32±2.23 |
| 3. | Ethanolic extract | 87.63±2.03 | 85.42±2.81 | 81.35±2.42 |
| 4. | Methanolic extract | 76.31±2.21 | 74.61±2.33 | 70.39±2.17 |

Cytotoxicity measured by MTT Assay of *Curcuma amada* rhizome of aqueous extract at 10mg/ml was found 69%, at 1mg/ml the cytotoxicity was found 67% and at 10µgm/ml the cytotoxicity was 64%. Whereas, ethanolic extract of *Curcuma amada* rhizome at 10mg/ml was found 84%, at 1mg/ml the cytotoxicity was found 82% and at 10µgm/ml the cytotoxicity was 80%. Methanolic extract of *Curcuma amada* rhizome at 10mg/ml was found 74%, at 1mg/ml the cytotoxicity was found 71% and at 10µgm/ml the cytotoxicity was 70%.(Table 1) Acetone extract of *Curcuma amada* rhizome at 10mg/ml was found 41%, at 1mg/ml the cytotoxicity was found 40% and at 10µgm/ml the cytotoxicity was 38%.

Cytotoxicity measured by NR Assay of *Curcuma amada* rhizome of aqueous extract at 10mg/ml was found 69%, at 1mg/ml the cytotoxicity was found 68% and at 10µgm/ml the cytotoxicity was 65%. Whereas, ethanolic extract of *Curcuma amada* rhizome at 10mg/ml was found 87%, at 1mg/ml the cytotoxicity was found 85% and at 10µgm/ml the cytotoxicity was 81% (Table 2). Methanolic extract of *Curcuma amada* rhizome at 10mg/ml was found 76%, at 1mg/ml the cytotoxicity was found 74% and at 10µgm/ml the cytotoxicity was 70%. Acetone extract of *Curcuma amada* rhizome at 10mg/ml was found 43%, at 1mg/ml the cytotoxicity was found 41% and at 10µgm/ml the cytotoxicity was 39%.

## 4. DISCUSSION

Today there is a wide range of medicinal plant parts which include the flowers, leaves, stem, fruits and root extracts which are used as powerful raw drugs possessing a variety of antimicrobial and healing properties. The phytochemical screening of the rhizome of Curcuma amada showed the presence of secondary metabolites including phenols, saponins, tannins and coumarins which had great medicinal properties. In addition, there are several reports to show *Curcuma amada* species for having potent antimicrobial chemicals (Kannan et al., 2013).

Animal studies involving rats and mice, as well as in vitro studies utilizing human cell lines, have demonstrated *Curcuma amada* rhizome extract ability to inhibit carcinogenesis at three stages: tumor promotion, 18 angiogenesis,(Thaloor et al., 1998) and tumor growth (Limtrakul et al., 1997). In two studies of colon and prostate cancer, *Curcuma amada*

rhizome extract inhibited cell proliferation and tumor growth (Hanif et al., 1997; Dorai et al., 2001). Curcuma amada rhizome extract and curcumin are also capable of suppressing the activity of several common mutagens and carcinogens in a variety of cell types in both in vitro and in vivo studies (Mehta and Moon, 1991; Soudamini and Kuttan, 1989; Azuine and Bhide, 1992; Boone et al., 1992).

**Fig 1.:** Rhizome of *Curcuma amada*

**Fig. 2:** Normal BHK-21 cells

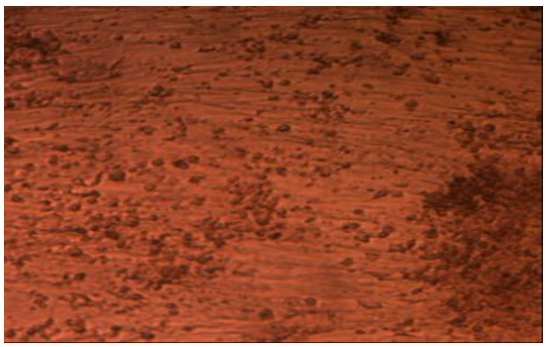

**Fig. 3:** Cells treated with extract of rhizome of *Curcuma amadas*

The anticarcinogenic effects of *Curcuma amada* rhizome extract and curcumin are due to direct antioxidant and free-radical scavenging effects, as well as their ability to indirectly increase glutathione levels, thereby aiding in hepatic detoxification of mutagens and carcinogens, and inhibiting nitrosamine formation. Numerous animal, *in vitro*, and *in vivo* studies have demonstrated the anti carcinogenic effects of *Curcuma amada* rhizome extract and its flavonoid component curcumin against colon (Chauhan, 2002; Reddy and Rao, 2002), breast, (Ramachandran et al., 2002; Somasundaram et al., 2002; Shao et al., 2002) and prostate cancers, as well as melanoma (Hour et al., 2002). A human study of 25 individuals at high risk of neoplasia or with pre-malignant lesions noted histologic improvement in one of two patients with recently resected bladder cancer, two of seven patients with oral leukoplakia, one of six patients with intestinal metaplasia of the stomach, one of four patients with cervical intraepithelial neoplasm, and two of six patients with Bowen's disease. More clinical trials need to be performed to further elucidate the potential of this botanical in cancer prevention and treatment (Bush et al., 2001; Cheng et al., 2001). Curcuma zedoaria and Curcuma amada rhizome solvent extracts were evaluated for their anticancer and antioxidant activity. The isopropyl extract of C. zedoaria exhibited high anticancer activity compared to acetone extract of C. amada. Crude protein of C. zedoaria showed good anticancer activity when compared to crude protein of C. amada. Acetone extract of C. zedoaria showed high radical scavenging activity of 88.7% and superoxide scavenging activity recording 83.15%. Acetone extract of C. zedoaria showed 82.5% hydroxyl radical scavenging activity (Kumar et al., 2012). Muthu kumar et al. (2012) had reported the anticancer and antioxidant activity of *Curcuma zedoaria* and *Curcuma amada* rhizome extracts. The isopropyl extract of C. zedoaria exhibited high anticancer activity compared to acetone extract of *C. amada*. Crude protein of C. zedoaria showed good anticancer activity when compared to crude protein of *C. amada*. Acetone extract of *C. zedoaria* showed high radical scavenging activity of 88.7% and superoxide scavenging activity recording 83.15%. Acetone extract of *C. zedoaria* showed 82.5% hydroxyl radical scavenging activity.

Thus, in the present investigation, on the basis of observed encouraging cytotoxic effects by the *in-vitro* bioassays, it can be revealed that methanolic and ethanolic extracts of *Curcuma amada* rhizome extracts had promising anticancer bio efficacy than its ethanolic extract and must have some phytochemical

moiety in the leaves of this plant which might be responsible for observed beneficial effects. It is suggested that the detailed *in-vivo* studies should be carried out in animal experimental model of cancer to further prove the anticancerous activity of *Curcuma amada* rhizome extracts. Moreover, the inhibitory effect of all the solvent extract and aqueous extract of *Curcuma amada* rhizome were maximal at 24 hours. These observations suggest that active principle might get metabolized or get inactivated during culture process and is no more available to impose growth inhibitory effect. However, these assumptions need further investigation. In contrast to *Curcuma amada* rhizome aqueous and acetone extract showed a minor growth promoting activity in dose range 10gm/ml -10 µg/ml. These results, suggest accumulation of cell growth promoter sin ethanolic extract that may have coexisted in the plant rhizome along with cytotoxic activity. However, there is no report which specifically describes any tumor growth promoting activity associated with this plant.

## 5. CONCLUSION

The active phytochemical constituents present in *Curcuma amada* rhizome extract imparts high therapeutic properties that can prevent, various infection, flatulence, jaundice, menstrual difficulties, hematuria, hemorrhage, and colic and other ailments. The aqueous, methanolic, acetone and ethanolic extracts were tested on BHK-21 cells, which showed cytotoxic effects on the cancer cells. Assessing its anticancer activity in our study indicates for the first time that *Curcuma amada* rhizome extract act as an cytotoxic inducing property against BHK-21 cells. It therefore provides an important lead for development of anti-cancer therapeutics for management of cancer.

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ANKITA JOSHI, M Sc, PDCR, ACPPM, MBA, PhD (Biotechnology; Thesis submitted).
She is currently working as a contractual scientist (PDF) in Institute of Biotechnology, G.B. Pant university of Agri. & Tech., Patwadangar. She has 5 books published and 13 research articles in national and international journal of repute. She is working on anti-cancer therapy using traditional herbs present in Himalayas and distilled cow urine of kumaon region. Her specialization is on animal cell culture, in which she has organized several training programme for young biotechnologist. One of her book, Nanotechnology for beginners is very popular among the students and researchers. She is the lfe time member and treasurer of Society of Immunology and Immunopathology (SIIP).

Prof. R.S Chauhan, BVSc&AH, MVSc, PhD, FNAVS, FSIIP, FIAVP, Diplomat ICVP, PDCR, ACPPM, OCTT, MBA.
During his tenure as academisian and scientist, he has written 62 books, most of are very popular among the students world over. He contributed 64 chapters in different books and published 185 research articles in various national and international journal of repute. Besides, he participated in 15 international and 57 national conferences/ symposia/ seminars and presented 147 papers in different occasions. He also popularizes scientific research among the common man by publishing 320 semi technical articles in various magazines. Based on this contribution and scientific achievements he has been awarded nationally and internationally.