In vitro antioxidant and cytotoxicity studies of *Curcuma amada* Roxb. (Mango ginger)

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Abstract: *Curcuma amada* Roxb, of *Zingiberaceae* is an important active spice used in traditional system of medicine. They also used in the manufacture of pickles, chutney, salad and jam. Phosphomolybdenam method was used to measure the total antioxidant capacity (TAC) of *C. amada* ethanolic rhizome extract (CAEREt). The free radical scavenging capacity of CAEREt was also carried out by ABTS and DPPH methods. The cytotoxicity effect of CAEREt was determined by Trypan blue dye exclusion method and MTT assay using two different cell lines such as Dalton Lyphoma Ascites (DLA) and Human Cervical Cancer Cell Line (HeLa). The results showed that the rhizome extract of *C. amada* rich in phytocompounds and have exhibited better antioxidant and cytotoxicity properties.

Keywords: *Curcuma amada*; Cytotoxicity; Free radical scavenging capacity; Dalton lymphoma ascites; Total antioxidant capacity

1. Introduction

Many human diseases, including accelerated ageing, cancer, cardiovascular disease, neurodegenerative disease and inflammation are associated to excessive amounts of free radicals. Free radicals are highly reactive oxygen compounds; causes oxidative damage to cellular macromolecules leads various chronic disorders (5). The antioxidants may protect our body from diseases and chronic disorders due to their free radical scavenging capacity (12). The bioactive compounds of plants have showed strong antioxidant activities, but the synthetic antioxidants possess some adverse effects. Now a day, more attentions are taken to searching natural antioxidants from medicinal and dietary plants to prevent oxidative damage (28). The antioxidants are scavenging reactive oxygen and nitrogen free radical species, metabolizing lipid peroxides to non-radical products, chelating metal ions to prevent formation of free radicals, etc. Plant foods like fruits, vegetables, greens and spices are rich in antioxidants provide health promoting activity and also represented as good chemo protective agent against various types of malignancies.

Natural sources play a vital role in cancer prevention and treatment. A considerable number of antitumor agents currently used in the medicine are of natural origin (7). Phytocompounds are known to be useful in the treatment of inflamed or ulcerated tissues and they have been shown the remarkable activity in cancer prevention and treatment (1). Phenolic compounds have also been investigated as antioxidants and expose a wide range spectrum of medicinal properties such as anticancer, anti-inflammatory and antidiabetes (14). Reactive oxygen species (ROS) and oxygen compounds generated through general metabolic pathways in the body are known to induce oxidative damages in proteins, fats, and DNA. Natural antioxidants like polyphenolic compounds from medicinal plants are effective in the scavenging of ROS by the action of their phenolic hydroxyl groups (11), and also act as strong electron donars (6). Antioxidants are not affected by the exposure of sunlight and are stable under severe oxygen stress (19).

In fact, there are many medicinal plants, which are being consumed traditionally for the prevention of cancer because of their antioxidant property. Recently spices have been very much attracted by the scientists to investigate their antitumor potential. *Curcuma amada* is an active spice, belongs to the family *Zingiberaceae*, popularly known as mango ginger. It is a perennial, rhizomatous aromatic herb, used in Siddha, Ayurveda and Unani system of medicine. They are used in food industry as pickles, preservatives, candies, sauces and curries salad. They are also used against scabies, lumbago, stomatitis, inflammation in the mouth and ear, gleets and ulcers on the male sex organs (8, 21). But there are no studies on antioxidant and cytotoxic activities of *C. amada* ethanolic rhizome extract (CAEREt). So the present study was aimed to investigate the antioxidant and cytotoxic properties of *C. amada* ethanolic rhizome extract.

2. Materials and Methods

2.1. Preparation of *C. amada* rhizome extracts

The rhizome of *C. amada* was collected from Kulumani Village, Tiruchirappalli District, Tamilnadu, India. It was shade dried at room temperature and powdered in a mechanical grinder. 100g of *C. amada* rhizome powder was soaked in water and ethanol separately and then kept it in electrical shaker for 48 hours. The aqueous and ethanolic extracts were filtered and the filtrates were poured into beaker and kept at 40^{o}C using hot air oven for complete evaporation of solvent. The brown and yellow residues were obtained and the residues were used for further studies.

2.2. Source of Chemicals

Trypan blue (TB), Ethylene diamine tetraacetic acid (EDTA), Dimethylsulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazil (DPPH), 2,2'-azino bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and phosphomolybdenam were purchased from Sigma Chemical Company, MO, St. Louis, USA. All other chemicals and reagents used in the present study were of analytical reagent grade with high purity and were purchased from Loba Chemie (P) Ltd., Mumbai, India, Nice Chemicals, Kerala, India, Fischer Chemicals, Chennai, India and E-Merck Chemicals (P) Ltd., Mumbai, India.

2.3. DLA and HeLa Cell Lines

DLA cell lines were obtained from Amala Cancer Institute, Thrissur, Kerala, India. They were maintained by intraperitoneal inoculation of $1X10^6$ cells/mouse. The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science (NCCS), Pune, India and grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37^{o}C, 5% CO_2, 95% air and 100% relative humidity.

2.4. Preliminary phytochemical analysis

Preliminary qualitative phytochemical analysis was carried out by standard procedures (3) for the identification of phytocompounds in *C. amada* aqueous rhizome extract (CAAREt) and *C. amada* ethanolic rhizome extract (CAEREt).

2.5. Phosphomolybdenam method

The total antioxidant capacity was determined by phosphomolybdenam method (17). Various concentrations of extracts (200-1000 µg/ml) were prepared and 0.2ml of the extract from this was added to 1.8ml of distilled water and 2 ml of phosphomolybdenam reagent. The mixture was incubated at 95^{o}C for 90 mints and then cooled at room temperature. The absorbance of all samples was measured at 695nm. Ascorbic acid was used as a standard and the antioxidant capacity was expressed as ascorbic acid equivalent per gram extract (µg/g of AE).

2.6. $ABTS^+$ method

Various concentrations (25-500 µg/ml) of extracts were prepared and 900µl of extract from this was added to 5ml of ABTS solution and mixed for 10 seconds. The absorbance was measured at 734 nm against distilled water blank. BHT was used as reference compound. Antioxidant activity was expressed based on the percentage of ABTS radical reduction (20).

2.7. $DPPH^+$ method

Different concentrations (25-500 µg/ml) of extracts were prepared and 2.0 ml of the extract from this was added to 0.5 ml of DPPH radical solution. The reaction mixture was vortexed for 10 seconds and then allowed to stand for 30 minutes at room temperature. After 30 minutes, the absorbance was recorded at 517 nm. The experiment was repeated three times and BHT was used as standard. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals (10).

% of activity = Control - Test/Control x 100

2.8. Trypan blue dye exclusion method

The procedure of Trypan blue dye exclusion method was approved by the institutional animal ethics committee. Aspirated tumor cells (DLA cell lines) from the peritoneal cavity of mice were added to the test tube containing phosphate buffer solution (PBS) which was then dipped in ice. Washed the cells with PBS and centrifuged 3 times. Cells were then suspended in 1ml of PBS. Two different concentrations of the CAEREt like 6 mg/ml and 12mg/ml were prepared. From this 10µl of the extract was added to 10µl of Trypan blue dye and 10µl of tumor cell line separately. After 12 hrs, 24hrs and 36hrs of incubation, the viability of tumor cells were measured and compared with Dalton Lymphoma Ascites cell line.

2.9. MTT Assay

The *in vitro* cytotoxicity analysis was performed by MTT assay (13). 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme of living cells, succinate dehydrogenase cleaves the tetrazolium ring and then converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^{o}C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The percentage (%) of cell inhibition was determined using the following formula.

% cell Inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between % of cell inhibition and Log_{10} concentration and IC_{50} was determined using Graph Pad Prism Software.

3. Results

3.1. Preliminary qualitative phytochemical analysis

The phytochemicals like reducing sugars, alkaloids, terpenoids, phenolic compounds and flavonoids were identified in both aqueous and ethanolic rhizome extract of *C. amada* (CAAREt and CAEREt). The phytocompouns such as steroids, tannins and sterols were absent in both extracts. CAEREt showed higher concentrations of phytocompounds than the CAAREt and based on this result, the CAEREt was selected for further studies. The results are represented in Table 1.

Table 1. Preliminary phytochemical screening of CAAREt and CAEREt.

Phytochemicals	CAAREt	CAEREt
Steroids	-	-
Tannins	-	-
Reducing sugar	+++	+++
Alkaloids	+	++
Phenolic compounds	+	++
Terpenoids	++	+++
Flavanoids	+++	+++
Volatile oil	++	+++
Sterols	-	-

CAAREt - *C. amada* aqueous rhizome extract, CAEREt - *C. amada* ethanolic rhizome extract

- Absent, + Present (+ trace quantity, ++ medium quantity, +++ high quantity)

3.2 .Total Antioxidant Capacity (TAC)

The total antioxidant capacity (TAC) was determined by phosphomolybdenam method using different concentration of CAEREt. The TAC was increased from lower to higher concentration of CAEREt. The TAC of different concentration of CAEREt like 200,400, 600, 800 and 1000 µg/ml were found as 119.26, 207.74, 310.48, 382.01 and 487.09 µg/g of ascorbic acid equivalents, respectively (Table 2).

Table 2. Total antioxidant capacity (TAC) of CAEREt by phosphomolybdenam method.

Concentration of CAEREt in µg/ml	TAC µg/g of ascorbic acid equivalents
200	119.26
400	207.74
600	310.48
800	382.01
1000	487.09

3.3. ABTS$^+$ and DPPH$^+$

The free radical scavenging capacity of CAEREt was found by both ABTS and DPPH methods and the percentage of free radical scavenging capacity is represented in Figure 1 and 2. In the ABTS absorption inhibition (%) study, different concentrations of CAEREt were used like 25, 50, 100, 200, 300, 400 and 500 µg/ml and the absorption inhibition were observed as 13.2, 25.6, 29.2, 50.8, 76.8, 89.2 and 99.6%, respectively. Among these seven concentrations, 500µg/ml extract had more absorption inhibition activity as 99.6 % than other concentrations. In DPPH method, the different concentrations like 25, 50, 100, 200, 300, 400 and 500 µg/ml of CAEREt showed different levels of radical scavenging activity like 0.4, 1.46, 6.4, 10.8, 22, 37 and 54 % of inhibition, respectively with the IC_{50} value of 475 µg/ml. ABTS radical cation decolonization assay showed quite similar results, when compared to those obtained in the DPPH reaction.

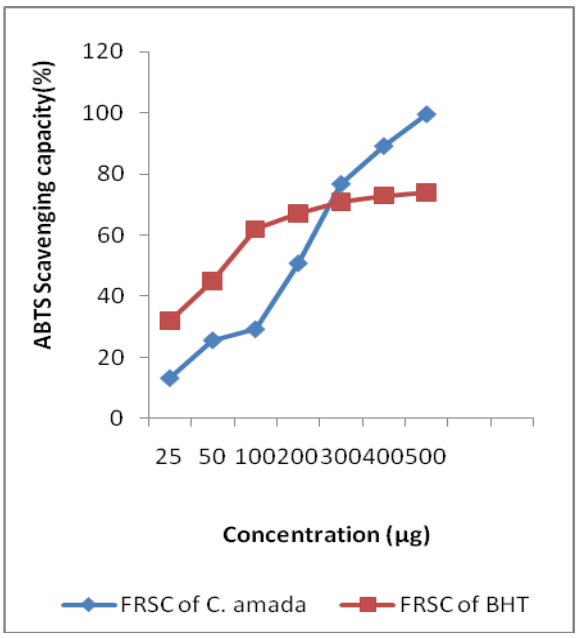

Figure 1. ABTS Scavenging capacity of CAEREt.

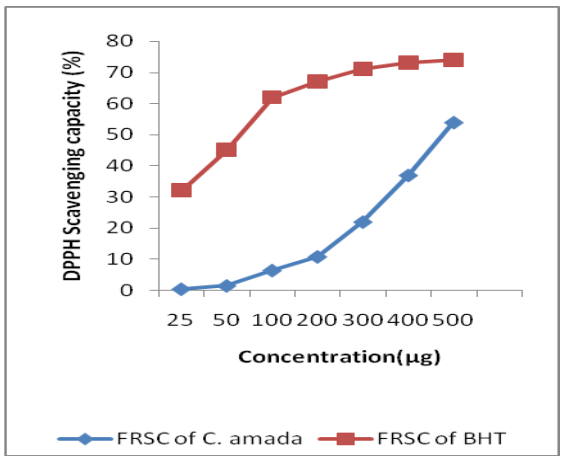

Figure 2. DPPH Scavenging capacity of CAEREt.

3.4. Trypan blue dye exclusion method

The *in vitro* cytotoxicity analysis was carried out by Trypan blue dye exclusion method using two different concentrations like 6 mg/ml and 12 mg/ml of CAEREt on DLA cell lines. The increased level of cell death was observed at 6 mg/ml than the 12 mg/ml of CAEREt. In this study, the results showed that the high concentration of CAEREt has less effect on DLA cell lines.

3.5. MTT assay

The cytotoxic effect of different concentrations like 18.75µg, 37.5µg, 75µg, 150µg, and 300µg of CAEREt was determined by MTT assay as 7.51, 24.49, 79.13, 99.86 and 100 % of cell inhibition, respectively using human cervical cancer cell lines (HeLa) and the results are represented in Figure 3 and Plate - 1 From the results IC_{50} value was calculated as 51.1µg/ml. The percentage of cell inhibition is gradually increased with increasing the concentrations of CAEREt and 100 % of cell inhibition was observed at 300µg of CAEREt.

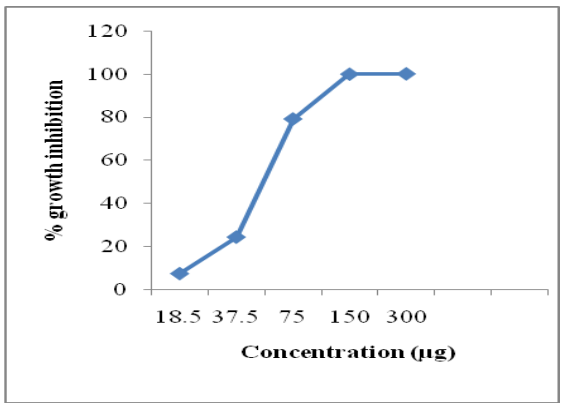

Figure 3. Effect of CAEREt on HeLa cell line by MTT Assay (*In vitro* cytotoxicity).

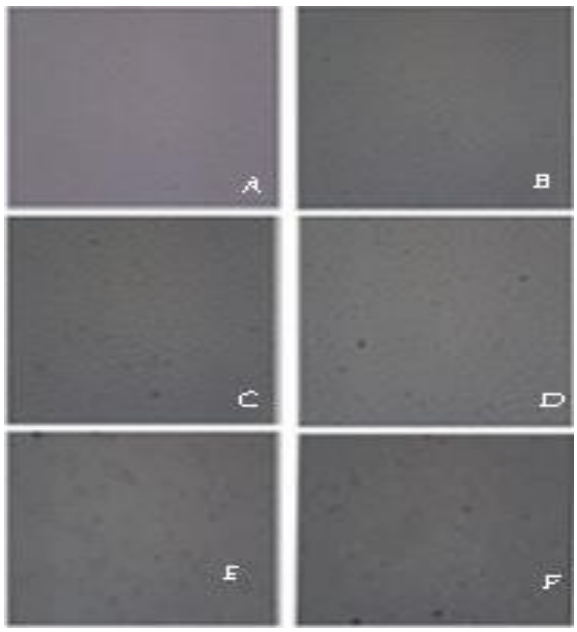

Plate 4: Effect of CAEREt on cell viability (Cytotoxic effect) by MTT assay using HeLa cell line.

A. HeLa Cell Line (control), B. HeLa + 18.5µg of CAEREt. C. HeLa +37.5µg CAEREt. D. HeLa +75µg of CAEREt. E. HeLa +150µg CAEREt. F. HeLa + 300µg CAEREt.

4. Discussion

Plants are rich in nutrients, vitamins, alkaloids, tannins, polyphenolic compounds and flavonoids. In the present study, we observed the presence of phytocompounds like alkaloids, terpenoids, phenolic compounds and flavonoids in the rhizome of *C. amada*. The antioxidant property of plants is mainly due to the presence of polyphenolic compounds and flavonoids. The antioxidant activity has been associated with many diseases including cancer. Spices are economically important and rich in phenolic compounds and flavonoids, which are easily absorbed by our body and do not have toxic effects (4). Flavonoids and phenolic compounds are the most important groups of secondary metabolites and bioactive compounds of plants (24). Flavonoid rich food has been advised to reduce neurodegenerative diseases (23). Many varieties of fruits are known to be rich in vitamins, minerals and antioxidants, which they consumed either for their nutrients or for their medicinal values (18).

C. amada is an important active spice and closely resemble to turmeric rich in many phytochemicals of therapeutic interest. The antioxidant activity of aqueous methanolic extract of leaves and rhizome of *C. amada* has been evaluated by β – carotene bleaching method (16). Similar results were observed in the present study and it confirmed the TAC of CAEREt, which may be due to the presence of antioxidants. It may react with the phosphate group of sodium phosphate to form green colored complex. The antioxidant activity of sequential extracts of *C. amada* was increased with increasing polarity of the solvents (15). The distribution of both free and bound form of phenolic acids like caffeic, ferulic, gentisic, gallic, cinnamic, procatechuic, syringic and p-

coumaric acids of *C. amada* were reported (22). The polyphenolic acids of various grains, fruits, vegetables and herbs have been reported and exhibits antioxidant and anticarcinogenic activities. So, the antioxidant property of CAERE t may be due to the presence of flavonoids and polyphenolic compounds.

The phytocompounds of fruits and vegetables possess medicinal and health promoting properties. It has been recognized that there is an inverse association between consumption of some fruits and vegetables and morbidity and mortality from degenerative diseases, which could be partly attributed to their antioxidants (2). The health promoting effects of antioxidants from natural sources have been widely used in food industry to extend the shelf life (27).

The FRSC of CAERE t was determined by both ABTS and DPPH methods and they have also been popular free radial scavenging methods for natural products. In these methods, a stable free radical such as ABTS and DPPH are formed and reduced to colorless compounds which have been used to investigate the FRSC of the plant extracts (9). The antioxidant agents are act as a hydrogen or electron donar and the reduction of free radical is mainly due to the antioxidants properties of flavonoids and phenolic compounds. Antioxidants from natural sources possessed wide range of biological activities such as prevention of ROS formation, scavenging of free radicals either directly or indirectly and modification of intracellular redox potential. Recently, the phenolic compounds including flavonoids in *W. somnifera* root and leaf extracts were reported (25) and their antioxidant activity may be responsible for the reduction of blood glucose level in alloxan-induced diabetic rats (26).

In vitro cytotoxicity of *C. amada* was investigated by both Tryphan blue dye exclusion method and MTT assay using DLA and HeLa cell lines, respectively. MTT assay is a quantitative and more sensitive test used to determine the cytotoxic effect of CAERE t. CAERE t showed the potent cytotoxic effect on HeLa cell line with the IC_{50} value of 51.1µg/ml. This may be due to the conversion of 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) into purple formazan by the cellular mitochondrial enzyme reductase of living cells. In Trypan blue dye exclusion method, the maximum cell death was observed at low concentration of CAERE t, which confirmed that the cytotoxic effect of CAERE t.

The present study results showed that the antioxidant and cytotoxic activities of CAERE t. The antioxidant effect of *C. amada* will be protected our body from oxidative damage, when taking this spice along with our daily food. Plant foods like fruits, vegetables and spices are rich in antioxidants and save our health from cancer and other deleterious effects. From this study, we concluded that the rhizome of *C. amada* possesses antioxidant and cytotoxic activities and there is also needed further study to exploit a novel anticancer drug from the rhizome of *C. amada*.

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