INVITRO CYTOTOXICITY, FREE RADICAL SCAVENGING AND ANTIOXIDANT ACTIVITY OF ETHANOL EXTRACT OF *Curcuma amada*.

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**ABSTRACT**

Medicinal plants have been identified and used throughout human history. They have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions, and to defend against attack from predators such as insects, fungi and herbivorous mammals. They also mediate their effects on the human body through processes identical to those already well understood for the chemical compounds in conventional drugs. Mango ginger (*Curcuma amada* Roxb.) is a unique spice having morphological resemblance with ginger but imparts a raw mango flavour. The major chemical components include starch, phenolic acids, volatile oils, curcuminoids and terpenoids like difurocumenono, amadannulen and amadaldehyde. The present study has been done to evaluate the free radical scavenging activity and to estimate the *invitro* antioxidant activities and cytotoxicity by MTT assay, Cell Viability Assay (Tryphan Blue Method) in the ethanol extract of rhizome of *Curcuma amada*. The results showed the rhizome’s ability to scavenge free radicals like Superoxide radical, Hydroxyl radical, Nitric oxide radical, DPPH radical and Lipid per oxidation inhibiting activity followed by Ferric reducing antioxidant power and Metal chelating activity and its cytotoxic activity at the concentration of 164.77µg/ml. From the results it was concluded that extract had good *invitro* cytotoxic activity, free radical scavenging ability and can be used as radical inhibitor or scavenger.

**Key Words :** *Curcuma amada, invitro antioxidants, free radicals, invitro cytotoxicity.*
INTRODUCTION

The use of herbs to treat disease is almost universal among non-industrialized societies, and is often more affordable than purchasing expensive modern pharmaceuticals. The World Health Organization (WHO) estimates that 80 percent of the populations of some Asian and African countries presently use herbal medicine for some aspect of primary health care (Fabricant and Farmsworth., 2001). Free radical generation is directly related with oxidation in foods and biological systems. Reactive oxygen species, an entire class of highly reactive molecules derived from the metabolism of oxygen, including superoxide radicals, hydroxyl radicals, and hydrogen peroxide, are often generated as by products of biological reactions or from exogenous factors. In vivo, some of these ROS play positive roles in cell physiology; however, they may also cause great damage to cell membranes and DNA, inducing oxidation that causes membrane lipid peroxidation, decreased membrane fluidity and DNA mutations leading to cancer, degenerative and other diseases (Mensor et al., 2001).

The antioxidant effect of plant products is mainly due to radical scavenging activity of phenolic compound such as flavonoids and terpenes (Rahman and Moon, 2007). Mammalian cells possess elaborate defence mechanisms of metabolic enzymes like superoxide dismutase (SOD), catalase (CAT) glutathione peroxidase (GPX) and nonenzymic molecules like thioredoxin and thiols which play important roles in antioxidant defence systems for radical detoxification. Some of the compounds are of an exogenous nature and are obtained from food, such as tocopherol, carotene, ascorbic acid and some micronutrient elements like zinc and selenium (Hasan et al., 2009). Antioxidant-based drugs/formulations for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer’s disease, and cancer have appeared during the last three decades (Hodzic et al., 2008). Several herbs and spices have been reported to exhibit antioxidant activity due to the presence of the active antioxidant compounds like flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans.

Cancer is the abnormal growth of cells in our bodies that can lead to death. Medicinal plants play an important role in cancer treatment and indeed, their chemical constituents and derivatives have been utilized for combating cancers over the last half-century (Jariya Alongkornsopit et al., 2011). HeLa cell, an immortal cell line used in scientific research is the oldest and most commonly used human cell line derived from cervical cancer cells taken from Henrietta Lacks, a patient who eventually died of her Cancer on October 4, 1951.
Cytotoxicity is the cell-killing property of a chemical compound (such as a food, cosmetic, or pharmaceutical) or a mediator cell (such as a cytotoxic T cell), independent from the mechanisms of death. Cells of the immune system [such as cytotoxic T cells, natural killer (NK) cells, and lymphokine activated (LAK) cells] can recognize and destroy damaged, infected and mutated target cells. Although the recognition machinery used by these cells is very different, their mechanism of target cell destruction may be very similar. Dead cells are unable to metabolize various tetrazolium salts. This allows the use of the colorimetric assays MTT to measure cell survival (Hans-Jurgen Rode, 2008).

Trypan blue, diazo dye, is a vital stain used to selectively color dead tissues or cells blue. Live cells or tissues with intact cell membranes are not colored. Since cells are very selective in the compounds that pass through the membrane, in a viable cell trypan blue are not absorbed; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue color under a microscope. Since live cells are excluded from staining, this staining method is also described as a dye exclusion method. Trypan blue is commonly used in microscopy (for cell counting) and in laboratory mice for assessment of tissue viability (Wainwright, 2010).

Mango ginger (*Curcuma amada* Roxb.) is a unique spice having morphological resemblance with ginger but imparts a raw mango flavour. It is botanically related to neither mango nor ginger, but to turmeric (*Curcuma longa*). Morphologically it is similar to turmeric, but has shorter crop duration of six months. The rhizomes are pale yellow inside with lighter colour outside, have sweet smell of unripe mango when crushed. (Yu-Wen Lin *et al*., 2008). It is an under-exploited spice crop, which grows luxuriantly in tropical soils with good drainage. The main use of mango ginger rhizome is in the manufacture of pickles and culinary preparations. Ayurveda and Unani medicinal systems have given much importance to mango ginger as an appetizer, alysteric, antipyretic, aphrodisiac, diuretic, emollient, expectorant and laxative and to cure biliousness, itching, skin diseases, bronchitis, asthma, hiccup and inflammation due to injuries (Policegoudra *et al*., 2011). The major chemical components include starch, phenolic acids, volatile oils, curcuminoids and terpenoids like difurocumenol, amadannulen and amadaldehyde (Parida and Das., 2004).

**MATERIALS AND METHODS**

**Collection and Preparation of Sample:** The fresh rhizomes of *Curcuma amada* were collected from local market, Coimbatore and were authenticated at Botanical Survey of India,
Tamilnadu Agricultural University, Coimbatore. The collected samples were cleaned to remove adhering dust and were cut into pieces and shade dried. Then the rhizomes were powdered and 5 gram was extracted in soxhlet apparatus using ethanol. The extract was then concentrated, vacuum dried and used for the study.

**Invitro Cytotoxicity**

**MTT assay**

**Chemicals:** 3-(4,5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco’s Modified Eagle’s Medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd., Mumbai, India.

**Cell lines and Culture medium:** HeLa cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO2 at 37 ºC until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

**Preparation of Test samples:** For cytotoxicity studies, weighed test drug was separately dissolved in DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

**Principle:** The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used (Francis and Rita, 1986).
Procedure: The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM containing 10% FBS. Later to each well of the 96 well microtitre plate, polymer were placed in triplicates, later 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 4 h, to each well 150µl of DMEM supplemented with 2% FBS was added to the wells. The plate was then incubated at 37 °C for 3 days in 5% CO2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and plate was washed with PBS and then media containing MTT (5 mg/ml) was added each well. The plates were gently shaken and incubated for 3 h at 37 °C in 5% CO2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The solubilized formazan was then transferred into clean plate and absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC50) values is generated from the dose-response curves for each cell line.

\[
\text{% of Growth Inhibition} = \frac{100 - \text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100
\]

Cell Viability Analysis (Trypan Blue Method) (Warren Strober, 2001)

Trypan Blue is a vital dye. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable.

Cell line and Culture  HeLa cell lines was obtained from National centre for cell sciences Pune (NCCS). The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 μg/ml CO2 at 37 °C.

Reagents : MEM was purchased from Hi Media Laboratories Fetal bovine serum (FBS) was purchased from Cistron laboratories, Trypsin, methylthiazolyl diphenyl- tetrazolium bromide (MTT), and Dimethyl sulfoxide (DMSO) were purchased from (Sisco research laboratory chemicals Mumbai). All of other chemicals and reagents were obtained from Sigma Aldrich Mumbai.
Procedure: Trypan Blue Staining of Cells

1. Place 0.5 ml of a suitable cell suspension HeLa cells (dilute cells in complete medium without serum to an approximate concentration of 1 x 10⁵ to 2 x 10⁵ cells per ml) in a screw cap test tube.
2. Add 0.1 ml of 0.4% Trypan Blue Stain. Mix thoroughly.
3. Allow to stand 5 min at 15 to 30°C (room temperature).
4. Fill a hemocytometer as for cell counting.
5. Under a microscope, observe if non-viable are stained and viable cells excluded the stain.

Free radical Scavenging Assays

Superoxide radical scavenging activity: The assay was based on the capacity of the sample to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system. Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, 12 mM EDTA, 0.1 mg NBT and various concentrations (100-500µg) of sample extracts. Reaction was started by illuminating the reaction mixture with sample extract for 90 seconds. Immediately after illumination the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture kept in dark served as blank. The percentage inhibition of superoxide anion generation was calculated as:

\[
\text{% inhibition} = \left( \frac{\text{control OD} - \text{sample OD}}{\text{control OD}} \right) \times 100
\]

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC₅₀) under the assay condition was calculated from the graph of inhibition percentage against sample concentration (Beauchamp and Fridovich, 1971).

Hydroxyl radical scavenging activity: The scavenging activity of the sample on hydroxyl radical was measured according to the method of Klein et al., 1991. Different concentrations of the extract (100-500µg) were added with 1 ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%), and 1 ml of dimethyl sulfoxide (DMSO) (0.85%V/V IN 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80-90⁰C for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1 ml of ice-cold TCA (17.5% W/V). Three millilitres of Nash reagent (75.0g of ammonium acetate, 3 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled
water) was added and left at room temperature for 15 min. The intensity of the colour formed was measured spectroscopically at 412 nm against reagent blank. The % hydroxyl radical scavenging activity was calculated as follows:

\[ \% \text{HRSA} = \left( \frac{\text{control OD} - \text{sample OD}}{\text{control OD}} \right) \times 100 \]

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC\textsubscript{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

**Nitric oxide radical scavenging activity:** (Srejayan and Rao 1997). 3 ml of 10 mM sodium nitroprusside in 0.2 M phosphate buffered saline (pH 7.4) was mixed with different concentrations (100-500 µg) of solvent extracts and incubated at room temperature for 150 min. After incubation time, 0.5 ml of Griess reagent (1% sulfanilamide, 0.1% naphthylenediaminedihydrochloride in 2% H\textsubscript{3}PO\textsubscript{4}) was added. The absorbance of the chromopore formed was read at 546 nm. Percentage radical scavenging activity of sample was calculated as follows:

\[ \% \text{NO radical scavenging activity} = \left( \frac{\text{control OD} - \text{sample OD}}{\text{control OD}} \right) \times 100 \]

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC\textsubscript{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

**DPPH radical scavenging activity:** The antioxidant activity of the sample was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to the method of Blois 1958. The sample extracts at various concentration (100-500 µg) was taken and the volume was adjusted to 100 µl with methanol. 5 ml of 0.1Mm methanol solution of DPPH was added and allowed to stand for 20 min at 27\degree c. The absorbance of the sample was measured at 517 nm. Percentage radical scavenging activity of the sample was measured at 517 nm. Percentage radical scavenging activity of the sample was calculated as follows:

\[ \% \text{DPPH radical scavenging activity} = \left( \frac{\text{control OD} - \text{sample OD}}{\text{control OD}} \right) \times 100 \]

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC\textsubscript{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration. Blois (1958)
Lipid peroxidation inhibiting activity: (Ohkawa et al 1979). Goat liver was washed thoroughly in cold phosphate buffer saline (pH 7.4) and homogenized to give a 10% homogenate. The homogenate was filtered and centrifuged at 10000 rpm for 10 min and the supernatant used to carry out the assay. To 0.5 ml of 10% homogenate, 0.5 ml of the sample (100-500µg) was added. To this, 0.05 ml of 0.07M ferrous sulphate was added incubated at room temperature for 30 min. To the incubated solution, 1.5 ml of 20% acetic acid (PH 3.5) and 1.5 ml of 0.8%TCA (in 1% SDS) were added. The tubes were incubated at 100°C for 1 hr and cooled to room temperature. About 5 ml of butanol was added and centrifuged at 3000 rpm for 10 min. The upper layer was used to read the absorbance at 532 nm. The percentage inhibition was calculated as follows:

\[
\text{% inhibition} = \left(\frac{\text{control OD} - \text{Sample OD}}{\text{Control OD}}\right) \times 100
\]

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC₅₀) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Ferric reducing antioxidant power assay: The FRAP assay was used to estimate the reducing capacity of the sample, according to the method of Benzie and Strain, 1996. The FRAP reagent contained 2.5 ml of a 10 mM TPTZ solution in 40 mM HCL, 2.5 ml of aqueous ferric chloride and 25 ml of 300 mM acetate buffer (pH 3.6). It was freshly prepared and warmed at 37°C. 900 µl FRAP reagent was mixed with 90 µl water and 10 µl of the extract. The reaction mixture was incubated at 37°C for 30 minutes and the absorbance was measured at 593 nm.

Metal chelating activity: The chelating activity of ferrous ions by the sample was estimated by the method of Dinis et al., 1994. 50µl of 2mM Ferric chloride was added to sample extracts. The reaction was initiated by the addition of 0.2 ml of 5mM ferrozine solution. The mixture was vigorously shaken ad left to stand at room temperature for 10 min. The absorbance of the solution was thereafter measured at 562nm. The analysis was performed in triplicate and the results were expressed as EDTA equivalent.

Invitro Antioxidant assays

Enzyme extract: The sample was homogenized in a pre chilled mortar and pestle with 50mM phosphate buffer at 1 - 4°C and centrifuged. Re-extracted the sediment with cold phosphate buffer, allowed to stand in the cold with occasional shaking and then repeated the
extraction once or twice. The combined supernatants were used for the assay. Fresh extract was used for the following enzymatic assays.

**Estimation of superoxide dismutase activity** (Beauchamp and Fridovich, 1971): The incubation medium contained a final volume of 3ml, 50mM potassium phosphate buffer (pH 7.8), 45μM Methionine, 5.3μM Riboflavin, 84μM Nitro Blue Tetrozolium (NBT), 20mM 0.1 mM EDTA and 0.1 mL of enzyme extract. The tubes were placed in aluminium Foil – lined box maintained at 25°C and equipped with 15W fluorescent lamps. Reduced NBT was measured spectrophotometrically at 600nm after exposure to light for 10 minutes. The 50% inhibition of the reaction between the riboflavin and NBT in the presence of methionine was taken as 1 unit of SOD activity. The enzyme activity was expressed as units/mg protein.

**Estimation of catalase activity** (Luck, 1974) 3ml of 0.1 mM H2O2 in 50 mM phosphate buffer was mixed with 0.1ml of enzyme extract. Noted the time it required for a decrease in absorbance from 0.45–0.4. This value was used for calculations. The rate of decomposition of hydrogen peroxide after the addition of the enzyme was noted at 240nm at an interval of 30 sec for 1 minute. The concentration of H2O2 was calculated using the extinction coefficient 0.036μ mole/ml.

**Estimation of Vitamin C** (Sadasivam and Manickam, 2009) 5ml of standard ascorbic acid solution was pipetted out into a conical flask and 10ml of 4% oxalic acid was added into it. The contents in the conical flask were titrated against the DIP dye solution. The end point was noted as V1. Vitamin C from the sample was extracted by making up 5ml of aqueous extract to 100ml with 4% oxalic acid. This was centrifuged to obtain a clear supernatant. 5ml of this supernatant was added to 10ml of 4% oxalic acid and titrated against DIP dye solution. The end point was noted as V2. The amount of ascorbic acid in the sample was calculated as follows:

Amount of ascorbic acid, mg/100g sample = (0.5/ V1)*(V2/5)* (100/Wt. of sample)*100.

**RESULTS**

Results in Table 1 show the highly significant superoxide radical scavenging activity of the rhizome extract when compared to Lipid per oxidation inhibiting activity. DPPH radical scavenging was found to be lower than the nitric oxide and hydroxyl radical scavenging activity of the extract. From Table 2, it was found that, the chelating activity for ferrous iron was found to be higher than the ferric reducing activity of the extract. The level of
antioxidants were observed as 4.81, 4.34 and 0.135 units for SOD, Catalase and Ascorbic acid respectively as shown in Table 3.

**Table 1: Free radical scavenging activity of ethanol extract of *Curcuma amada***

<table>
<thead>
<tr>
<th>Parameters (%)</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide radical scavenging activity</td>
<td>524.61 ± 10.21</td>
</tr>
<tr>
<td>Hydroxyl radical scavenging activity</td>
<td>174.72 ± 3.48</td>
</tr>
<tr>
<td>Nitric oxide radical scavenging activity</td>
<td>156.69 ± 0.08</td>
</tr>
<tr>
<td>DPPH radical scavenging activity</td>
<td>146.59 ± 0.07</td>
</tr>
<tr>
<td>Lipid Per oxidation inhibiting activity</td>
<td>40.84 ± 0.45</td>
</tr>
</tbody>
</table>

Values are means of three independent analyses of the sample ± standard deviation (n=3).

**Table 2: Metal chelating activity and FRAP assay of ethanol extract of *Curcuma amada***

<table>
<thead>
<tr>
<th>Parameters</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metal chelating activity (mg EDTA /g extract)</td>
<td>160.26 ± 1.55</td>
</tr>
<tr>
<td>FRAP mmol (Fe(II)/g extract)</td>
<td>3.47 ± 0.13</td>
</tr>
</tbody>
</table>

Values are means of three independent analyses of the sample ± standard deviation (n=3).

**Table 3: Level of *in vitro* antioxidants in the ethanol extract of *Curcuma amada* rhizome.**

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide Dismutase#</td>
<td>4.81 ± 0.28</td>
</tr>
<tr>
<td>Catalase</td>
<td>4.34 ± 0.37</td>
</tr>
<tr>
<td>Ascorbic Acid*</td>
<td>0.195 ± 0.48</td>
</tr>
</tbody>
</table>

Values are means of three independent analyses ± standard deviation (n = 3).

Units: # amount causes 50% reduction in the extent of NBT oxidation/g sample.
* amount of enzyme required to decrease the optical density by 0.05 units / g sample.
@ mg/ g sample.

**MTT assay:** Table 4 and Plate 1 show the percentage of cell inhibition for various concentration of ethanol extract of *Curcuma amada* rhizome against the HeLa cell lines in a dose dependent manner. 50% of the cytotoxicity was observed at the concentration of 164.77µg/ml.

**Cell viability Analysis (Trypan blue assay):** The Cell Viability for various concentration of ethanol extract of *Curcuma amada* against the HeLa cell lines in a dose dependent manner is shown in Table 5 and Figure 1. As the concentration of the extract increased, the percentage of cell viability decreased. This may be due to the presence of terpenoids in the ethanol extract of *Curcuma amada* rhizome that caused cytotoxicity to the cancer cells.
Plate 1: Cytotoxic property of ethanol extract of *Curcuma amada* rhizome against HeLa cell line by MTT ASSAY

Table 4: Percentage of cell inhibition for various concentration of ethanol extract of *Curcuma amada*.

<table>
<thead>
<tr>
<th>Test Concentration (µg/ml)</th>
<th>% Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.5</td>
<td>6.48±1.3</td>
</tr>
<tr>
<td>125</td>
<td>40.30±0.2</td>
</tr>
<tr>
<td>250</td>
<td>67.79±1.0</td>
</tr>
<tr>
<td>500</td>
<td>93.82±0.1</td>
</tr>
<tr>
<td>1000</td>
<td>94.19±0.2</td>
</tr>
</tbody>
</table>

Table 5: Cell Viability analysis of *Curcuma amada* on HeLa cell line

<table>
<thead>
<tr>
<th>S. No</th>
<th>Concentration (µg/ml)</th>
<th>Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1000</td>
<td>16.94</td>
</tr>
<tr>
<td>2</td>
<td>500</td>
<td>21.29</td>
</tr>
<tr>
<td>3</td>
<td>250</td>
<td>29.27</td>
</tr>
<tr>
<td>4</td>
<td>125</td>
<td>32.31</td>
</tr>
<tr>
<td>5</td>
<td>62.5</td>
<td>42.58</td>
</tr>
<tr>
<td>6</td>
<td>31.2</td>
<td>55.51</td>
</tr>
<tr>
<td>7</td>
<td>15.6</td>
<td>63.49</td>
</tr>
<tr>
<td>8</td>
<td>7.8</td>
<td>80.22</td>
</tr>
<tr>
<td>9</td>
<td>Cell control</td>
<td>100</td>
</tr>
</tbody>
</table>
DISCUSSION

The rhizome selected for the present study belongs to the family of Zingeberacea, which is composed of 70-80 species of rhizomatous annual herbs. In recent years, several reports have been published concerning the composition and the biological properties of these extracts (Nguefack et al., 2004). So, the present study has been done to evaluate antioxidant, free radical scavenging and invitro cytotoxic activities.

Free radical induced oxidative stress is the root cause for many human diseases. Naturally occurring antioxidant supplements from plants are vital to counter these oxidative damage in cells (Rao et al., 2010). When the generation of free radicals exceeds the scavenging capacity of the cell, the excess free radicals seek stability through electron pairing with biological macromolecules such as proteins, lipids, and DNA in healthy human cells resulting in induction of lipid peroxidation which leads to cancer, atherosclerosis, cardiovascular diseases, ageing and inflammatory diseases (Chotimarkon et al., 2008).

Superoxide anion plays an important role in formation of reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA. It is a highly toxic species, which is generated by numerous biological and photochemical reactions that are more detrimental due to their role as second messengers in fibroblast proliferation in inflammation and mediators of tissue destruction. The superoxide radical scavenging activity of the ethanol extract of Curcuma amada showed...
the maximum scavenging activity of 15.13% with IC50 value of 524.61 μg/ml. Similar studies were observed in the methanol extract of Curcuma amada that showed superoxide scavenging activity (Sivaprabha., 2011).

Hydroxyl radical species is considered as one of the quick initiators of the lipid oxidation process, abstracting hydrogen atoms from unsaturated fatty acids. Therefore, the removal of hydroxyl is probably one of the most effective defences of a living body against various diseases. The extract showed highest scavenging activity at 36.64% with IC50 of 174.72 μg/ml (Table 1). The result correlates with the results of (Sivaprabha., 2011) that the administration of methanol extract of Curcuma amada reversed the damage caused by H2O2.

Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological processes. However, its excess production is associated with several diseases, thereby producing the highly damaging peroxynitrite (Mondal et al., 2006). In the present study, the nitrite produced by the incubation of solutions of sodium nitroprusside in administration of ethanol extract of Curcuma amada reversed the damage caused by H2O2. Phosphate buffer was reduced by the ethanol extract of Curcuma amada with maximum activity of 46.96% with IC50 of 156.69 μg/ml. This may be due to the antioxidant principles in the extracts which compete with oxygen to react with nitric oxide and thus inhibit the generation of nitrite. Similar study was observed in methanol extract of leaves and rhizomes of Curcuma amada that efficiently inhibited the nitric oxide generation invitro followed by chloroform and aqueous extract (Sivaprabha., 2011).

DPPH test, which is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants, is a direct and reliable method for determining radical scavenging action. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517 nm and also for a visible deep purple colour. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. The maximum radical scavenging activity was observed in the extract at 146.59 that was similar to the methanol extract of both leaves and rhizomes that showed higher ability to scavenge DPPH followed by chloroform and aqueous extract (Sivaprabha., 2011).

Free radicals induce lipid peroxidation in polyunsaturated lipid rich areas like liver and brain. In this study, lipid peroxidation was induced invitro and the extract showed
concentration dependent prevention towards generation of lipid peroxides. The maximum capability of lipid per oxidation inhibiting activity of the extract was found to be highest at its concentration of 500μg with IC50 value as 40.84. Similar result was observed by (Policegoudra., 2010) that except water extract all other mango ginger extracts showed potential lipid per oxidation inhibitory activity.

The method of metal chelating activity is based on chelating property of Fe2+ ions by the reagent ferrozine which is a quantitative formation of a complex with Fe2+ ions (Halliwell et al., 1995). In the presence of other chelating agents, the complex formation is disrupted and as a result the red colour of the complex is decreased. Measurement of the rate of colour reduction therefore allows estimation of the chelating activity of the coexisting chelator. EDTA is used as the standard as it is known metal ion chelator. The Metal chelating activity and Ferric reducing antioxidants power of ethanol extract of Curcuma amada was found to be 160.26 ± 1.55 and 3.47 ± 0.13 respectively. These results were found to be similar to the results of (Vishnupriya et al., 2011) that shows the metal chelating activity of aqueous extract of Curcuma amada.

Antioxidants and their derivatives are of great importance in our daily life. These are compounds that are capable to trap free radicals (Singh Priya et al., 2010) or inhibit the oxidation of lipids or other bio molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Mansor Hakimen and Mahmood Maziah, 2009) before vital molecules are damaged. The level of antioxidants like Superoxide Dismutase (SOD), Catalase and Ascorbic acid (Vitamin C) in the fresh rhizome were observed as 4.81, 4.34 and 0.135 respectively (Table 3). This was similar to the study of (Policegoudra et al., 2011) who reported that high antioxidant activity and cytotoxicity activity of different Curcuma amada rhizome extracts can be correlated with their phenolic content and bioactive constituents like terpenoids which is known for their health beneficial properties. The mango ginger extracts can be used as natural source of phenolic and terpenoid compounds.

The cytotoxicity results indicate that the ethanol extract of Curcuma amada possesses anticancer activity as shown in Table 4 and Plate1(MTT Assay) and in Table 5and Figure1 (Tryphan Blue Assay) that may be due to the presence of terpenoid bioactive compounds (Chitra and Thoppil, 2002), that exhibit cytotoxicity against a variety of tumor cells and possess anticancer efficacy (Roslin and Anupam, 2011). This is similar to the study of (Policegoundra et al., 2010) that the Curcuma amada extracts showed potential platelet
aggregation inhibitory activity and cytotoxicity properties of the n-hexane, chloroform, ethyl acetate, methanol and water extracts against Vero cell (African green monkey kidney) and A-549 (human small cell lung carcinoma) cells by MTT assay.

The present study is similar to the study of (Policegoudra et al., 2008) who showed that the hexane, chloroform, ethyl acetate, acetone and methanol extracts of mango ginger showed the higher toxicity towards cancer cells

**CONCLUSION**

*Curcuma amada*, commonly known as mango ginger, is a perennial, rhizomatus, aromatic herb belonging to the family Zingeberaceae composed of 70-80 species of rhizomatous annual or perennial herbs. It appears to be highly potential and has an array of secondary metabolites like phenols, terpenoids, alkaloids and flavonoids. The biological activities of mango ginger include antioxidant activity, antibacterial activity, antifungal activity, anti-inflammatory activity, platelet aggregation inhibitory activity, cytotoxicity, antiallergic, hypotriglyceridemic activity, brine-shrimp lethal activity, enterokinase inhibitory activity, CNS depressant and analgesic activity. From the present study it was observed that the ethanol extract of rhizome possess good antioxidant activity and showed ability to scavenge free radicals like Superoxide radical, Hydroxyl radical, Nitric oxide radical, DPPH radical and Lipid per oxidation inhibiting activity followed by Ferric reducing antioxidant power assay and Metal chelating activity. Thus it is concluded that *Curcuma amada* ethanol extract have good free radical scavenging ability and can be used as radical inhibitor or scavenger, acting possibly as primary antioxidants. MTT assay showed the *invitro* cytotoxicity exhibited by the extract that may be due to presence of terpenoids. The study can be further extended that may lead to the development of active compounds responsible for mechanisms and pathways for antioxidant activity.

**CONFLICT OF INTEREST**

We declare that we have no conflict of interest.

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