Chapter 6

Cytotoxicity and anti-tumor activity of turmeric essential oil
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6.4. Discussion
6.1. Introduction

Cancer is one of the leading causes of death in the world. Chemotherapy, surgery and radiatiotherapy are the important treatment methods, even though their side effects are difficult to tolerate. Hence, more attention is necessary towards alternative treatment methods using natural products for the cancer treatment. Effective cancer chemotherapeutic drugs such as vinblastine, vincristine, camptothecin, docetaxel and paclitaxel that have their origin from the plant sources (Wall et al., 1966) and nowadays, there is significant impact of plant derived drugs for cancer treatment. Essential oils are concentrated volatile, aromatic, lipophilic compounds which are mainly monoterpenes and sesquiterpines. Three hundred different plant species are used for the production of essential oils, in addition to their use in food industry, which are rich sources of natural products with fewer side effects (Shoeb, 2006 and Sacchetti et al., 2005). The essential oils isolated from the spices have many bio pharmacological properties including antioxidant, anti-inflammatory and anticancer activities and hence became excellent research materials for the scientific community (Sacchetti et al., 2005).

Turmeric is commonly used to enhance the colour, aroma and flavor of food in most Asian countries. Although there are several studies on the oleoresin of turmeric and its active ingredient curcumin, less work has been reported on its essential oil. GC/MS analysis showed that the active ingredients in TEO are ar-turmerone and curlone which exhibit significant pharmacological and anticancer properties including antineoplastic activity (Won et al., 1992 and Kyong et al., 1993). TEO has been reported to reduce cytogenetic damage in patients suffering from oral submucousal fibrosis (Deepa et al., 2010). Clinical and histopathological evaluation in oral submucosal fibrosis (OSMF) patients after treatment with turmeric oil showed significant improvement when compared with untreated patients (Deepa et al., 2010). TEO showed antiproliferative activity against both human mouth epidermal carcinoma (KB) and murine leukemia (P388) cell lines (Manosroi et al., 2006). In the present study, we have conducted short term cytotoxic activity of TEO using DLA and EAC cancer cell lines. We have also evaluated the antiproliferative and apoptotic effect of TEO on various cell lines. Moreover, we have examined the DLA induced antitumour activity of TEO using ascites and solid tumour models in mice.
6.2. Materials and Methods

6.2.1. Preparation of turmeric essential oil (TEO)

To analysis cytotoxicity and MTT assay TEO was dissolved in DMSO (0.1%). For oral administration, TEO was dissolved in paraffin oil which was prepared fresh every time. (Chapter 2, section 2.1.1)

6.2.2. Animals

Young adult male Swiss albino mice (Average weight 20-25 g/6-8 weeks) were used for antitumor study. (Chapter 2, section 2.1.5)

6.2.3. Cell Lines

Dalton’s Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cells were initially procured from Adayar Cancer Institute, Chennai and propagated as transplantable tumors in the peritoneal cavity of Balb/C mice. L929 (mouse lung fibroblast), Vero cell lines (Normal African green monkey kidney cells), HepG2 (Human hepatocellular Liver Carcinoma cell lines) and HeLa (Cervical cancer cells) cell lines were obtained from National Centre for Cell Science (NCCS), Pune.

6.2.4. Cell viability assay

6.2.4.1. Maintenance of cells

Maintenance of cells was described in Chapter 2 Section 2.2.2.3.1 and 2.2.2.3.2.

6.2.4.2. Short term cytotoxic activity of TEO

Short term cytotoxic activity of different concentrations of TEO (10, 25 and 50 µg/ml) was done by determining the percentage of viability of Daltons Lymphoma Ascites (DLA) and Ehrlich’s Ascites Carcinoma (EAC) cells using trypan blue dye exclusion technique (Chapter 2 section 2.2.2.4.1).

6.2.4.3. Anti-proliferating activity of TEO using cell lines (MTT assay)

We used MTT assay to evaluate the anti-proliferating activity of TEO to different cell lines at different concentrations (1, 5, 10, 25, 50 and 100 µg/ml) (Cole, 1986;
Campling et al., 1991). L929 (mouse lung fibroblast), Vero cell lines (Normal African green monkey kidney cells), HepG2 (Human hepatocellular Liver Carcinoma cell lines) and HeLa (Cervical cancer cells) cell lines were used for this study (Chapter 2, section 2.2.2.4.2). After incubation the cells were observed under phase contrast microscope to find out the morphological characters of treated and untreated cells are photographed.

6.2.5. Morphological changes of HepG2 cells after treated with TEO and dual staining

HepG2 cells (1×10^6) were seeded in 6 well plates and treated with 50 μg/ml concentration of TEO. After 12 h incubation the cells were observed under phase contrast microscope to find out the morphological characters of treated and untreated cells are photographed.

To evaluate the apoptotic cell characters, the differential staining was done with acridine orange/ethidium bromide. Cells were washed with cold PBS and acridine orange/ethidium bromide (1mg/ml; 1:1 ratio) was added to the cell suspension at final concentration of 10 μg/ml and observed under fluorescence microscopy and photographed. Hoechst stain (5 μl) was added from 1mg/ml stock to cell suspension and incubated for 5 minutes to observe nuclear condensation.

6.2.6. Effect of TEO on apoptotic gene expression in HepG2 cell lines.

6.2.6.1. cDNA synthesis from the HepG2 cells.

HepG2 cells were treated with 50 μg/ml TEO for 24 h and the cDNA is synthesised using cDNA DirectTM kit (GeNei). The cells were harvested by adding enough trypsin to cover the mono layer of attached cells, incubated for 5 min at 37°C and added 2X volume of serum containing fresh medium. Spinded the cell suspension to remove the medium and washed with cold PBS. The cell density was adjusted to 5000 cells/μl. Then the 100 μl of the lysis buffer (provided by the kit manufacturer) is added to 2 μl of cell suspension and incubated at 75°C for 14 minutes. DNaseI (4 μl) was added to this mixture and incubated for 15 min at 37°C to remove the genomic DNA contamination followed by heating 75°C for 5 minutes. This cell lysate (5 μl) was treated with 2 μl of random hexamer provided and the total volume is made upto 10 μl with
nuclease free water. The suspension is incubated at 70°C for 4 min in a thermal cycler and transferred to ice for 2 min. The tube is centrifuged briefly to collect the condensation and added the following reagents, 8 µl of cDNA direct RT mix, 1 µl of RNase inhibitor and 1 µl of AMV RT enzyme. Mixed gently and incubated at 42°C for 60 min followed by 95°C for 10 min to inactivate reverse transcriptase enzyme and stored the cDNA at -20°C.

6.2.6.2. Apoptotic gene expression profile analysis using RT PCR


The PCR reactions are performed using SYBR Premix Ex Taq (Tli RNaseH Plus) from TaKaRa. PCR reaction mix (25 µl) contained 12.5 µl of 2x SYBR Premix (contains TaKaRa Ex Taq HS, dNTP Mixture, Mg2+, Tli RNase H, and SYBR Green I), 1 µl (0.2 µM) of both forward (F) and reverse primers (R), 0.5 µl of ROX Reference Dye (50 X), 5 µl of cDNA synthesized and total 25 µl was make up with distilled water. The amplification was done by following the thermal cycling sequence: PCR standard protocol.

Stage 1: 95°C for 30 sec.
Step 2: 95°C for 5 sec and 60°C for 34 sec (40 cycles).
Step 3: 95°C for 27 sec, 60°C for 1 min, 95°C for 15 sec and 4°C for 5 min.

Following thermal cycling for the relative quantification, the threshold was set to 0.20000 using the SDS software and CT values were calculated automatically.
**Data analysis**

The endogenous control used in the study was β-actin, which is a housekeeping gene (highly conserved and the expression won’t change). The apoptotic genes like Caspase 3, 8, 9 and P53 from both the untreated and treated were used as the target gene. The data from qRT-PCR consist of CT values for both these target and endogenous control genes irrespective of the untreated or treated cells. The target gene from the untreated control cells will act as the ‘calibrator’, from the treated cells is the ‘test’ and endogenous control β-actin is the reference gene.

1. Normalization of the target gene to the CT of the reference gene for both calibrator and test.
   \[ \Delta CT \text{ (Calibrator)} = CT \text{ (Target of calibrator)} - CT \text{ (reference gene)} \]
   \[ \Delta CT \text{ (test)} = CT \text{ (Target of test)} - CT \text{ (reference gene)} \]
2. Normalization ΔCT of test sample to the ΔCT of calibrator.
   \[ \Delta \Delta CT = \Delta CT \text{ (Test)} - \Delta CT \text{ (Calibrator)} \]
3. Calculation of fold difference
   \[ \text{Fold difference} = 2^{\Delta \Delta CT} \]

**6.2.7. Effect of TEO on the survival rate of ascites tumor bearing mice**

Swiss Albino mice (20-25 gm/6-8 weeks) were divided into six groups and each group contained six animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control (Treated with DLA cells alone),</td>
</tr>
<tr>
<td>II</td>
<td>Vehicle control (DLA cells + paraffin oil),</td>
</tr>
<tr>
<td>III</td>
<td>DLA cells + Cyclophosphamide 10 mg/kg body weight.</td>
</tr>
<tr>
<td>IV</td>
<td>DLA cells + TEO 100 mg/kg body weight.</td>
</tr>
<tr>
<td>V</td>
<td>DLA cells + TEO 500 mg/kg body weight.</td>
</tr>
<tr>
<td>VI</td>
<td>DLA cells + TEO 1000 mg/kg body weight.</td>
</tr>
</tbody>
</table>

Viable DLA cells (1 x 10⁶ cells) in 0.1 ml of PBS were injected into the peritoneal cavity of mice. Drugs were administered orally at different concentration for 10 days after tumor injection. Cyclophosphamide (10 mg/kg body weight) was used as standard. The death
pattern of animals due to tumor burden was noted and the percentage increase in life span calculated.

\[
\text{Percentage increase in life span} = \frac{T - C}{C} \times 100
\]

Where ‘T’ and ‘C’ are mean survival of treated and control mice respectively.

6.2.8. Effect of TEO on the solid tumour development in mice

Swiss albino mice (20-25 gm /6-8 weeks) weighing 25-30 g was divided into six groups of six animals each. DLA cells (1\times10^6 cells/animals) were injected subcutaneously on the right hind limb of mice to produce solid tumour.

Group I : Control (Treated with DLA cells alone),
Group II : Vehicle control (DLA cells + paraffin oil),
Group III : DLA cells + Cyclophosphamide 10 mg/kg body weight.
Group IV : DLA cells + TEO 100 mg/kg body weight.
Group V : DLA cells + TEO 500 mg/kg body weight.
Group VI : DLA cells + TEO 1000 mg/kg body weight

At 24 h, after tumour inoculation, different doses of the drugs were given and drug administration was continued for 10 consecutive days. Cyclophosphamide (10 mg/kg body weight) was used as standard drug. The diameter of the hind limb was measured using vernier calipers from the 7th day onwards every 3rd day up to 31st days. The tumour volume was calculated using the formula:

\[
V = \frac{4}{3} \pi r_1^2 r_2
\]

Where \(r_1\) was the minor diameter and \(r_2\) major diameter. The survival of the animals was recorded for up to 31st days.
6.3. Results

6.3.1. Short term cytotoxic activity of TEO

TEO showed significant cytotoxic activity in both DLA and EAC cell lines in a dose dependent manner (Fig. 6.1). The concentration of TEO required for 50% death of DLA cell lines (IC\textsubscript{50}) was found to be 8 µg/ml and 24 µg/ml for EAC cells (Table 6.1).

6.3.2. Anti-proliferating activity of TEO in cancer cell lines (MTT assay)

MTT assay indicated TEO exhibited dose-dependent decrease of cell proliferation in L929, HeLa, HepG2 cancer cell lines with IC\textsubscript{50} values of 32 µg/ml, 42.3 µg/ml, 53.7 µg/ml respectively (Table 6.1 and Fig..2). TEO exhibited weak cytotoxicity towards normal cell line (Vero cells) (IC\textsubscript{50} value > 100 µg/ml) (Fig.6 .3). Figure 6.3 shows morphological changes of cell lines L929, HeLa, HepG2 and Vero after treated with TEO using phase contrast microscopy.

6.3.3. Morphological changes of HepG2 cells after treated with TEO

Morphological analysis of TEO (50 µg/ml) treated and untreated HepG2 cells were done by different staining method. The cytoplasmic leakage, cytoplasmic granulation and cell shrinking were observed in TEO treated cells when compared with control (Fig. 6.4). Moreover the cell density was very much lesser when compared with untreated cells, which indicated the anti-proliferative activity of TEO. Apoptotic characters such as nuclear condensation and blebbing was also observed in treated cells (Fig.6.4).

In acridine orange and ethidium bromide staining, TEO treated cells showed more than 50% cells were in early apoptotic phase as indicated by the orange colour nucleus. Fluorescent red coloured nucleuses were observed in less than 10% cells indicating the necrotic cell death (Figure 6.4a). Apart from that, nuclear condensation was also seen in TEO treated HepG2 cells (Hoechst staining) (Fig. 6.4b).

6.3.4. Apoptotic gene expression profile

The gene expression is calculated by relative quantification using ΔΔCT method. A positive ΔΔCT indicates down-regulation and a negative ΔΔCT indicates up-
Table 6.1: Cytotoxicity and antiproliferative activity of TEO against normal and cancer cell lines (IC\textsubscript{50} values)

<table>
<thead>
<tr>
<th>No.</th>
<th>Type of cells</th>
<th>Cell lines</th>
<th>IC\textsubscript{50} values (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dalton’s Lymphoma Ascites\textsuperscript{1}</td>
<td>DLA</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>Ehrlich Ascites Carcinoma\textsuperscript{1}</td>
<td>EAC</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>Mouse lung fibroblast\textsuperscript{2}</td>
<td>L929</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>Cervical cancer cells\textsuperscript{2}</td>
<td>HeLa</td>
<td>42.3</td>
</tr>
<tr>
<td>5</td>
<td>Human hepatocellular Liver\textsuperscript{2}</td>
<td>HepG2</td>
<td>53.7</td>
</tr>
<tr>
<td>6</td>
<td>Normal cells\textsuperscript{2}</td>
<td>Vero</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Cytotoxicity assay

\textsuperscript{2} MTT assay
Fig 6.1. *In vitro* cytotoxic activity of turmeric essential oil (TEO) on DLA and EAC cell lines

Fig 6.2. Antiproliferative activity of turmeric essential oil (TEO) on L929, HeLa, HepG2 and Vero cell lines
Fig: 6.3. Phase contrast microscopic view of TEO essential oil treated L929, HeLa, HepG2 and Vero cell lines morphology.
Fig: 6.4. Morphology of HepG₂ cells treated with turmeric essential oil

A: Acridine orange/Ethidium bromide staining (Fluorescent microscopic view);
B: Hoechst staining (Fluorescent microscopic view);
C: Phase contrast microscopic view

Control | TEO (50 μg/ml) treated
Fig: .6.5. The apoptotic gene expression profile of HepG2 cells treated with TEO

The cDNA synthesised from the untreated and treated cells were amplified using specific primers of apoptotic gene. The gene expression is calculated by relative quantification using ΔΔCT method. A positive ΔΔCT indicates down regulation and a negative ΔΔCT indicates up-regulation.
Table 6.2: Effect of TEO on the survival rate of DLA induced ascites tumour bearing mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean survival days</th>
<th>Increase in life span (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16 ± 1.4</td>
<td>-</td>
</tr>
<tr>
<td>Paraffin oil</td>
<td>17 ± 1.2</td>
<td>-</td>
</tr>
<tr>
<td>Cyclophosphamide (5 mg/kg b.wt.)</td>
<td>26 ± 2.4</td>
<td>62.5</td>
</tr>
<tr>
<td>TEO 100 mg/kg</td>
<td>22 ± 2.7**</td>
<td>37.5</td>
</tr>
<tr>
<td>TEO 500 mg/kg</td>
<td>24 ± 3.3***</td>
<td>50</td>
</tr>
<tr>
<td>TEO 1000 mg/kg</td>
<td>25 ± 2.7***</td>
<td>56.25</td>
</tr>
</tbody>
</table>

TEO: turmeric essential oil.
DLA: Daltons Lymphoma Ascites
** P<0.01; **P<0.001; *p<0.05
Table 6.3: Effect of TEO on solid tumor in mice at 31st day

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tumor volume (cm²)</th>
<th>Percentage inhibition of solid tumor at 31st day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.61±0.33</td>
<td>-</td>
</tr>
<tr>
<td>Vehicle Control</td>
<td>3.75±0.35</td>
<td>-</td>
</tr>
<tr>
<td>Cyclophosphamide 10 mg/kg body weight</td>
<td>0.69±0.08</td>
<td>81.6</td>
</tr>
<tr>
<td>TEO 100 mg/kg body weight</td>
<td>1.92±0.19</td>
<td>48.8</td>
</tr>
<tr>
<td>TEO 500 mg/kg body weight</td>
<td>1.48±0.14</td>
<td>60.5</td>
</tr>
<tr>
<td>TEO 1000 mg/kg body weight</td>
<td>1.23±0.18</td>
<td>67.20</td>
</tr>
</tbody>
</table>
Fig: 6.6. Effect of turmeric essential oil (TEO) on solid tumour development
It is found that caspase 9, caspase 3 and P53 are up regulated. The Δ ΔCT values obtained is plotted as the bar diagram and shown in the figure 6.5. The extent of target gene expression in treated cells showed as fold difference from that of untreated. Caspase 9 and Caspase 3 genes are up-regulated significantly than Caspase 8.

The amplification plot from q-PCR shows that the expression of caspase 3, caspase 8, caspase 9 and p53 were up-regulated in TEO treated cells compared to the non-treated control cells. β-actin which is taken as the standard didn’t show a significant change in the amplification pattern in treated and untreated cells.

6.3.5. Effect of TEO on the survival of ascites tumor bearing animals

The mice with DLA induced ascites tumour survived for a period of 16.3 ± 1.4 days. The administration of standard drug cyclophosphamide (10 mg/kg body weight) increased in life span to 26 ± 2 days. Treatment with TEO at doses 100, 500 and 1000 mg/kg body weight increased average life span of animals to 22 ± 2, 24 ± 3 and 25 ± 3 days, respectively (Table 6.2).

6.3.6. Effect of TEO on the solid tumour development

The animals injected with DLA cancer cells alone (Vehicle control) produced solid tumour volume of 3.75 ± 0.35 cm³ on the 31th day. The standard cyclophosphamide (10 mg/kg body weight) treated mice showed the reduced tumour volume 0.69 ± 0.08 cm³ on the same day. TEO treated groups (100, 500 and 1000 mg/kg body weight) significantly decreased the tumour volume to 1.92 ± 0.19, 1.48 ± 0.14 and 1.23 ± 0.18 cm³ respectively (Fig. 6.6). Percentage reduction of solid tumour volume after oral administration of TEO 1000 mg/kg body weight was found to be 67.2% (Table 6.3).

6.4. Discussion

Many natural products are gifted with potent anticancer activity. Curcumin, from turmeric, is a well-known natural compound showing significant antioxidant and anticancer properties. Ar-turmerone, an active ingredient in turmeric essential oil (TEO) from turmeric, exhibits significant pharmacological activity and anticancer properties (Won et al., 1992 and Kyong et al., 1993). TEO exhibited significant free radical
scavenging activity and could also enhance the antioxidant enzymes levels in living system. Treatment with TEO showed significant improvement in cancer patients (Deepa et al., 2010) and it exhibited antiproliferative activity against cancer cell lines (Manosroi et al., 2006). In the present study, we have evaluated the cytotoxicity and antiproliferative potential of TEO using tumour cells, DLA and EAC as well as other selected cell lines such as L929, HeLa and HepG2. Moreover, in vivo antitumour studies were also conducted in mice using DLA cells.

Essential oils are easily permeable through cytoplasmic membrane and can induce cytotoxicity to cancer cells (Nguefack et al., 2004). TEO exhibited significant cytotoxicity to DLA and EAC cell lines. MTT assay revealed that TEO has no significant cytotoxic action on normal Vero cell line. The same assay, when performed on L929, HeLa and HepG2 cancer cell lines, showed significant morphological changes and cytotoxicity in a dose dependent manner. Moreover apoptotic characters such as nuclear condensation and blebbing were also observed in HepG2 cells.

Compounds exhibiting cytotoxicity towards cancer cells may also show antitumour activity in experimental animals (Ruby et al., 1995). Transplanted tumours in animal models are effective methods to investigate the antineoplastic effects of drugs. Dalton’s ascites lymphoma (DLA) is a poorly differentiated transplantable, malignant tumour which grows as both ascites and solid tumour in mice. It was found that oral administration of TEO significantly increased the life span of DLA induced ascites tumour bearing mice. Our study revealed that TEO decreased DLA induced solid tumour volume in mice model. Different mechanisms are responsible for the anticancer properties of essential oil. In cancer cells, there is no control of cellular proliferation. Elimination of damaged or malignant cells through cell cycle inhibition or induction of apoptosis is some of the mechanisms of cancer therapeutic agents (Srivasthava et al., 2006). Generally, when normal cells get damaged, it will undergo apoptosis. But in the case of cancer cells, mutations may have occurred that prevent cells from undergoing apoptosis. TEO exhibits an antiproliferative effect in HepG2 cells by inducing apoptosis. This growth inhibition is associated with caspase 3, 9 and p53 gene activation. It was therefore concluded that TEO induced apoptosis in HepG2 cells may be via an intrinsic pathway. As apoptosis is an important anti-cancer therapeutic target, these results suggest
a potential of TEO as a chemotherapeutic agent. Our study provide insight into the molecular mechanisms underlying TEO induced apoptosis in HepG2 cells that are worthy of further study. Reports suggest that ar-turmerone, the major compound present in TEO, can induce apoptosis and significantly reduce the proliferative activity of different cancer cells (Ji et al., 2004, Kim et al., 2013 and Cheng et al., 2012). Another report revealed that apoptotic effect of ar-turmerone on cancer cells involves caspase-3 activation through the induction of Bax and p53 (Lee, 2009).

In conclusion, TEO was found to have significant in vitro cytotoxic activity and antiproliferative potential against several cancer cell lines by apoptosis. Oral administration of TEO was found to significantly increase the life span of DLA induced ascites tumour bearing mice. Besides, significant reduction in solid tumour volume in mice also indicates that TEO is an effective anticancer agent. Thus, the in vivo and in vitro anticancer property of TEO demonstrated the potential of TEO to be developed as an effective antineoplastic agent in future.