Chapter - 9

IN VITRO CYTOTOXICITY ASSAY OF ZERUMBONE AND MDM\textsubscript{3:1}

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9.1 INTRODUCTION

Cytotoxicity assay using cell lines is a valuable tool in identifying compounds that might pose certain health risks in human. *In vitro* test systems represent first phase in the evaluation on the toxicity of a drug. Cell culture models that employ liver cells could be potent tools for predictive studies on the toxicity and metabolism of drugs in pharmaceutical industry (Saad et al., 2006). Chang liver cell line is a human cell line which expresses liver function markers such as albumin, uridine diphosphate glucuronosyltransferase and cytochrome P450 3A4. Hence cytotoxicity of purified compounds zerumbone and MDM$_{3:1}$ was tested by MTT assay using Chang liver cell lines.

Micro culture tetrazolium assay using 3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) is one of the best known method for studying cell viability. MTT assay measures the reduction of yellow 3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. MTT that enters the cells passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The formazan crystals are then solubilised with organic solvents such as dimethyl sulfoxide and read spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of
reduction and hence the formation of formazan crystals is a measure of the viability of the cells.

9.2 CHAPTER OBJECTIVE

- Determination of *in vitro* cytotoxicity of zerumbone and MDM\(_{3:1}\) against normal liver cell line

9.3 MATERIALS AND METHODS

9.3.1 Test materials

- Test materials- Zerumbone, MDM\(_{3:1}\)

- Chang normal liver cell line – purchased from NCCS, Pune

- Dulbecco’s Modified Eagle’s Media, Trypsin, Ethylene diamine tetraacetic acid and 3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) – purchased from HiMedia Laboratories, Mumbai

- Fetal bovine serum (FBS) – Invitrogen, India

9.3.2 Determination of *in vitro* cytotoxic effect on cultured Chang liver cell lines – MTT assay

Chang liver cell line was maintained in Dulbecco’s modified Eagle’s media supplemented with 10% FBS and grown to confluency at 37°C and 5% CO\(_2\) in a humidified atmosphere in a CO\(_2\) incubator (NBS, Eppendorf, Germany). The cells were trypsinized (0.025% Trypsin in PBS/ 0.5mM EDTA
solution) and were placed into 96 well tissue culture plates at a density of $1 \times 10^5$ cells/well and incubated (24 hours) to assure 70-80% confluency. Test materials were added to grown cells at a final concentration of 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL and 200 µg/mL and incubated for 24 hours.

The cells were then washed with 1x PBS and added fresh media along with 30 µL of MTT solution (MTT -5mg/mL dissolved in PBS). It was then incubated at 37°C for 3 hours. MTT was removed by washing with 1x PBS and 200 µL of DMSO was added to the culture. Incubation was done at room temperature for 30 minutes in order to dissolve the formazan crystals formed. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 minutes to precipitate cell debris. Optical density was read at 570 nm using DMSO as blank in a microplate reader (ELISASCAN, ERBA) (Arung et al., 2006, Al-Qubaisi et al., 2011). Percentage viability was calculated as follows,

$$\text{Percentage viability} = \left( \frac{OD \text{ of Test}}{OD \text{ of Control}} \right) \times 100$$

Control and tests were done in triplicate and the results are expressed as mean ±SD, where n=3 (Calculated using Microsoft Office Excel 2007). Doxorubicin (anti-cancer drug) was used as the reference drug (concentration range tested was 0.1 -0.6 µg/mL). IC$_{50}$ value was calculated by plotting percentage viability of cells against concentration of test materials.
9.4 RESULT

Effect of zerumbone, MDM<sub>3:1</sub> and doxorubicin on percentage viability of Chang liver cells at different concentrations is depicted in Figure 9.1 and IC<sub>50</sub> values are given in Table 9.1.

Figure 9.1 - Percentage viability of Chang liver cells in presence of zerumbone, MDM<sub>3:1</sub> and doxorubicin (concentration range tested for doxorubicin was 0.1 -0.6 µg/mL only)
zer – zerumbone, MDM – mikanolide – dihydromikanolide mixture, doxo - doxorubicin

The percentage viability of Chang liver cells was very high in presence of zerumbone and MDM<sub>3:1</sub> compared to the standard doxorubicin. At lowest tested concentration of 6.25 µg/mL, the percentage viability of Chang liver cells in presence of zerumbone and MDM<sub>3:1</sub> was 90.77±1.18 and 92.29±0.62 respectively. (At the lowest tested concentration 0.1 µg/mL of doxorubicin the
percentage viability was 56.66±1.52 only). Then there was slight decrease in viability with increase in concentration of both test materials. At the concentration of 12.5µg/mL the percentage viability was 82.35±0.64 and 82.69±0.54 respectively for zerumbone and MDM3:1. At higher concentrations (100µg and 200µg) MDM3:1 was found to be more cytotoxic than zerumbone.

Table 9.1
IC\textsubscript{50} of test materials against the viability of normal Chang liver cells

<table>
<thead>
<tr>
<th>Test materials</th>
<th>Zerumbone</th>
<th>MDM\textsubscript{3:1}</th>
<th>Doxorubicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC\textsubscript{50} in µg</td>
<td>150</td>
<td>121</td>
<td>0.31</td>
</tr>
</tbody>
</table>

MDM\textsubscript{3:1} – mikanolide – dihydromikanolide mixture

IC\textsubscript{50} of zerumbone and MDM\textsubscript{3:1} was very high compared to doxorubicin. This indicated comparatively low toxicity of the materials towards Chang liver cells.

9.5 DISCUSSION

The MTT assay provides a quantitative measurement of viable cells by determining the amount of formazan crystals produced by metabolically active cells. From the MTT assay, cytotoxicity of zerumbone and MDM\textsubscript{3:1} was to be very low compared to standard anticancer agent doxorubicin. IC\textsubscript{50} of zerumbone, MDM\textsubscript{3:1} and doxorubicin were 150,121 and 0.31 µg respectively.

Ibrahim \textit{et al}, (2010) have studied acute toxicity of zerumbone in Sprague Dawly rats. They have reported that median acute toxicity (LD\textsubscript{50}) of
In vitro Cytotoxicity Assay of Zerumbone and MDM

Zerumbone is 1.84 g/kg body weight. Ibrahim et al., (2010) have also reported that single doses of zerumbone at 100-200 mg/kg had no adverse effect towards the liver and renal tissues of Sprague Dawly rats. But they found that higher dose of zerumbone (500 mg/kg) induced hepatocellular and nephrocellular damage. Rahman et al., (2014) have also investigated acute toxicity of zerumbone loaded nanostructured lipid carrier on BALB/c mice model. The lipid carrier was used to improve the solubility of zerumbone and hence the bioavailability. They have reported that at oral doses of 100 and 200 mg/kg of zerumbone loaded nanostructured lipid carrier there was no sign of toxicity or mortality. In the present study the IC$_{50}$ of zerumbone against the viability of normal Chang liver cells was 150 µg. Zerumbone is an anti-cancer agent that can induce apoptosis (Wahab et al., 2009). The decrease in viability of cells might be due to this property.

In the present study MIC of zerumbone was 8µg/mL against *Epidermophyton floccosum* and *Microsporum canis* and 16µg/mL for *Microsporum gypseum* and *Trichophyton rubrum*. MFC of zerumbone was 64µg/mL against *Epidermophyton floccosum* and *Microsporum canis* and 128µg/mL for *Microsporum gypseum* and *Trichophyton rubrum*. From the MTT assay and earlier reports on acute toxicity study it can be concluded that zerumbone might not be hepatotoxic at these concentrations.
No previous reports are available on the hepatotoxic effect of mikanolide or dihydromikanolide. In the present study the percentage viability of MDM\textsubscript{3:1} was equal to that of zerumbone up to 50µg/mL. At higher concentrations the cytotoxic effect was slightly higher than zerumbone. This suggests that MDM\textsubscript{3:1} also might not be toxic to liver at lower concentrations tested. MIC of MDM\textsubscript{(3:1)} was 32, 16, 64 and 32 µg/mL for \textit{Epidermophyton floccosum}, \textit{Microsporum canis}, \textit{Microsporum gypseum} and \textit{Trichophyton rubrum} respectively. MFC of MDM\textsubscript{(3:1)} was 128 µg/mL for both \textit{Epidermophyton floccosum} and \textit{Microsporum canis}. MDM\textsubscript{(3:1)} was not fungicidal towards \textit{Microsporum gypseum} and \textit{Trichophyton rubrum} at the maximum tested concentration 256 µg/mL. Hence it can be concluded that at fungistatic concentration MDM\textsubscript{(3:1)} might not be hepatotoxic. But it may be hepatotoxic at concentration required for fungicidal activity.

However toxicity is not a serious matter for topical applications because of the minimum serum absorption (Gupta and Cooper, 2008). Treatment with topical preparations is the first choice in dermatophytosis therapy. Treatment with oral antifungal agent is usually required in Tinea unguium and extensive dermatophytosis only. Agents with antidermatophytic as well as anti-inflammatory activity are preferred for topical usage. Zerumbone and MDM\textsubscript{(3:1)} have both the activities and hence are very good candidates for preparing topical medications for dermatophytoses. MTT assay and earlier reports on
In Vitro Cytotoxicity Assay of Zerumbone and MDM

Acute toxicity study suggests that zerumbone might not be hepatotoxic at fungicidal concentration in oral use. However, determination of therapeutic index (ratio between lethal dose and pharmacologically effective dose – LD50/ED50) and clinical studies are required before confirmation. Earlier reports are not available on the toxicity study of mikanolide or dihydromikanolide or their mixture. MTT assay suggests that MDM (3:1) may not be toxic at fungistatic concentration in oral use. But further studies on toxicity are required using animal models.