CHAPTER IV

IN VITRO CYTOTOXIC ACTIVITY OF CRUDE PLANT EXTRACTS AGAINST ORAL CANCER CELL LINE

4.1 INTRODUCTION

Oral cancer is one of the common disorders throughout the world and it causes nearly 1,35,000, deaths annually and is associated with uncontrolled growth of cells [282]. In India, the incidence of oral cancer accounts for both men and women in the ratio of 2: 1 [283]. Currently the rate of oral cancer is high because of the late discovery and diagnosis, only in the later stages of cancer [284]. Oral cancer can be diagnosed when it is metastasized to the lymph node of the neck.

4.2 CAUSES OF ORAL CANCER

4.2.1 EPIGENETIC FACTORS

4.2.1.1 TOBACCO

Tobacco consumption is main cause of many cancers like oral, pharynx, renal, pancreas and larynx etc. The relationship of oral cancer with smoking was confirmed by many researchers [285]. The carcinogens present in tobacco were aromatic hydrocarbon benzpyrene and the tobacco specific nitrosamines and it binds with the DNA of keratinocyte cells [286,287].

4.2.1.2 BETEL QUID
Betel quid also called Paan are highly associated with pre-oral cancers and oral cancers [288]. Many researches had been carried out to prove that, betel quid is a causative agent of oral cancer [289].

4.2.1.3 ALCOHOL

The consumption of alcohol causes various types of cancer particularly in combination with tobacco smoking [290,291]. The independent role of alcohol in causing oral cancer still remains unclear. It was proposed that the permeability of oral mucosa has increased leading to the entry of carcinogens into oral cavity [292].

4.2.2 ENVIRONMENTAL FACTORS

4.2.2.1 VIRAL INFECTIONS

Viruses such as Herpes and Human Papilloma Virus (HPV) are strongly associated with oral cancer particularly in the oral squamous epithelium [293]. The association of EBV in oral cancer is unknown whereas it causes oral hairy leukoplakia in immune-compromised patients. The HPV is highly associated with oral carcinogens especially in squamous epithelium. The HPV16,18,31,33,35 and 39 are highly associated with oral cancer. The protein E6 and E7 produced by HPV bind and destroy the tumor suppressor genes p53 and Rb, disturb the cell cycle which causes the loss of control of DNA replication which further causes the loss in DNA repair and apoptosis [294].

4.2.2.2 FUNGAL INFECTIONS
The *Candida albicans*, a commensal in the oral cavity have been converted to an opportunistic pathogen during immuno suppression or systemic infection which leads to oral pre-malignant lesions particularly in the untreated conditions. The relationship between oral yeast and epithelial dysplasia have been proved by many researchers and the role of yeast is unknown [256].

### 4.2.2.3 IMMUNOSUPPRESSED CONDITIONS

The immune compromised patients are highly prone to develop oral cancers. The exposure to UV light causes lip cancer. The dental factors like poor oral hygiene and dental equipment also causes tongue cancer [295].

### 4.2.3 GENETIC FACTORS

The individual unable to metabolize the tobacco can cause DNA damage which leads to the genetic polymorphism in p450 and XME enzymes and leads to head and neck cancers [296].

### 4.2.4 MTT ASSAY

There are many *in vitro* and *in vivo* assays used for evaluating the anticancer activity of a compound. The common *in vitro* methods used were tryphan blue dye exclusion assay, XTT assay, MTT assay, Lactic dehydrogenase assay and Sulforhodamine B. Among these MTT and Sulforhodamine B were the most commonly used assays.
The MTT assay is based on the principle of conversion of purple formazan crystals from yellow tetrazolium salt by mitochondrial dehydrogenase of active cells which reveals the active cell cycle in the living organisms [297].

4.3 MATERIALS AND METHODS

4.3.1 CELL LINE

The mouth cancer KB cell line was obtained from NCCS, Pune, India. The obtained oral cancer cell lines (KB) were cultivated in Eagles minimum essential medium which contains fetal bovine serum (FBS) in 10% concentration. The cell lines were maintained in 5% CO\textsubscript{2} at 37\textdegree C in humidified environment. The passaging of cell lines were performed once in a week and the media was changed two times per week.

4.3.2 CELL TREATMENT

The KB cells in the monolayer were detached with EDTA and the viable cells were counted using a hemocytometer. The cells were prepared in the concentration of 1x10\textsuperscript{5} per ml by diluting with the medium containing 5% phosphate buffered saline (PBS). Hundred micro litres of the cell suspension were added in each well of 96-well plates containing 10,000 cells per well. The plate was then incubated at 37\textdegree C with 5% CO\textsubscript{2} in a humidified environment for 24 hrs for cell attachment.

4.3.3 PREPARATION OF PLANT EXTRACTS
The prepared samples were dissolved and diluted in DMSO and serum free medium respectively. It was again serially diluted four times to make five different concentrations.

After the incubation period, the attached cells were treated with 100 µl of five different concentrations of the six selected medicinal plant extracts. The plates were then incubated for another 48 hours at the above mentioned incubation conditions. The wells with medium and without plant extracts were served as a control. All the tests were performed in triplicates.

### 4.3.4 IN VITRO CYTOTOXICITY THROUGH MTT ASSAY

MTT, a yellow colored tetrazolium salt. The succinate dehydrogenase enzyme present in the mitochondria of living cells converts the MTT in to a formazan which is insolubla and purple in color. The formazan produced by cleaving the tetrazolium ring is directly proportional to the total number of living cells.

Fifteen microliters of MTT from the stock solution of 5mg/ml was added to the well plate and incubated for 4 hrs at room temperature. After incubation, the medium was decanted and the formazan formed were dissolved in DMSO and the absorbance was measured at 570 nm [297, 298].

The cell inhibition was determined by

\[
\text{percentage of cell inhibition} = 100 - \frac{\text{Abs(Sample)}}{\text{Abs(Control)}} \times 100
\]
4.4 RESULT AND DISCUSSION

The rates of growth inhibition for the selected medicinal plants were shown in the table 20 and figure 10. All the selected plants showed a dose dependent cell inhibition. The maximum inhibition were found in *M. chamomilla* (98%) followed by *E. alba* (96 %) and *A. racemosus* (73%) at 300 µg/ml concentration. The least activity expressed by *M. koenigii* (25.01%) followed by *M. alba*(33.04%). Moderate activity was observed in *G. glabra*(71.1%).

Table 4.1: Percentage of cell growth inhibition of the selected medicinal plants on KB cell line

<table>
<thead>
<tr>
<th>PLANTS</th>
<th>G. glabra</th>
<th>M. chamomilla</th>
<th>E. alba</th>
<th>M. alba</th>
<th>A. racemosus</th>
<th>M. koenigii</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONCENTRATION (µg/ml)</td>
<td>18.75</td>
<td>0.13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>37.5</td>
<td>0.8</td>
<td>1.5</td>
<td>0.04</td>
<td>0</td>
<td>1.68</td>
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<tr>
<td></td>
<td>75</td>
<td>5.29</td>
<td>15.1</td>
<td>13.4</td>
<td>1.23</td>
<td>10.08</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>33.72</td>
<td>45</td>
<td>41.07</td>
<td>12.6</td>
<td>24.41</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>71.1</td>
<td>98</td>
<td>96</td>
<td>33.04</td>
<td>73</td>
</tr>
</tbody>
</table>
The KB cell lines were treated with the five concentrations of the selected medicinal plants and its morphological observations were carried out by light microscopy. The cell death was visualized clearly in *M. chamomilla, E. alba* and *A. racemosus* at higher concentrations. The control cells showed a clear spherical morphology whereas the cell shapes were disturbed in the treated cell line which clearly indicates the cell death.
Figure 4.1: Cytotoxicity of G. glabra on oral cancer KB cell line
Figure 4.2: Cytotoxicity of *M. chamomilla* on oral cancer KB cell line
Figure 4.3: Cytotoxicity of *E. alba* on oral cancer KB cell line
Figure 4.4: Cytotoxicity of *M. alba* on oral cancer KB cell line
Figure 4.5: Cytotoxicity of *A. racemosus* on oral cancer KB cell line
G. glabra showed 71.1% cell inhibition at 300 µg/ml concentrations and the morphological damage were observed in the
KB cells treated with *G. glabra* extract. The anticancer activities of *G. glabra* for many cancers have been reported. Flavonoids from this plant possess antioxidant, antiulcer and anticancer activities. Licochalcone, a phenolic compound isolated from the roots of *G. glabra* were effective against stomach, liver, kidney, lung, breast and colon cancers [299].

*M. chamomilla* showed the highest percentage of cell death in a dose dependent manner. Akram Ranjbar et al 2014, represented the *in vivo* anticancer activity of *M. chamomilla* against lung cancer at the concentration of 50 mg/kg body weight [300].

The anticancer activity of *E. alba* was first reported by Lira prapamongko et al, (2008) [301]. It was also reported to be effective against the cancer cell lines of liver, kidney and glioma[302].

Shatavarins, an anticancer compound from *A. racemosus* were found to be active against breast, kidney and colon cancers [303].

Eventhough the anticancer activities of selected medicinal plants were studied by many researchers, the activity against oral cancer is still to be determined. Among the six selected plants, *M. chamomilla* showed the highest percentage of cell inhibition in *in vitro* conditions. Hence *M. chamomilla* was selected for the further analysis in the *in vivo* conditions.
4.5 CONCLUSION

All the selected medicinal plants showed the significant anticancer activity against the KB oral cancer cell lines. The maximum activity was found in *M. chamomilla* followed by *E. alba*. 