CHAPTER III

ANTI-CANCER ACTIVITY OF THE ISOLATED FLAVONOID GLYCOSIDES
INTRODUCTION

Cancer is a growing public health problem whose estimated worldwide new incidence is about 6 million cases per year. It is the second major cause of deaths after cardiovascular diseases. Cancer is a general term applied for a series of malignant diseases that may affect different parts of the body. These diseases are characterized by a rapid and uncontrolled growth of cells, which may mass together to form a growth or tumour, or proliferate throughout the body, initiating abnormal growth at other sites. If the process is not arrested, it may progress until it causes the death of the organism. These cells are born due to imbalance in the body and by correcting this imbalance the cancer many be treated.\textsuperscript{376}

Causes of cancer

Modern medicine attributes most cases of cancer to changes in DNA that reduce or eliminate the normal controls over cellular growth, maturation, and programmed cell death. These changes are more likely to occur in people with certain genetic backgrounds (as illustrated by the finding of genes associated with some cases of cancer and familial prevalence of certain cancers) and in persons infected by chronic viruses (e.g. viral hepatitis may lead to liver cancer; HIV may lead to lymphoma). The ultimate cause, regardless of genetic propensity or viruses that may influence the risk of the cancer, is often exposure to carcinogenic chemicals (including those found in nature) and/or to radiation (including natural cosmic and earthly radiation), coupled with a failure of the immune system to eliminate the cancer cells at an early stage in their multiplication. The immunological weakness might arise
years after the exposure to chemicals or radiation. Other factors such as tobacco smoking, alcohol consumption, excess use of caffeine and other drugs, sunshine, infections from such oncogenic virus like cervical Papilloma viruses, Adenoviruses, Karposis sarcoma (HSV) or exposure to asbestos.

These obviously are implicated as casual agents of malignant cancers. However a large population of people is often exposed to these agents. Consequently, cancer cells continue to divide even in situations in which normal cells will usually wait for a special chemical transduction signal. The tumor cells would ignore such stop signals that are sent out by adjacent tissues. A cancer cell also has the characteristic of immortality even in vitro whereas normal cells stop dividing after 50–70 generations and undergoes a programmed cell death (apoptosis). Cancer cells continue to grow invading nearby tissues and metastasizing to distant parts of the body. Metastasis is the most lethal aspect of carcinogenesis.

**TYPES OF CANCER\(^{377}\)**

1. **Cancers of blood and lymphatic systems:**
   a) Hodgkin’s disease; b) Leukemias; c) Lymphomas;
   d) Multiple myeloma; e) Waldenstrom’s disease

2. **Skin cancers:**
   a) Malignant melanoma

3. **Cancers of digestive systems:**
   a) Esophageal cancer; b) Stomach cancer;
   c) Cancer of the pancreas; d) Liver cancer;
   e) Colon and rectal cancer; f) Anal cancer
4. Cancers of urinary system:
   a) Kidney cancer; b) Bladder cancer; c) Testis cancer; 
d) Prostate cancer

5. Cancers in women:
   a) Breast cancer; b) Ovarian cancer; c) Gynecological cancer; 
d) Choriocarcinoma

6. Miscellaneous cancers:
   a) Brain cancer; b) Bone cancer; c) Carcinoid cancer; 
d) Nasopharyngeal cancer; e) Retroperitoneal cancer; 
f) Soft tissue cancer; g) Thyroid cancer

Objective

The purpose of this study was to assess the cell growth inhibition property of the flavonoid glycosides G1, G2, G3, G4, G5, and G6 in HeLa human cancer cell lines.

MATERIALS AND METHODS

1. CO₂ incubator-Thermo Fisher, USA
2. Multimode microplate reader-BioTek, USA
3. Refrigerated centrifuge-Eppendorf Germany
5. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide))
6. Fetal bovine serum
7. Trypsin
8. Penicillin
9. RPMI1640 medium
10. DMSO
Media preparation (Sigma)

The Sachest 12.0g was dissolved in 800ML of sterile distilled water to which 2.5g of sodium bicarbonate was added. The beaker was covered with aluminum foil and stirred using magnetic stirrer for 10 minutes. The medium pH was adjusted to 7.2 using 0.1M NaOH. The volume of the medium was made to 1000ML and filtered through sterile 0.2μ membrane filter unit. The medium quality control was checked by incubating 5ML of filtered medium in the CO₂ incubator for 2 days. The antibiotics and serum was added before it was used for cell culture.

Cell culture and MTT assay Procedure

The HeLa human cancer cell line was purchased from NCCS Pune. The cells were grown in a RPMI1640 medium supplemented with 10% fetal bovine serum and antibiotics as mentioned earlier. Cell proliferation (MTT) assay was performed and percentage of cell viability was determined by spectrophotometric determination of accumulated formazan derivative in treated cells at 570nm in comparison with the untreated ones.

For the MTT assay, the cells were grown in 25cm×25cm×25cm tissue culture flasks containing RPMI1640 medium as culture medium supplemented with 10% FCS, 100U/ml penicillin, 100µg/ml streptomycin (GIBCO) and grown at 37°C under a humidified atmosphere of 95% air and 5% CO₂. Cells were regularly passaged and maintained before including for the experiment.
When a cell density in a culture flask reached 70–80% confluence, they were trypsinized and seeded in 96-well plates at varying cell number according to the size and shape of the HeLa were seeded in the density of 3500 cells per well in 100µl and incubated for 24 hours at CO₂ incubator.

The glycosides G1, G2, G3, G4, G5, and G6 were prepared as 1mg/ml stocks by adding directly in to the DMEM medium. The working stock of 2X (200mg, 20, 2, 0.2 and 125µg) concentration to the cell in 100µl volume and the final concentration range were: 100, 10, 1, 0.1, and 0.01µg/ml. Since the compounds are soluble in medium, the compounds are weighed an appropriate amount and dissolved directly in to the L15 media and further dilutions were in the media. 100µl of stock was added to the cell. The plates were further incubated for 48 hrs in the CO₂ incubator.

MTT solution was composed of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) at 5mg/ml in phosphate buffered saline (1.5mM KH₂PO₄, 6.5mM Na₂HPO₄, 137mM NaCl, 2.7mM KCl; pH 7.4). From this solution 50µl was pipette out into each well to achieve 1g/mL as final concentration. The plate was further incubated for 2.30 hours in incubator and the medium was carefully decanted. The formazan crystals were air dried in dark place and dissolved in 100µl DMSO and the plates were mildly shaked at room temperature and the OD was measured using Synergy H4 microplate reader at 570nm.

From the optical densities the percentage growths were calculated using the following formula:
Percentage growth = 100×[(T-To)/(C-To)]

if T is greater than or equal to To, and if T is less than To,
Percentage growth = 100×[(T-To)/To],

Where T is the optical density of test,
C is the optical density of control,
To is the optical density at time zero.

From the percentage growths a dose response curve was generated and GI$_{50}$ values were interpolated from the growth curves.

**Cell Imaging**

At the end of 48 hours time point, the images were captured before adding the MTT. Different concentration treated cells were observed under microscope for cell morphology analysis and images of each concentration was captured and recorded.

**RESULTS**

**Cell growth inhibition property**

Among the flavonoid glycosides, G2 exhibited moderate inhibition in HeLa cell lines with GI$_{50}$ of 33.6µg. The other flavonoid glycosides does not show cell growth inhibition. The flavonoid glycosides of each concentration was performed in quadruplicate and cumulative variation were maintained less than 20% between the data points. Three set of cell lines were tested in a 96-well plate as described in the below 96-well format.

The results and raw data have been illustrated in Tables 3.1 and 3.2.
### TABLE – 3.1

Percentage growth of HeLa against the flavonoid glycosides

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Percentage Growth</th>
<th>Growth Inhibition in µg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100µg</td>
<td>10µg</td>
</tr>
<tr>
<td>G1</td>
<td>99</td>
<td>95</td>
</tr>
<tr>
<td>G2</td>
<td>20</td>
<td>83</td>
</tr>
<tr>
<td>G3</td>
<td>101</td>
<td>102</td>
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<tr>
<td>G4</td>
<td>69</td>
<td>105</td>
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<td>G5</td>
<td>47</td>
<td>94</td>
</tr>
<tr>
<td>G6</td>
<td>89</td>
<td>105</td>
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<table>
<thead>
<tr>
<th></th>
<th>1µg</th>
<th>0.1µg</th>
<th>0.01µg</th>
<th>GI50</th>
<th>TG1</th>
<th>LC50</th>
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</thead>
<tbody>
<tr>
<td>G1</td>
<td>104</td>
<td>99</td>
<td>100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>G2</td>
<td>110</td>
<td>103</td>
<td>33.6</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>G3</td>
<td>99</td>
<td>97</td>
<td>96</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>G4</td>
<td>109</td>
<td>104</td>
<td></td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>G5</td>
<td>108</td>
<td>107</td>
<td>86.0</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>G6</td>
<td>110</td>
<td>99</td>
<td></td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

### TABLE – 3.2

GI50 value of compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>GI50 (µg)</th>
<th>Hela</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>33.6</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>G5</td>
<td>86.0</td>
<td></td>
</tr>
<tr>
<td>G6</td>
<td>&gt;100</td>
<td></td>
</tr>
</tbody>
</table>

- **G1** --- 7-methoxy quercetin-3-O-glucuronide
- **G2** --- 6-acetoxy-4'hydroxy isoflavone-7-O-rhamnopyranoside
- **G3** --- Kaempferol 3-O-β-D (4" coumaroyl) rhamnoside
- **G4** --- Eriodictyol 7-O-β-D (6"malonyl) neohesperidoside
- **G5** --- quercetin-3-O-(6"acetyl)-galactosyl glucoside
- **G6** --- 4'-methoxy isoflavone 7-O-rhamnopyranoside
Graph 3.1. Percentage growth curve of HeLa against the flavonoid glycosides
Fig. 3.1. HeLa cells treated with the flavonoid glycoside G1 for 48 hours.

G1-treated HeLa Cells
Fig. 3.2. HeLa cells treated with the flavonoid glycoside G2 for 48 hours.
Fig. 3.3. HeLa cells treated with the flavonoid glycoside G3 for 48 hours.

G3-treated HeLa Cells

100 µg

10 µg

1 µg

0.1 µg

0.01 µg

Vehicle Control
Fig. 3.4. HeLa cells treated with the flavonoid glycoside G4 for 48 hours.

G4-treated HeLa Cells

100 ng

10 ng

1 ng

0.1 ng

0.01 ng

Vehicle Control
Fig. 3.5. HeLa cells treated with the flavonoid glycoside G5 for 48 hours.

G5-treated HeLa Cells
Fig. 3.6. HeLa cells treated with the flavonoid glycoside G6 for 48 hours.