Chapter - VII

Immunological mechanism of action of Polyphenolic compounds in mice
7.1 INTRODUCTION

Immunocompetence of the host is one of the major defences against cancer development. Tumours are probably outgrowths of transformed cells that have successfully escaped destruction by the immune system. Immunotherapy usually involves the use of immunomodulating agents with the aim of general stimulation or suppression of the activity of the stimulated cells towards particular antigen.

In the present chapter, the immunological mechanism of action of some polyphenolic compounds, which possess antimetastatic activity are studied. The effect of these compounds on antibody production, antibody producing cells and macrophage activation were screened.

7.2 MATERIALS AND METHODS
7.2.1 Test Compounds

The polyphenolic compounds curcumin, catechin, rutin and genistein were used for the present study.

7.2.2 Animals

Inbred BALB/c mice, and C57BL/6 mice, 6-8 weeks old (female, 20-25 g body weight) were used for the experiments. L-929 cells were maintained in MEM (2.2.3b). Fresh sheep blood was collected from the slaughter house and processed (2.2.26).

7.2.3 Determination of circulating antibody titre

Three groups of BALB/c mice (6 nos/group) were used for the experiment. The compounds were given orally (200 μmoles/dose/kg body
weight, suspended in 1% gum acacia) for five consecutive days. Along with the last dose of drug, all animals received antigen (SRBC, 20% 0.1 ml) intraperitoneally. Blood was collected every 3rd day for one month, sera separated and heat inactivated at 56°C for 30 minutes. The sera was serially diluted and checked for agglutination of antigen SRBC (2.2.28).

### 7.2.4 Determination of antibody producing cells

Three groups of Balb/c mice (7 per group) were used for the study. Each group was treated with the respective compounds (200 μmoles/kg bodyweight, suspended in 1% gum acacia), intraperitoneally for 5 days. Along with the last dose, antigen (SRBC 2.5 x 10^8 cells) was injected intraperitoneally. Animals were sacrificed on various days, spleen was processed (2.2.24) and used to determine the number of plaque forming cells by plaque assay (2.2.29).

### 7.2.5 Effect of polyphenolic compounds on the production of TNF-α by macrophages

Sodium caseinate elicited macrophages were activated \textit{in vivo} as well as \textit{in vitro}.

\textbf{(a) In vivo activation of macrophages}

Test compounds (catechin and rutin) were given intraperitoneally (200 μmoles/kg body weight) on five consecutive days to BALB/c mice. Along with the second dose of drug treatment, all animals received 0.5% sodium caseinate intraperitoneally to elicit macrophages. Five days later the animals were sacrificed by cervical dislocation, and peritoneal macrophages were harvested (2.2.25) and 2.5 x 10^4 macrophages/well were incubated with
L-929 cells (5x10³/well). The cytotoxicity was determined morphologically (2.2.30), after staining with crystal violet.

(b) **In vitro activation of macrophages**

Peritoneal macrophages were elicited by injecting 0.5% sodium caseinate into the peritoneal cavity of BALB/c mice. Five days later, the animals were sacrificed, and peritoneal macrophages were harvested. The macrophages were cultured in medium containing FCS in the presence of test compounds for 24 h at 37°C in 5% CO₂ atmosphere. The supernatant was collected and dispersed to titre plate wells containing L-929 cells (5 x 10³/well) and incubated further for 48 h. The cell lysis was determined after staining the cells with crystal violet dye (2.2.30).

7.2.6 **Effect of polyphenolic compounds on the serum TNF-α levels in metastatic tumour bearing animals**

Five groups of C57BL/6 mice, were injected with B16F-10 melanoma cells (1 x 10⁶/animal) through the caudal vein. Simultaneously polyphenolic compounds (200 μmoles/kg body weight) were administered orally for 10 alternate days. At various days (5th day, 10th day and 21st day), the animals were sacrificed, and the serum was collected and used for L-929 bioassay. Serum (100 μl) was added to wells containing L-929 cells (5 x 10³/0.1 ml well) and incubated at 37°C for 48 h in a 5% CO₂ atmosphere. The cells were washed with PBS, fixed with 5% formaldehyde for 20 minutes and stained with crystal violet. The cytotoxicity was observed by the morphology of tumour cells.
7.3 RESULTS

7.3.1 Effect of polyphenolic compounds on circulating antibody titre

The effect of polyphenols on circulating antibody titre is given in Table 20. Catechin and rutin did not enhance the production of antibodies against SRBC, to a significant level. The titre value was almost similar to that of untreated animals.

7.3.2 Effect of polyphenolic compounds on antibody producing cells

The effect of polyphenols on antibody producing cells is given in Table 21. The maximum number of plaque forming cells for catechin (455.8) and rutin (431.7) were found on 6th day which was not highly significant compared to control (302.5).

7.3.3 Effect of polyphenolic compounds on macrophage activation

Administration of rutin and catechin, did not activate macrophages to liberate TNF-α. In *in vitro* experiments, catechin and rutin did not activate normal macrophages to liberate TNF-α. The polyphenolic compounds rutin and catechin did not activate macrophages (either *in vivo* or *in vitro*) to liberate TNF-α.

7.3.4 Effect of polyphenolic compounds on the serum TNF-α production in metastatic tumour bearing animals

When serum from the 5th day control group was added, 50% L-929 cells were dead. But addition of serum samples from curcumin, catechin and rutin treated group did not produce any cell lysis. When the serum samples
**Table - 20**

*Effect of polyphenolic compounds on the circulating antibody titre in mice*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days after Immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
</tr>
<tr>
<td>Rutin</td>
<td>16</td>
</tr>
<tr>
<td>Catechin</td>
<td>8</td>
</tr>
</tbody>
</table>

Balb/c mice, were administered orally (200 μmoles/kg body weight) with compounds, for five consecutive days. Along with the last dose of SRBC was intraperitoneally administered. Blood was collected every third day for one month, and serum was checked for agglutination.
Table - 21

Effect of polyphenolic compounds on antibody producing cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plaque forming cells (PFCs)/10^6 spleen cells</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>126.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±</td>
</tr>
<tr>
<td>Catechin</td>
<td></td>
<td>160</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±</td>
</tr>
<tr>
<td>Rutin</td>
<td></td>
<td>140</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±</td>
</tr>
</tbody>
</table>

Balb/c mice were treated with test compounds, (200μmoles/kg body weight, suspended in 1% gum acacia) intraperitoneally for 5 days. Antigen was injected along with the last dose. Animals were sacrificed on various days, spleen processed and Jerne's plaque assay was carried out.

Values are expressed as ± SD.
of control group of 10th day and 21st day (Fig. 12A) produced 100% cell lysis, curcumin (Fig. 12B) and catechin (Fig. 12C) treated samples did not produce any cell lysis. Addition of serum samples of 10th day of rutin treated group did not produce any cell lysis but sample from 21st day (Fig. 12D) produced 50% cytolysis of L-929 cells. The results indicate that curcumin, catechin and rutin reduced the levels of TNF-α in serum of metastatic tumour bearing animals.

7.4 DISCUSSION

The main objectives of immunotherapy is to modulate immune responses for selected objectives, which include augmentation of cell-mediated immunity, cytotoxic antibody and macrophage activation. In the present chapter some of the immunological mechanisms of action of polyphenolic compounds were studied.

Administration of either catechin or rutin did not increase the circulating antibody titre.

Modulation of TNF-α induced cytotoxicity by polyphenols is reported (129). Macrophages can mediate cytotoxicity either causing necrosis of tumour cells or by inducing apoptosis by various mechanisms. Administration of polyphenolic compounds did not activate macrophages to liberate tumour necrosis factor, and thereby no lysis of TNF-sensitive L-929 cells were observed.
Effect of polyphenolic compounds on the serum TNF-α levels of metastatic tumour bearing animals (on 21st day of tumour inoculation)

B16F-10 melanoma cells (1 x 10^6 cells/animal) were injected through the tail vein. The animals were sacrificed at various time intervals. The serum was collected and L929 bioassay was carried out.

Serum samples from

A. Control animals
B. Curcumin treated animals
C. Catechin treated animals
D. Rutin treated animals
Fig. 12.

L-929 CELLS

A
TNF level was found to be enhanced during metastasis. Control metastatic tumour bearing animals had enhanced TNF level in the serum. Lowered levels of serum TNF-α was observed in metastatic tumour bearing animals treated with curcumin, catechin and rutin at various time intervals of tumour growth. But in the case of catechin, curcumin and rutin treated groups, no TNF production was observed at various time intervals (except for rutin on 21st day).

Eventhough the compounds, catechin, rutin and genistein showed antimetastatic activities (in vivo and in vitro), these compounds did not have much effect on the tumoral immune response. TNF production during metastasis was significantly inhibited by curcumin, catechin and rutin and is correlated with its activity in the inhibition of metastasis.