CHAPTER 2

- INHIBITION OF THE GENOTOXIC EFFECTS OF BENZO(A)PYRENE BY SPICES AND LEAFY VEGETABLES.
INTRODUCTION

Inhibition of genotoxic effects in germ and somatic cells provides a fundamental strategy, complementary to the control of environmental and lifestyle risk factors, aiming at extending life expectancy and at preventing mutation related pathological conditions, the most important of which is cancer (1).

Most human malignancies and cancer prone conditions involve or are associated with specific chromosomal aberrations suggesting an important role of these endpoints in carcinogenesis (2). A number of consistent and specific chromosome aberrations have been detected in various types of human malignant cells (3,4). The activation of an oncogene and enhancement of its expression in experimental systems as well as in human tumours frequently involves chromosome rearrangements such as deletions, insertions and transpositions (5). In leukaemias and lymphomas, specific chromosomal translocations result in the transposition and subsequent activation of the associated oncogene (6). Many mutagens and clastogens have been identified in the human diet (7). These clastogens give rise to primary DNA lesions, some of which are further processed during replication and/or repair of DNA into secondary lesions which develop into various types of chromosome aberrations in the metaphase cell (2).

It therefore becomes imperative to identify compounds that can inhibit the deleterious effects of various
genotoxic compounds present in the diet such as polycyclic aromatic hydrocarbons (PAHs), pyrrolizidine alkaloids, flavonoids like quercetin, phenolics etc. (7). The 10 plant products—cumin seeds, poppy seeds, asafoetida, turmeric, kandathipili, neem flowers, drumstick leaves, manathakkali leaves, basil leaves and ponnakanni leaves—which increased GST activity by more than 78% in the stomach, liver and oesophagus of Swiss mice were tested for their capacity to suppress chromosome aberrations (CA), induced by 3,4-benzo(a)pyrene (BP). BP is a potent carcinogen and clastogen (8) and a widespread environmental pollutant present in the atmosphere, drinking water and food (9).

MATERIALS AND METHODS

Chemicals: 3,4-benzo(a)pyrene, colchicine and Giemsa powder were purchased from Sigma Chemical Company, U.S.A. All other chemicals were of ANALAR grade.

Plant products: The following plant products were used to assess their chemopreventive potential—cumin seeds, poppy seeds, asafoetida, kandathipili, turmeric, neem flowers, basil leaves, drumstick leaves, manathakkali leaves and ponnakanni leaves. They were purchased from the local market processed and used as mentioned in Materials and Methods and Table 1.1 of Chapter 1.

Animals: Groups of 5 male Swiss mice (6-8 weeks of age) weighing 25±3.8 g were used. Powdered diet containing the
various spices/leafy vegetables prepared according to the protocol in Table-1.1, Chapter 1, were given to other groups. After 14 days of feeding, the mice were administered BP (ip., 60 mg/kg body weight) taken in ground nut oil (0.5ml). One group of mice, fed standard pellet diet and administered BP, served as the positive control. One group of mice fed standard pellet diet, served as the negative control.

Analysis of chromosome aberrations:

The bone marrow cells in a state of active division in vivo were used to assess the chromosome damage (10). The cells at metaphase were arrested using a spindle inhibitor colchicine. Chromosome preparations were then observed under a microscope for aberrations.

Reagents:

1. Potassium chloride - 0.075 M.
2. Saline.
3. Colchicine solution - A 1 mg/ml stock solution of colchicine in saline was prepared and stored at -20 C.
4. Fixative: Methanol - acetic acid solution (3:1,v/v) prepared fresh just before use.
5. Giemsa stain: One g of the powder was dissolved in 66 ml glycerol using a magnetic stirrer, at 55 C for 90 min. The solution was cooled to room temperature and 66 ml of methanol was added and mixed thoroughly using a magnetic stirrer at room
temperature. The solution was filtered using a Whatman no:3 filter paper.

Stock Giemsa was diluted to 4% by adding 2 ml of 10% disodium hydrogen phosphate (pH 6.4) and 2 ml of stock Giemsa solution to 46 ml of distilled water. This was used for staining the slides.

Method:

The mice were injected intraperitoneally, 0.1 ml of the colchicine solution, 18 h after injection of BP. The animals were sacrificed after 90 min and the bone marrow cells aspirated from the long bones (femur and humerus) with saline. The saline containing the cells was centrifuged in a table top centrifuge at 100 g for 10 min and supernatant discarded. The pellet was resuspended in 10 ml of 0.075M KCl pre-warmed to 37 C. The cell suspension was incubated at 37 C for 20 min and again centrifuged at 100 g for 5 min. The supernatant was removed and the cell pellet again resuspended in 0.5 ml of 0.075 M KCl. The resuspended cells were transferred to a 15 ml centrifuge tube containing 10 ml of freshly prepared ice-cold fixative (methanol-acetic acid, 3:1, v/v), and gently mixed using a pasteur pipette. After 10 min, the suspension was centrifuged for 10 min at 100 g and the supernatant discarded. Ten ml of fresh fixative was added, the cell pellet resuspended and left undisturbed for 10 min, centrifuged and the pellet resuspended in fixative. This procedure was repeated for a third time. The cells were then suspended in 0.5 ml of fixative. One drop of the cell
suspension was dropped using a pasteur pipette onto a clean acid washed microscopic slide from a height of 5-8 cm. The slide was air dried. The slide was stained with Giemsa working solution and left undisturbed for 10 min. The slides were then washed thoroughly in distilled water to remove excess stain and were then air dried. Permanent slides were prepared using DPX mountant. The slides were then examined under a Nikon microscope for chromosome aberrations (CA). Fifty metaphase cells per mouse were analysed.

Gaps were defined as incomplete interruptions of the continuity of one or both chromatids not exceeding the width of a chromatid and breaks as the discontinuity greater than the width of a chromatid, irrespective of whether or not the distal fragment was dislocated (11). Cells with multiple CA were defined as cells in which the number of CA were too great to count. Cells were classified according to the most severe damage that had occurred and were placed in one of the 4 categories (i) cells with gaps only, (ii) cells with breaks, (iii) cells with exchanges (iv) cells with multiple CA. The incidence of aberrant cells was expressed as the percent of damaged cells in the total population of cells analysed. Cells with gaps were not included in the category of damaged cells because it is generally believed that gaps are not good indicators of chromosome damage (11).

CA were also analysed at various times (8 h, 16 h, 24 h, 48 h and 72 h) after BP injection in normal mice. The
effects of feeding different doses of plant products (which caused maximum suppression of CA) viz., cumin seeds, poppy seeds, manathakkali leaves and ponnakanni leaves, on BP induced CA were also determined.

The different doses of spices employed were 80 mg/g diet, 120 mg/g diet, 160 mg/g diet and 200 mg/g diet. The effects of the two leafy vegetables on BP induced CA were determined at the following doses - 520, 560, 600 and 640 mg/g diet.

Statistical analysis:
Students "t" test was performed to analyse the significance of the results (12).

RESULTS

The effect of time after which the mice were sacrificed following BP injection on chromosome aberrations is shown in Figure 2.1. The percentage of aberrant cells observed 8 h after injection of BP was 25% It increased to 66.5% at 18 h and thereafter decreased at the periods studied- 55% at 24 h, 32% at 48 h and 20% at 72 h. This study revealed that the best time to study the incidence of CA was 18 h after the injection of BP.

BP induced CA consisted mainly of gaps, breaks and multiple aberrations, (19%, 43% and 21% respectively, Table 2.1). Figure 2.2 shows different types of CA seen in BP injected mice.

The results of the study on the effects of
Figure 2.1

Effect of time on BP-induced CA
Figure 2.2

The different types of CA seen in BP injected mice

A: Shows an example of chromosomal break

B: Reveals gap formation in chromosome
Figure 2.2

C : Shows a chromosomal exchange
pretreatment with different plant products on BP induced CA are presented in Table 2.1. Cumin seeds and poppy seeds reduced the incidence of aberrant cells by 80-83%. The reduction of BP induced multiple aberrations effected by cumin and poppy seeds were 92% and 77%, respectively, while the incidence of breaks were brought down by 80% and 85% respectively.

Asafoetida, kandathipili and turmeric exhibited moderate suppressive action against BP induced CA (66%, 50% and 54%, respectively). The three spices reduced multiple aberrations by about 56%. Asafoetida decreased the incidence of breaks by about 73%, while kandathipili and turmeric lowered the incidence by 49% and 55% respectively. However all the three failed to reduce the incidence of exchanges, significantly. Neem flowers had no effect on the incidence of aberrant cells induced by BP. Among the spices, cumin seeds alone significantly decreased the incidence of exchanges induced by BP (p < 0.01).

Among the leafy vegetables, ponnakanni leaves and manathakkali leaves suppressed the incidence of CA by about 65%, while in the case of basil leaves and drumstick leaves, the reduction was around 20%. Ponnakanni and manathakkali leaves reduced the incidence of gaps by about 52-69%. Basil leaves did not significantly decrease the incidence of breaks while drumstick leaves had no effect on the incidence of multiple aberrations caused by BP. Among the leafy
Table 2.1: Suppression of the genotoxic effect of BP by plant products

<table>
<thead>
<tr>
<th>Test substances</th>
<th>Per centage of cells with</th>
<th>Per cent incidence of aberrant cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gaps</td>
<td>Breaks</td>
</tr>
<tr>
<td>None</td>
<td>1.4 ± 0.3</td>
<td>3.5 ± 1.0</td>
</tr>
<tr>
<td>Benzo(a)pyrene (BP)</td>
<td>18.5 ± 3.2</td>
<td>43.3 ± 5.4</td>
</tr>
<tr>
<td>Cumin seeds + BP</td>
<td>2.1 ± 0.4a</td>
<td>8.5 ± 4.7a</td>
</tr>
<tr>
<td>Poppy seeds + BP</td>
<td>6.1 ± 1.2a</td>
<td>6.3 ± 2.2a</td>
</tr>
<tr>
<td>Asafoetida + BP</td>
<td>7.2 ± 0.5a</td>
<td>11.5 ± 3.4a</td>
</tr>
<tr>
<td>Turmeric + BP</td>
<td>5.2 ± 0.6a</td>
<td>19.5 ± 2.1a</td>
</tr>
<tr>
<td>Kandathipili + BP</td>
<td>9.5 ± 3.2a</td>
<td>22.0 ± 3.9a</td>
</tr>
<tr>
<td>Neem flowers + BP</td>
<td>17.1 ± 8.1</td>
<td>42.3 ± 4.8</td>
</tr>
<tr>
<td>Manathakkali leaves + BP</td>
<td>8.9 ± 1.4a</td>
<td>13.8 ± 2.8a</td>
</tr>
<tr>
<td>Drumstick leaves + BP</td>
<td>14.2 ± 2.6</td>
<td>34.3 ± 4.0c</td>
</tr>
<tr>
<td>Basil leaves + BP</td>
<td>10.3 ± 4.1c</td>
<td>37.5 ± 3.9</td>
</tr>
<tr>
<td>Ponnakanni leaves + BP</td>
<td>6.7 ± 1.2a</td>
<td>13.3 ± 2.2a</td>
</tr>
</tbody>
</table>

Values are Mean ± SD  
\[ n = 5 \]
\[ a : p < 0.001 \quad b : p < 0.01 \quad c : p < 0.05 \]
vegetables, ponnakanni leaves alone significantly (p < 0.05) decreased the incidence of exchanges.

The effects of feeding different doses of cumin seeds, poppy seeds, manathakkali leaves and ponnakanni leaves on BP induced CA are shown in Figures 2.3 and 2.4, respectively.

Cumin seeds and poppy seeds when fed to mice at a dose of 80 mg/g diet suppressed BP-induced CA by 26% and 25% respectively. At a dose of 120 mg/g diet, cumin seeds and poppy seeds showed suppressive effects of 64% and 55%, respectively. At a dose of 200 mg/g diet, cumin seeds suppressed BP induced CA by 85% while poppy seeds exhibited suppressive effects of around 84%. Both spices showed dose-dependent suppression of BP induced CA.

The leafy vegetables also showed dose-dependent decrease of BP-induced CA. At a dose of 520 mg/g diet ponnakanni leaves and manathakkali leaves suppressed BP induced CA by 12% and 15%, while at a dose of 560 mg/g diet, per cent suppression was 26% and 35%, respectively. At a dose of 640 mg/g diet, ponnakanni and manathakkali leaves suppressed BP induced CA by 70% and 68%, respectively.

DISCUSSION

Nine out of the ten plant products tested- cumin seeds, poppy seeds, asafoetida, turmeric, kandathipili, basil leaves, drumstick leaves, manathakkali leaves and ponnakanni leaves significantly reduced the genotoxic effects of BP.
Figure 2.3
Suppressive effects of cumin seeds and poppy seeds on BP-induced CA.

CS - Cumin seeds
PS - Poppy seeds
Figure 2.4
Suppressive effects of manathakkali leaves and ponnakanni leaves on BP-induced CA.
ML - Manathakkali leaves
PL - Ponnakanni leaves
The positive correlations between CA, mutagenicity and carcinogenicity as well as between DNA repair and CA have been widely recognised (13). Accordingly, the number of viable cells with subtle genetic changes might be increased in proportion to the increase of aberrant cells induced by chemical carcinogens. The present results therefore suggest that these plant products may suppress chemically induced cancers.

Ito et al (11) have reported that many vegetables like onion, burdock, cabbage etc. suppress CA induced by dimethylbenz(a)anthracene (DMBA). They have also reported that glutathione suppressed DMBA-induced CA significantly. Glutathione as a non-critical nucleophile has the capability to scavenge electrophilic forms of carcinogens. The 9 plant products significantly increased GSH levels (p < 0.001) in the stomach, liver and oesophagus (vide chapter 1, Table-1.3). This may constitute one of the mechanisms by which these plant products inhibit the genotoxicity of BP.

These plant products also increased significantly the GST levels in stomach, liver, and oesophagus when administered to mice (Chapter 1, Table-1.2). Since these enzymes effectively detoxify a wide variety of electrophiles (which bind to DNA causing CA leading to cancer), this may constitute another mechanism by which these plant products suppress CA. Thus, increased levels of GSH and GST may act synergistically to inhibit CA caused by BP by detoxifying the ultimate carcinogens (electrophiles).
The biochemical and molecular events leading to chromosomal changes are not known (2). Chemical agents that interact with DNA and especially those which give rise to DNA strand breaks and DNA adducts are usually effective in causing CA (2). There are a number of such genotoxic compounds present in the diet and they include protein pyrolysates, PAHs such as dimethylbenzanthracene (DMBA) and heterocyclic amines formed during cooking (7). Some spices like ginger, cinnamon and mace, which are widely used, have been reported to possess genotoxic properties (14). The plant products which suppress CA could hence have a role in the neutralisation of genotoxic effects of such compounds present in the diet.

Fresh turmeric is reported to induce elevated frequencies of micronuclei and chromosomal anomalies in mice (15). Turmeric extract is also reported to cause dose and time dependent induction of chromosome aberrations in several mammalian cell lines (16) and in plant cells (17). Vijayalakshmi (18) has however reported that curcumin, the chief constituent of turmeric did not induce elevated frequencies of micronuclei or chromosomal aberrations in mice bone marrow. Jensen (19) has also reported that dried turmeric extract, its oleoresins and curcumin are non mutagenic in the Ames test. Nagabhushan and Bhide (20) have reported that turmeric and its chief constituent curcumin are antimutagenic and anticarcinogenic. Our results which show that turmeric significantly reduced the incidence of CA
induced by BP are in accordance with the conclusions of the latter reports, that turmeric has potent chemopreventive properties.

Hosono et al (14) have reported kandathipili to be mutagenic when assayed with a streptomycin dependent mutant of Salmonella typhimurium TA 98, the SD 510 strain. Unnikrishnan and Kuttan (21) have reported that extracts of kandathipili had antitumour effects. Our results clearly indicate that kandathipili significantly decreased the genotoxic effects of BP.

Neem flowers failed to significantly reduce the incidence of CA induced by BP. But it was found to increase GST levels by more than 78% and GSH levels significantly. Neem flowers may cause enhancement of certain cytochrome P-450 dependent reactions including BP hydroxylation and increases in the enzyme epoxide hydratase. These reactions may thus neutralise the effects of GST and GSH in detoxification and account for the failure of neem flowers to decrease the incidence of CA induced by BP.

Thus the effects of these spices/leafy vegetables on chemically induced carcinogenesis cannot be predicted mainly on the response of the detoxification enzymes, since monooxygenases, epoxide hydratases, cytochrome P-450 are also inducible by dietary substances like flavones, indoles etc. (22). Therefore it is necessary to use other short-term tests and also long-term animal carcinogenicity tests to confirm the chemopreventive capabilities of any
substance. Nevertheless, the use of GST induction for screening anticarcinogens is a useful tool since 9 out of 10 plant products which increased GST levels by more than 78% were able to significantly suppress BP induced CA.
SUMMARY

1. The effect of the 10 plant products (cumin seeds, poppy seeds, asafoetida, turmeric, neem flowers, kandathipili, basil leaves, drumstick leaves, manathakkali leaves and ponnakanni leaves) on benzo(a)pyrene (BP) induced chromosomal aberrations in bone marrow cells of Swiss mice were investigated.

2. Nine of them, except neem flowers, significantly reduced the incidence of BP-induced chromosomal aberrations (CA).

3. Neem flowers failed to inhibit BP induced CA.

4. Cumin seeds, poppy seeds, manathakkali leaves and ponnakanni leaves showed dose-dependent inhibition of the genotoxic effects of BP.
REFERENCES

1. De Flora S.
Mechanisms of inhibitors of genotoxicity, Relevance in preventive medicine.


3. Mitelman F.
Catalogue of chromosome aberrations in cancer.

4. Varmus H.E.
The molecular genetics of cellular oncogenes.

5. Yunis J.
The chromosomal basis of human neoplasia.

6. Klein G. and Klein E.
Evolution of tumours and impact of molecular oncology.

7. Miller E.C. and Miller J.A.
Carcinogens and mutagens that may occur in foods.
8. Dipple A.
Formation, metabolism and mechanism of action of polycyclic aromatic hydrocarbons.

9. Benford D.J. and Bridges J.W.
Carcinogenic polycyclic aromatic hydrocarbons in food.
In: Food Toxicology- Real or imagined problems, (eds.),
Gibson G.G. and Walker R., Taylor and Francis, London,

10. Macgregor H. and Varley J.
Working with animal chromosomes, John Wiley and Sons,

11. Ito Y., Maeda S. and Sugiyama T.
Suppression of 7,12-dimethylbenz(a)anthracene induced chromosome aberrations in bone marrow cells by vegetable juices.

12. Bailey N.T.J.
Statistical methods in biology, Hodder and Stoughton,

13. Ito Y., Maeda S., Souno K., Ueda N. and Sugiyama T.
Induction of hepatic glutathione-S-transferase and suppression of 7,12-dimethyl benz(a)anthracene induced chromosome aberrations in rat bone marrow cells by Sudan III and related dyes.
Mutagenicities of selected spices and desmutagenic compounds in regard to spice induced mutagenicity.

15. Gururaj E. and Uma O.
Cytogenetic effects of turmeric (Curcuma longa) extract on male mice.

16. Good pasture C.E. and Arrighi P.E.
Effects of food seasoning on the cell cycle and chromosome morphology of mammalian cells in vitro: with special reference to turmeric.

17. Abraham S., Abraham S.K. and Radhamony G.
Mutagenic potential of condiments, ginger and turmeric.

18. Vijayalakshmi.
Genetic effects of turmeric and curcumin in mice and rats.

19. Jensen N.J.
Lack of mutagenic effect of turmeric oleoresin in Salmonella/mammalian microsome test.

20. Nagabhushan M. and Bhide S.V.
Nonmutagenicity of curcumin and its antimutagenicity
versus chilli and capsaicin.

21. Unnikrishnan M. C. and Kuttan R.
Antitumour and anticarcinogenic activity of spices.
In: Ninth annual convention of Indian Association for
cancer research and national symposium on cancer

22. Kapitulnik J., Poppers P.J., Buening M.K.,Fortner J.G.
and Conney A.H.
Activation of monooxygenases in human liver by 7,8-
benzoflavone.
Clinical Pharmacology and Therapeutics, 22, 475-485,