Materials and Methods
3.1 MODULATION OF EXPERIMENTAL CERVICAL CARCINOGENESIS

3.1.1 Chemicals

3-Methylcholanthrene (MCA) was obtained from Sigma Chemical Company, St. Louis, USA. Hematoxylin was procured from Merck, Germany and eosin from BDH, England. Yellow variety of beeswax was obtained from Mysore (India). *Trigonella foenum-graecum* L. seeds, *Piper betle* L. leaves and *Punica granatum* L. fruit were purchased from the local market. *Hippophae rhamnoides* L. fruit was a generous gift from Dr V. Singh, Himachal University.

3.1.2 Animals

Random bred, 8-9 weeks old virgin Swiss albino mice were supplied by Animal facility, JNU, New Delhi. Animals were maintained in an airconditioned environment with a regulated 12 hr light/12 hr dark cycle. These were given (unless otherwise stated) standard food pellets (Lipton India Ltd.) and tap water, *ad libitum*.

3.1.3 Preparation of Modulators for Feeding

*Artemisia annua* L.: 200 gms of leaves were ground in 300 ml of ethanol + distilled water (1:1). This was filtered and then lyophilised. Of this lyophilised extract, a preweighed quantity was added to 5 ml of distilled water to give the desired concentration w/v.

Curcumin: Prewaighed curcumin was dissolved in 500 µl of corn oil (vehicle) to give the desired concentration w/v.

*Hippophae rhamnoides* L.: The seeds were discarded and a preweighed quantity of the fruit was homogenised in 10 ml of distilled water to give the desired concentration w/v.

*Piper betle* L.: 200 gms of leaves were ground in 300 ml of ethanol + distilled water (1:1). This was filtered and then lyophilised. Of this lyophilised extract, a preweighed quantity was added to 5 ml of distilled water to give the desired concentration w/v.

*Punica granatum* L.: The juicy aril was crushed and filtered and, the desired quantity given by oral gavage.

*Trigonella foenum-graecum* L.: The seeds and the standard animal food pellets were powdered separately. A preweighed quantity of the modulator was added to the animal
feed to obtain the desired concentration in diet.

Doses of modulators and their route of delivery:

<table>
<thead>
<tr>
<th>Modulator</th>
<th>Vehicle</th>
<th>Dose 1</th>
<th>Dose 2</th>
<th>Route of delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Artemisia annua</em> L.</td>
<td>Water</td>
<td>12.5 mg</td>
<td>15 mg</td>
<td>Oral gavage</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Corn oil</td>
<td>7 mg</td>
<td>15 mg</td>
<td>Oral gavage</td>
</tr>
<tr>
<td><em>Hippopha rhamnoides</em> L.</td>
<td>Water</td>
<td>50 mg</td>
<td>100 mg</td>
<td>Oral gavage</td>
</tr>
<tr>
<td><em>Piper betle</em> L.</td>
<td>Water</td>
<td>10 mg</td>
<td>20 mg</td>
<td>Oral gavage</td>
</tr>
<tr>
<td><em>Punica granatum</em> L.</td>
<td>Water</td>
<td>50 μl</td>
<td>100 μl</td>
<td>Oral gavage</td>
</tr>
<tr>
<td><em>Trigonella foenum-graecum</em> L.</td>
<td>Pulverised standard animal feed</td>
<td>5 %</td>
<td>10 %</td>
<td>Diet</td>
</tr>
</tbody>
</table>

a: The value given is in terms of per kg body weight

3.1.4 Experimental Design

Animals were divided into various control and experimental groups as follows:

Group 1: Animals were treated with the vehicle used for the modulator. They were not given any intracervical treatment.

Group 2: Animals were given the vehicle used for the modulator by the oral route. After 2 weeks of oral treatment, the animals were operated upon and intracervically inserted the beeswax containing thread. The oral treatment was continued for 90 days.

Group 3: Animals were given the vehicle used for the modulator by the oral route. After 2 weeks of oral treatment, the animals were intracervically inserted the beeswax + carcinogen containing thread. The oral treatment was continued for 90 days.

Group 4: Animals were treated with dose 1 of the modulator by the oral route for 90 days. They were not given any intracervical treatment.

Group 5: Animals were given dose 1 of the modulator by the oral route. After 2 weeks of oral treatment, the animals were operated upon and intracervically inserted the beeswax containing thread. The oral treatment was continued for 90 days.

Group 6: Animals were given dose 1 of the modulator by the oral route. After 2 weeks of oral treatment, the animals were intracervically inserted the beeswax + carcinogen
containing thread. The oral treatment was continued for 90 days.

Group 7: Animals were treated with dose 2 of the modulator by the oral route for 90 days. They were not given any intracervical treatment.

Group 8: Animals were given dose 2 of the modulator by the oral route. After 2 weeks of oral treatment, the animals were operated upon and intracervically inserted the beeswax containing thread. The oral treatment was continued for 90 days.

Group 9: Animals were given dose 2 of the modulator by the oral route. After 2 weeks of oral treatment, the animals were intracervically inserted the beeswax + carcinogen containing thread. The oral treatment was continued for 90 days.

After 3 months of post- carcinogen exposure, the animals were sacrificed by cervical dislocation and their cervical tissues were fixed and processed for histopathology. Animals were weighed at the beginning of the experiment and at the time of sacrifice.

3.1.5 Tumor Induction

Murphy’s string method (Murphy, 1953) as described by Manoharan and Rao (1984) was followed for cervical tumor induction. This method comprises of the following steps.

3.1.5.1 Preparation of MCA - Beeswax Mixture and Threads

Cotton threads of suitable thickness were cut into pieces of 10- 12 cms each, washed in distilled water for 12 hours, and then immersed in ethyl alcohol for about 1 hour. The threads were knotted at one end and weighed. About 0.7 cm of each knotted end of the thread was impregnated with molten beeswax and weighed again. By subtracting post- impregnation weight from the pre- impregnation weight, the amount of wax in each thread was calculated. Now a known amount of 3- Methylcholanthrene (MCA) was mixed in a known amount of beeswax, kept exactly at its melting point (60°C), so that each impregnated thread should have 600 µg of MCA. The mixture was stirred in the molten state for about 70 hours at 60°C. The knotted end of each washed thread was immersed in the carcinogen- beeswax mixture and dried.
3.1.5.2 Intracervical Thread Insertion

Fig. 3.1 shows the insertion of thread into the canal of uterine cervix. For this, the animals were anaesthetized with anesthetic ether, and fixed on a wooden platform exposing the ventral portion of the body. A small slit was made in the abdominal wall and both the uterine horns were exposed gently by using a needle. A small blunt sewing needle carrying the thread was gently inserted through the vaginal opening and guided into the cervical canal smoothly. The tip of the needle was taken out through one uterine horn and the thread was drawn into the vagina until the wax-coated portion reached the cervical canal and the knot rests at the point where the vagina touches the external os. Then the free end of the impregnated thread was loosely tied around the uterine horn. This method assures the continuous exposure of the uterine cervix with the carcinogen.

3.1.6 Procedure for Histopathological Analysis

3.1.6.1 Fixation, Dehydration, Infiltration and Block Preparation

The uterine cervix of each animal was dissected out, freed from connective tissue and fatty tissue and fixed in Bouin’s fluid for 24-48 hours depending upon the volume of the tissue. The tissues were then dehydrated by passing through graded series of ethyl alcohol (30 minutes each in 50%, 70%, 90%, 100% I and 100% II). These were then cleared in xylene (two changes of 30 minutes each). The cleared tissues were placed for 10 minutes in xylene plus wax (1:1) mixture at 58-60°C. After giving two changes of molten paraffin (1 hour each), the tissues were embedded in fresh paraffin wax.

Embedded tissue was sectioned (5-6 μ thickness) on a microtome, in a plane parallel to the long axis of the organ. The sectioned slices were spread on albuminized glass slides. These were then dried at 35-40°C.

3.1.6.2 Staining Procedure

Sections were deparaffinized by dipping in xylene (two changes of 5 minutes each). The slides were then passed through graded concentrations of ethyl alcohol (2 minutes each in 100%, 95%, 70%, 50% and 30%), and then kept in running water for
Figure 3.1: Schematic drawing of thread insertion.
3 minutes. They were then put in Harris' hematoxylin for 1 minute and again washed in running water for about 10 minutes.Slides were then passed through 50% and 70% ethyl alcohol (2-3 minutes each), and subsequently put in eosin stain (prepared in 70% ethyl alcohol) for 2 minutes. These were then passed through graded series of ethyl alcohol (5 minutes each in 70%, 90%, 100%), then in 100% alcohol + xylene mixture (1:1), for 5 minutes, and finally given two changes of xylene of 3 minutes each.

3.1.6.3 Mounting of Stained Sections
The slides were mounted with DPX, covered with glass cover slips and kept for drying at room temperature.

3.1.7 Histopathological Analysis
In the present study the following precancerous and cancerous lesions were taken as parameters.

1. Hyperplasia
Characterised by an increase in the thickness of the epithelial layer due to the increased proliferative activity. Keratinization and flattening of the epithelial cells persist at the surface.

2. Dysplasia
The cells show varying degrees of atypia. They show characteristic nuclear enlargement and hyperchromatism. The abnormal epithelial cells are disorderly arranged. Three grades of dysplasia: mild, moderate and marked, can be envisaged according to the degree of cellular atypia and epithelial architecture.

2a. Mild Dysplasia
The lesion involves lower one-third of the epithelium and shows varying degrees of hyperplasia and focal areas of epithelial dysplasia. Loss of cellular polarity and regular stratification are minimal. Nuclei are always enlarged and darkly stained.

2b. Moderate Dysplasia
The atypia is seen spreading to the lower two-thirds of the epithelium. Hyperplasia,
hyperkeratinosis and dyskeratosis are present in a wide area of the epithelium. The degree of epithelial abnormality is intermediate between mild and marked dysplasia.

2c. Marked Dysplasia
The atypia is pronounced and there is loss of cellular polarity and the crowded cells have large darkly stained nuclei. The abnormal cells tend to be present in the upper third as well as middle and lower thirds of the epithelium. The lesion presents the picture of intraepithelial carcinoma except that there is a variable amount of differentiation of the superficial layer of the epithelium.

3. Squamous Cell Carcinoma
The squamous cell carcinoma lesions are further categorised as:

3a. Carcinoma in situ or Intraepithelial Carcinoma
It shows a marked proliferation of cells in the entire epithelium where nuclei are enlarged and hyperchromatic. There is an increased number of mitotic figures and crowding of atypical cells. However, the basement membrane remains intact and there is no invasion of the underlying stroma. Loss of stratification and polarity are seen.

3b. Invasive Carcinoma
It resembles the squamous cell carcinoma in situ in its cellular characteristics. In addition there is disruption of the basement membrane with sheets or islands of malignant squamous cells invading the underlying stroma. The invasive squamous cell carcinoma are further classified as:
   i. Differentiated: Tumors with much keratinization and pearl formation
   ii. Poorly differentiated: Tumors with some keratinization
   iii Undifferentiated: Tumors without any keratinization

3.1.8 Statistical Analysis
Chi square ($\chi^2$) test was applied for calculating the level of significance of difference in the tumor incidence between control and experimental groups.
Plate 1: Longitudinal section of the normal cervical region showing stratified squamous epithelium. (x 100).

Plate 2: Section of the cervical epithelium showing hyperplastic condition. (x 100).

Plate 3: Cervical squamous epithelium of mouse showing dysplasia. Disorderly arrangement of the cells, disturbed cellular polarity and hyperchromatic nuclei are prominent. (x 100).
Plate 4: Carcinoma *in situ* of the cervical epithelium. Loss of stratification and polarity are seen. No differentiation of the entire layer. Basement membrane is intact. (x 100).

Plate 5: Well differentiated invasive carcinoma of the cervical epithelium. Masses of cancerous cells have invaded the stroma. Keratinization and pearl formation is seen. (x 100).

Plate 6: Poorly differentiated invasive carcinoma of the cervical epithelium. (x 100).
3.2 BIOCHEMICAL STUDIES

3.2.1 Chemicals

Bovine serum albumin (BSA), 1-chloro 2-4-dinitrobenzene (CDNB), curcumin, 5,5-dithio bis-2-nitrobenzoic acid (DTNB), glutathione reductase (GR), reduced glutathione (GSH), oxidised glutathione (GSSG), reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma Chemical Co., St. Louis, USA. All other chemicals used were of analytical grade. *Trigonella foenum-graecum* L. seeds, *Piper betle* L. leaves and *Punica granatum* L. fruit were purchased from the local market. *Hippophae rhamnoides* L. fruit was a generous gift from Dr V. Singh, Himachal University.

3.2.2 Animals

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3.2.3 Dose of Modulators and their Route of Delivery

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<th>Dose 2</th>
<th>Dose 3</th>
<th>Route of delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Artemisia annua</em> L.</td>
<td>Water</td>
<td>5 mg</td>
<td>10 mg</td>
<td>15 mg</td>
<td>Oral gavage</td>
</tr>
<tr>
<td><em>Hippophae rhamnoides</em> L.</td>
<td>Water</td>
<td>25 mg</td>
<td>50 mg</td>
<td>100 mg</td>
<td>Oral gavage</td>
</tr>
<tr>
<td><em>Piper betle</em> L.</td>
<td>Water</td>
<td>2 mg</td>
<td>5 mg</td>
<td>10 mg</td>
<td>Oral gavage</td>
</tr>
<tr>
<td><em>Punica granatum</em> L.</td>
<td>Water</td>
<td>50 μl</td>
<td>100 μl</td>
<td>--</td>
<td>Oral gavage</td>
</tr>
<tr>
<td>*Trigonella foenum-</td>
<td>Pulverised standard</td>
<td>2 %</td>
<td>10 %</td>
<td>--</td>
<td>Diet</td>
</tr>
<tr>
<td>Graecum* L.</td>
<td>animal feed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*: The value given is in terms of per kg body weight
3.2.4 Experimental Design

Female mice were divided into four groups (6-8 animals in each group) as follows:
Group 1: Animals were treated with the vehicle and served as control
Group 2: Animals were given dose 1 of the modulator
Group 3: Animals were given dose 2 of the modulator
Group 4: Animals were given dose 3 of the modulator

The treatment was followed for 10 days. Animals were then starved O/N and sacrificed on the day 11 by cervical dislocation (The treatment period in case of *Trigonella foenum-graecum* L. was 20 days). Animals were weighed at the beginning of the experiment and at the time of sacrifice.

3.2.5 Preparation of Tissue Homogenates, Cytosolic and Microsomal Fractions

Livers were dissected out and perfused with ice cold 0.9% NaCl. A 10% homogenate (w/v) of the tissue was prepared in ice cold Tris KCl (pH 7.4) buffer. 0.5 ml of this was used for sulphydryl group estimation while the rest was centrifuged at 14,000g for 20 min. at 4°C. The pellet obtained was discarded. After removing the lipid layer by aspiration, the supernatant was further centrifuged at 1,05,000g for 1 hr at 4°C. The pellet and supernatant thus obtained represent the microsomal and cytosolic fractions respectively. The pellet was resuspended in 1 ml of Tris HCl (pH 7.4) buffer and was used for cytochrome b5 and cytochrome P450 estimations. The cytosolic fraction was used for assaying glutathione S-transferase, glutathione reductase and glutathione peroxidase. Protein content in both the microsomal and cytosolic fraction was estimated by Lowry's method using BSA as standard. Duplicate determinations were made for each sample.

3.2.6 Glutathione Estimation

Glutathione content was measured as acid soluble sulphydryl levels, assayed by the method described by Moron et al. (1979).

*Principle:* The acid soluble sulphydryl group (non protein thiols, of which more than 90% is reduced glutathione) generates a yellow colored complex (S-thio-2-
nitrobenzoate) with DTNB that absorbs at 412 nm.

Reduced Glutathione + DTNB $\rightarrow$ 5-thio-2-nitrobenzoate

**Assay:** 0.5 ml crude tissue homogenate was added to 0.1 ml of 25% TCA to precipitate the proteins. This was centrifuged for 10 minutes, thus allowing the precipitate to settle as pellet. 0.1 ml of supernatant was added to 0.9 ml of 0.1 M phosphate buffer (pH 8.0) and 2 ml of 0.6 mM DTNB. The absorbance was taken at 412 nm against a blank that contained 0.1 ml of 5% TCA in place of supernatant. The sulphydryl content was calculated with the help of standard graph made by using different concentrations of reduced glutathione and expressed as $\mu$mol/gm tissue.

### 3.2.7 Cytochrome b$_5$ Assay

Cytochrome b$_5$ was assayed by the method of Omura and Sato (1964).

**Principle:** The enzyme was estimated from its redox spectrum of NADH-reduced versus oxidised cytochrome, which peaks at 424 nm.

**Assay:** 0.5 ml of the microsomal fraction was added to 4.3 ml of 50 mM Tris HCl (pH 7.4) buffer and mixed thoroughly. This was equally divided between two matched cuvettes and scanned in a dual beam UV-160 Shimadzu spectrophotometer between 400 nm and 500 nm for baseline correction. To the test cuvette, 100 $\mu$l of 0.5% NADH (w/v) was added and 100 $\mu$l of Tris HCl (pH 7.4) buffer was added to the reference cuvette. Reaction was allowed to proceed for 2 minutes, at the end of which the redox spectrum between 400 nm and 500 nm was recorded. Cytochrome b$_5$ is determined as the absorption difference between 424 nm and 409 nm. The activity of the enzyme was calculated using the extinction coefficient 185 mM$^{-1}$cm$^{-1}$ and expressed as nanomol/mg protein.

### 3.2.8 Cytochrome P$_{450}$ Assay

The enzyme was assayed by the method of Omura and Sato (1964).

**Principle:** Reduced cytochrome P$_{450}$ combines with carbon monoxide to give a characteristic absorption maxima at 450 nm.

**Assay:** The sample used for cyt b$_5$ determination was remixed. To this, a pinch of
sodium dithionate was added to reduce the hemeprotein. This was then divided equally between two matched cuvettes and scanned between 400 nm and 500 nm for baseline correction. The contents of the test cuvette were gently bubbled with carbon monoxide for about 30 seconds and then scanned between 400 nm and 500nm. Cytochrome P<sub>450</sub> is determined as the absorption difference between 450 nm and 490 nm. The activity of the enzyme was calculated using the extinction coefficient 91 mM⁻¹cm⁻¹ and expressed as nanomol/ mg protein.

3.2.9 Glutathione S-Transferase Assay

Glutathione S-transferase (GST) was assayed by the method of Habig et al. (1974).

**Principle:** GST activity was determined by the rate of formation of conjugate between reduced glutathione and CDNB. The conjugate absorbs at 340 nm.

\[
\text{GST} \quad \text{RX} + \text{GSH} \quad \rightarrow \quad \text{HX} + \text{R-SG}
\]

**Assay:** Reaction mixture, containing final concentrations of 0.1 M phosphate buffer (pH 6.5), 1 mM CDNB and 1 mM GSH, was incubated at 37 °C for 5 min. The reaction was initiated by the addition of enzyme sample and enzyme activity was followed for 5 minutes at 340 nm. The specific activity was calculated using extinction coefficient 9.6 mM⁻¹cm⁻¹ and expressed in terms of μmol of CDNB-GSH conjugate formed/ mg protein.

3.2.10 Glutathione Reductase Assay

Glutathione reductase (GR) was assayed using the procedure described by Carlberg and Mannervik (1975).

**Principle:** GR activity is determined by the amount of NADPH consumed in the conversion of oxidised glutathione to reduced glutathione. This reaction is catalyzed by GR and can be monitored by the decrease in absorbance of NADPH at 340 nm.

**Assay:** The final concentrations in 1 ml of the reaction mixture were 0.2 M sodium phosphate buffer (pH 7.0) containing 2 mM EDTA, 1 mM GSSG and 0.2 mM NADPH. The reaction was initiated by adding 25 μl of cytosolic fraction and the decrease in OD/min. was followed for 5 min. at 340 nm. 6.22 mM⁻¹cm⁻¹, the extinction
coefficient of NADPH was used to calculate the enzyme activity. GR activity was expressed as nanomol of NADPH consumed/ min/ mg protein.

3.2.11 Glutathione Peroxidase Assay

Glutathione peroxidase (GPx) was assayed by the method by Paglia and Valentine (1967).

Principle: This procedure is an indirect measurement of GPx activity. Glutathione disulfide (oxidised form) produced as a result of GPx activity is immediately reduced by an excess glutathione reductase (GR), thereby maintaining a constant level of glutathione (reduced form) in the reaction system. The assay takes advantage of the depletion of NADPH by GR, which is measured at 340 nm.

\[
\text{GPx} \quad \text{H}_2\text{O}_2 + 2 \text{GSH} \quad \xrightarrow{\text{GR}} \quad 2 \text{H}_2\text{O} + \text{GSSG}
\]

Assay: The final concentrations in 1 ml reaction mixture were 50 mM sodium phosphate buffer (pH 7.0) containing EDTA, 0.24 U/ml yeast glutathione reductase, 0.3 mM glutathione, 0.2 mM NADPH, 1 mM sodium azide and 1.5 mM H\textsubscript{2}O\textsubscript{2} and cytosolic sample. Reaction was started by addition of NADPH and the decrease in absorbance was monitored at 340 nm for 5 min. 6.22 mM\textsuperscript{-1}cm\textsuperscript{-1}, the extinction coefficient of NADPH was used to calculate the enzyme activity. GPx activity was expressed as nanomol of NADPH consumed/ min/ mg protein.

3.2.12 Protein Determination

Protein content was determined by following the method of Lowry (1951).

Principle: Proteins react with Folin- Cicocalteau reagent to give a coloured complex. The colour so formed is due to the reaction of the phosphomolybdate with tyrosine and tryptophan.

Assay: Alkaline solution (50 ml of 2% Na\textsubscript{2}CO\textsubscript{3} in 0.1 N NaOH + 1 ml of 0.5% CuSO\textsubscript{4} in 1% Na, K tartarate) was prepared fresh. 5 ml of this solution was added to 1.0 ml of test solution. It was mixed thoroughly and allowed to stand at room temperature for
10 min. Subsequently 1 N Folin's reagent (0.5 ml) was added and allowed to stand for another 30 min. The absorbance was measured at 660 nm against the reference blank. The protein content of each sample was evaluated from the standard curve made with BSA and was expressed as mg/ml.

3.2.13 Statistical Analysis

Statistical significance between control and treated groups was determined by ANOVA (Analysis of variance) test.