MATERIALS AND METHODS
Breast cancer is multi-factorial disease where genetic susceptibility, environment, nutrition and other lifestyle risk factors interact. According to World Health Organization (WHO) 80% of all cancers are environmental in origin. Better identification of modifiable risk factors and risk reduction of breast cancer may allow implementation of useful strategies for prevention. Considering the above rationale, the present study on the “Breast Cancer Incidence and the Possible Causative Environmental Factors” was designed and selected for the present study.

3.1 Profile of the Study Area:

The state of Andhra Pradesh, known as “Rice bowl of India” is situated in the southern part of Peninsular India (Figure: 3.1). The present study was carried out in Guntur District, located in Andhra Pradesh along the east coast of the Bay of Bengal between 15° 18’ to 16° 50’ North latitude and 70° 10’ to 80° 55’ east longitude. Guntur district covers an area of 11,391 Sq.km, and has a population of 44, 65,144 of which 28.80% was confined to urban area as of 2001 AD (Figure: 3.2). Paddy, tobacco, cotton and chillies are the main agricultural crops cultivated in the district.

Figure: 3.1
The state of Andhra Pradesh, India
3.2 Study Design:

This study was carried out for a period of 4 years during 2008 to 2011, collected the data regarding the occurrence of breast cancer risk associated with residential proximity to wide areas of pesticide use in the Guntur District region. The study suspects a health risk (e.g., risk of breast, cervical and lung cancer) due to the exposure to pollution sources in the study area with special reference to pesticides. The data obtained from the questionnaire and clinical pathology was combined with statistical analysis to have a detailed insight into the local risk of breast cancer.

3.3 Sampling and Data Collection:

In the present study, 300 cases of various female breast cancer complaints were selected. Of these 300 cases included in the study, 96 were selected for the year 2008, 87 from 2009 and 117 from the years 2010 and 2011 from the Guntur district, Andhra Pradesh (the study area).
Government General Hospital and the Bommidala Cancer Institute, Pedakakani, Guntur. Approval was obtained for the recruitment of patients with various breast cancer complaints, the collection of information through a questionnaire, the collection and analysis of breast tissue and for the collation of questionnaire and analytical data (Annexure - II) (Permission Number – 2458b2).

3.4 Data Collection:

To evaluate the objectives of the study, the target population were interviewed personally by using a pre-tested questionnaire (Annexure- I). Broad questions about relevant issues were asked in sequence in Telugu (regional language) and they were encouraged to give more descriptive responses so as to obtain more in-depth perspectives. Their answers were noted and the data were analysed by using statistical methods.

3.5 Questionnaire and its parts:

A pre planned questionnaire according to the objectives of the present study containing both open-ended and close-ended questions was developed for this study. The questionnaire contained five sections as follows:

Part 1: Basic information of respondents regarding age, residence, body mass index, marital status, occupational characteristic, lifestyle factors.

Part 2: Contained question on the family history that search about cancer in family through first or second degree relative.

Part 3: Questions on the personal health history subdivided into general health history, gynaecological health history and obstetric history.

Part 4: Questions on the respondent’s exposure to chemicals.

Part 5: Questions on diet with focus on fat consumption.
Before going into the process of data collection the questionnaire was validated by distributing to 20 people in the same hospital, in order to make it relevant.

3.6 Collection of Breast Tissue and Blood Samples:

Breast adipose tissue and blood samples were obtained from surgeries of confirmed and suspected breast cancer patients from Guntur Government General Hospital and Bommidala Cancer Institute, Pedakakani, Guntur. The patient details were recorded from pathology records for all breast adipose samples subsequent to consent from the patients. In the instances where consent was not obtained from the patient, or if the consulting doctor decided that the patient was no longer appropriate for the study, the tissues and the details of such patients were discarded. The adipose tissue (approximately 1cm³) was excised from breast tissue and venous blood (5ml) was collected for pesticide residue analysis. Samples of breast tissue (approximately 1cm or greater) were wrapped in aluminium foil pre-treated with acetone, stored in labelled specimen containers. Blood samples were collected in residue free heparinized 10 ml glass vials containing 200 USP units of heparin in 0.2 ml solution with the help of sterilized syringe. The labels on the specimen containers contained information regarding the patient’s hospital, respondent number (RD) and surname, which was used to obtain pathology details, but upon collection the samples were assigned a breast cancer- respondent code (BC-RD) number.

3.6.1 Demographic Details of Patients:

The target population of this research was resident women (over the age of 24 years from both the agricultural and non-agricultural communities) of the Guntur District (study region), registered for treatment in the Guntur Government General
Hospital or Bommidala Cancer Institute, Pedakakani, Guntur with breast complaints. These two hospitals are the main source for the patients to get treatment.

### 3.6.2 Chemicals and Glassware:

Pesticide standards of organochlorines – α-HCH, β-HCH, γ-HCH and δ-HCH, alachlor, aldrin, α-endosulfan, β-endosulfan, endosulfan sulphate, dieldrin, pp’DDT, pp’DDD, pp’DDE and organophosphates – monocrotophos, atrazine, phorate sulfone, chlorpyrifos, ethion, malathion, phosphamidon were obtained from address of these pesticide standards with corresponding letters.

All the solvents - acetone, di-ethyl ether, hexane, methanol, and acetonitrile (HPLC grade) and other analytical grade reagents used for the analysis were purchased from Merck, India. Organic solvents were all glass contained, double distilled and checked for any spurious peaks.

The glassware used in the extraction and clean up was Borosil make and supplied by Vinay Scientific Company, Vijayawada. All glassware was washed with detergent, rinsed with water, dipped in chromic acid for 24 hours and finally rinsed with distilled water and then hexane. The glassware was sterilized at 120°C temperature in the hot air oven for overnight.

### 3.6.3 Pathology Data:

Pathology data were obtained from routine diagnostic tests including an assessment of malignancy by the pathologists operating in the two above referred hospitals. All the 300 women recruited for the present study were divided into two groups, benign (n = 120) or malignant cancer (n = 180). The results from the questionnaire and the pesticide levels of each patient were compared between two
groups to draw a conclusion regarding the role of pesticides in inducing the breast cancer.

The pathology of some tumours was classified further into a number of different categories. According to Dupont and Page (1985) and Bodian (1993) benign breast disease can be further classified according to the degree of epithelial proliferation, to produce more descriptive groups of benign breast disease. Dupont and Page (1985) also suggested that there is a continuum of events from a truly benign state to an invasive cancer with a poor prognosis. On this basis, all the tumours were reclassified into the categories listed below and the questionnaire and pesticide levels compared between the groups.

1) Normal (n = 28), as determined from breast mammoplasty.
2) Non-Proliferative Benign (n=36), included women diagnosed with fibroadenoma, cysts, apocrine metaplasia and lesions with mild atypia.
3) Proliferative Benign (n = 30), included women diagnosed with moderate or florid hyperplasia, intraductal papilloma and sclerosing adenosis
4) Atypical Hyperplasia (n =26), including both ductal and lobular types
5) In situ Carcinoma (n = 34), including both ductal and lobular types
6) Grade 1 Carcinoma (n = 24)
7) Grade 2 Carcinoma (n = 86)
8) Grade 3 Carcinoma (n = 36)

Given that some of these categories are precursors to breast cancer development, the subjects were further combined as truly benign or truly cancerous:

True Benign (combined groups normal, non-proliferative benign, proliferative benign and Atypical Hyperplasia) (n = 120). True Cancer (combined groups in situ Carcinoma, Grade 1, 2 and 3 carcinoma from above) (n = 180)
3.6.4 Transport:

The tissue and the blood samples were transported on dry ice to the laboratory of Acharya Nagarjuna University and the tissue samples were stored at -70°C whereas the blood samples were stored at – 20°C until extraction and analysis.

3.7 Extraction and cleanup of the samples:

3.7.1 Extraction from Blood:

Extraction was based on the method followed by Agarwal et al. (1976) with slight modifications. Blood (5 ml) was diluted by adding 25 ml of distilled water and 2 ml of saturated brine solution was added and transferred to a 125 ml capacity separatory funnel and extracted with hexane: acetone (1:1) (20ml) (thrice) by shaking the separatory funnel vigorously for 15-20 minutes by releasing the pressure intermittently. The layers were allowed to separate. The three combined extracts were passed through anhydrous sodium sulphate and concentrated to about 1-2 ml using a rotary vacuum evaporator.

3.7.2 Extraction from tissue:

The pesticides are lipophilic in nature and accumulate in tissue lipids; therefore for the extraction of pesticides, tissues macerated and extracted, and for such purpose the following procedures were adopted.

3.7.3 Soxhlet Extraction Method:

The extraction was carried out in a soxhlet apparatus. For the extraction of samples of adipose tissue, Holden and Marsden (1969) method was followed. A known quantity of samples weighing about 1 gm was macerated with Na₂SO₄ (anhydrous sodium sulphate) and was transferred into a thimble made of filter paper.
The thimble was then placed in the extractor which was fitted to the bolt head flask, containing 100 ml of n-hexane then fitted with a condenser, connected to the tap water for cooling. The apparatus was then placed in a water bath. The process of extraction was carried out for seven hours ensuring the 90% extracts were drawn into solvent. The tissue extracted solvent was then reduced to about 1 ml with the help of the rotary flash evaporator. For cleanup and separation of pesticides the column chromatography (sorption) was employed and the extracted material was passed three to four times through the column, to ensure the cleanup of the extract.

3.7.4 **Clean up procedures:**

The process of sorption was carried out in chromatographic columns of alumina (Holden et al., (1969) and silica (Kadoum (1967); (1968). Florisil, Alumina and Silica Columns.

The alumina column was used for the separation of tissue debris from pesticides which was used by Holden and Marsden (1969). The column was made of glass having length of 40-42 cm with an internal diameter of 6 mm. The column was filled with 2 grams of the florisil of 0.3 micron size (already activated at 180°C for overnight in a hot air oven and then partly deactivated by shaking with 5% by weight of water). The concentrated extract was re-dissolved in 1 ml of n-hexane (fractionated) and transferred to the surface of the florisil column. Now the pesticides adsorbed on the column were eluted with 12 ml of n-hexane and the eluted samples were then reduced to 1 ml by the rotary vacuum evaporator and were passed through a new column. The new column of the same size was packed with 2 grams of silica gel for column chromatography No. 60, 0.060 millimetre size (activated at 120°C for 2 hours, cooled and deactivated with 3.5% distilled water). For the removal of traces of moisture a layer of activated Na₂SO₄ was set on top of the silica gel. The elution of
pesticides was done by n-hexane and Acetone 3:1, 1:1, 1:3 basing on their R_f values obtained in TLC for concerned pesticides and standards. The fractions were concentrated to 1 ml, separated and identified qualitatively by TLC and quantified by GC.

### 3.7.5 Qualitative identification of pesticide residues by Thin layer Chromatography:

Thin layer chromatography (TLC) was used to identify the pesticide residues extracted from the blood as well as tissue samples. The samples were extracted into the organic solvents like acetone, hexane and methanol by following the standard methods of Mills and Olney (1977) and Edwards (1973). Tissue samples were ground in the mixer by adding anhydrous sodium sulphate and after thorough grinding the organic solvents were added and shaken on a shaker for half an hour. The samples were extracted thrice with organic solvents to ensure 90% of the extraction efficiency and made to 1ml concentration. For the identification of the Organochlorine samples Moats (1966) method was followed, and for the identification of the Organophosphates, Murthy et al, (1983) method was followed. The samples and standards of pesticides were spotted using a micropipette on the TLC plates. Basing on the R_f values the samples were identified.

#### 3.7.5.1 TLC analysis of organochlorine pesticide residues:

The method of Moats (1966) was followed. Silver nitrate impregnated silica gel borosil glass plates were prepared by adding silver nitrate 0.3% to the silica gel. The plates were dried and activated at 80°C for 15 minutes. The 1ml samples and 1 ml of standards were applied using a sample spotter and micro pipette. The plates were developed in TLC Chamber. The plates were removed from the chamber, air dried at room temperature for 15 minutes and exposed to UV light. Exposure to steam
improves the resolution of the spots. Hexane + Acetone (9.5: 0.5) was used as carrier solvents in the developing chamber. The $R_f$ values of the OC residues were noted down (Table: 3.1).

**Table: 3.1**

$R_f$ values of organochlorine pesticide standards in 95% Hexane + 5% Acetone solvent systems

<table>
<thead>
<tr>
<th>STANDARD</th>
<th>$R_f$ VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>p, p’ DDT</td>
<td>96</td>
</tr>
<tr>
<td>P, p’ DDE</td>
<td>93</td>
</tr>
<tr>
<td>$\alpha$ – Endosulfan</td>
<td>70</td>
</tr>
<tr>
<td>$\alpha$– HCH</td>
<td>98</td>
</tr>
<tr>
<td>$\beta$ – HCH</td>
<td>65</td>
</tr>
<tr>
<td>$\gamma$– HCH</td>
<td>99</td>
</tr>
</tbody>
</table>

3.7.5.2 **TLC analysis for organophosphate pesticide residues:**

Murthy *et al*, (1983), method was followed for identification of organophosphate pesticide residues. The method is based on conversion of organophosphate to its inorganic counterpart. The silica gel was washed with copious amounts of all glass triple distilled water to ensure the removal of inorganic phosphate. Inorganic phosphate free silica gel was spread on the plates and activated at 130°C in hot air oven. After cooling to room temperature, the samples and standards were applied using a sample spotter and micro pipette. The plates were developed in TLC Chamber. Removed from the developing chamber and dried at room temperature. On these plates 5ml of molybdate antimony tartrate and 2ml of ascorbic acid was sprayed. Blue spots were developed in the organophosphate sample
regions. The $R_f$ values of the OP residues in comparison with standards were calculated and noted down (Table: 3.2).

Table: 3.2

$R_f$ values of organophosphate pesticide standards in different solvent systems

<table>
<thead>
<tr>
<th></th>
<th>System-I</th>
<th>System-II</th>
<th>System-III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 Hexane : 1 Acetone</td>
<td>1 Hexane : 1 Acetone</td>
<td>3 Hexane: 1 Methanol</td>
</tr>
<tr>
<td>Malathion</td>
<td>76</td>
<td>68</td>
<td>Malathion</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>72</td>
<td>Phosphamidon</td>
<td>Chlorpyrifos</td>
</tr>
<tr>
<td>Monocrotophos</td>
<td>25</td>
<td>76</td>
<td>Standard</td>
</tr>
<tr>
<td>Phosphamidon</td>
<td>20</td>
<td></td>
<td>64</td>
</tr>
</tbody>
</table>

3.7.6 Gas-Liquid Chromatographic analysis:

Gas Chromatograph used for pesticide residue analysis was GC-MS [5975C Inert MSD] with 63 Ni selective Electron-Capture Detector with advanced software (Chromcard-32 bit) and Nucon –GC- 5765 series equipped with Nitrogen Phosphorus Detector. GC columns were capillary columns, HP - 1701 14%-Cyanopropyl-phenyl-methylpolysiloxane (HP-5MS, 0.25mm x 0.25µm x 30mtrs), HP - 17 50% phenol methyl polysiloxane (HP-5MS, 0.25mm x 0.25µm x 30mtrs), and DB-5, coated with 5% diphenyl and 95% dimethyl polysiloxane (HP-5MS, 0.25mm x 0.25µm x 30mtrs) J & W make for cross verification. Rotatory flash evaporator (Buchi type) was used to concentrate the extracted samples and a 10-µl syringe from Hamilton Co, was used to inject the extract into the column.
3.7.6.1 Qualitative and quantitative identification of pesticide residues (organochlorines and organophosphate) by Gas Chromatography-Mass Spectroscopy:

a. **Laboratory reagent blank:**

An aliquot of reagent grade water was treated exactly as a sample including exposure to all glassware, equipments, solvents, and reagents used with the sample matrix. No analytic peak was detected in laboratory reagent blank.

b. **Laboratory fortified blank:**

An aliquot of reagent grade water to which known amount of pesticides was added in the laboratory in ppb range was analysed exactly like the sample. The recovery of the pesticides over the background values obtained from unfortified samples was more than 80 per cent of all the pesticides.

c. **Laboratory fortified sample matrix:**

An aliquot of sample matrix (tissue and blood) was prepared to which known quantities of the pesticides were added in the laboratory in the ppb range. This laboratory fortified matrix was analysed exactly like the sample. 15-25% of the samples (minimum) were fortified with a known concentration of pesticides and percent recovery was calculated. Extraction and cleanup were done as mentioned and the recovery of the pesticides over the background values obtained from unfortified samples was more than 85 percent. Standard deviation and coefficient of variation was less than 10 indicating repeatability of the method. All calculations were done as described in US EPA method and the amount of residues in samples was obtained in mg/l (ppm) of adipose tissue and whole blood. The first run of each day consisted of a methanol injector followed by hexane, to clean out any residues that may have existed on the column or in the injection liner. 2µg/ml extracts of breast adipose tissue, blood
samples were run in sequence and individual peaks were identified by comparison with retention times in a standard mix containing each of the pesticides under investigation. The standard mix was injected into the gas chromatograph at the end of each series of runs. Once the peaks were identified in the breast adipose extracts and blood extracts, they were quantified by using the equations generated for the calibration curves of each corresponding pesticide. Gas Chromatographs equipped with ECD were checked for linearity. Instrumental limit of detection for GC- ECD was 0.01 ng / ml.

3.7.6.2 Analysis of organochlorine pesticides using Gas Chromatography-Mass Spectroscopy:

The organochlorine pesticides α, β, γ, δ HCH, alachlor, aldrin, α, β -endosulfan, endosulfan SO₄, dieldrin, DDT, DDD, DDE and organophosphate pesticide monocrotophos, atrazine, phorate sulfone, chlorpyrifos, ethion, malathion, phosphamidon in the breast adipose tissue was extracted and analysed by gas chromatography. Peak identification was performed by the GC – MS software. Calibration table set up with a relative retention time (RRT). The following conditions were followed for the analysis (Table: 3.3).

### Table: 3.3

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Organochlorines</th>
<th>Organophosphates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detectors</td>
<td>Electron capture</td>
<td>Nitrogen Phosphorus</td>
</tr>
<tr>
<td>Oven temperature (°C)</td>
<td>290</td>
<td>270</td>
</tr>
<tr>
<td>Detector temperature (°C)</td>
<td>290</td>
<td>280</td>
</tr>
<tr>
<td>Injection temperature (°C)</td>
<td>280</td>
<td>280</td>
</tr>
<tr>
<td>Flow rate (ml/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Carrier gas</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>b. Make-up gas</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Total run time (Minutes)</td>
<td>29.64</td>
<td>29.64</td>
</tr>
</tbody>
</table>
3.7.7 Steroid hormones:

Commercial Immunoassay kits were used to measure plasma concentrations of estradiol, testosterone, and progesterone. Conditional logistic regression analysis was used to examine the association between steroid hormones and breast cancer risk.

The quantitative determination of total estradiol, progesterone and testosterone concentration in human serum was estimated by Monobind Commercial Immunoassay Kit methods and the procedure was followed as given in the instruction leaflet (Abraham et al, 1981; Bergquist et al, 1983).

Procedure:

Before proceeding with the assay, all the reagents, serum reference and controls are brought to room temperature (20 – 27°C). Later the microplates, wells, were formatted for each serum reference control and patient’s specimens to be assayed in duplicate. Pipette 0.025 ml (20µl) of the appropriate serum reference, control or specimen into the assigned wells. To this 0.050 ml (50µl) of the estradiol biotin reagent was added to all wells, swirled the microplates gently for 20 – 30 seconds to mix and incubated for 30 minutes at room temperature. Then 0.050 ml (50µl) of the estradiol enzyme reagent was added to all wells, and swirled the microplate gently for 20 – 30 seconds to mix and incubated for 90 minutes at room temperature. Later the contents of the microplates were subjected for decantation/aspiration. Then 350 µl of wash buffer was added, decanted in an automatic plate washer. After that 0.100 ml (100 µl) of substrate solution was added to all wells and incubated at room temperature for 20 minutes. To this 0.050 ml (50 µl) of stop solution was added to each well and gently mixed for 15 – 20 seconds. The absorbents in each well were read at 450 nm (using a reference wavelength of 620 – 630 nm) within 20 minutes of adding the stop solution.
The same procedure was adopted for determining the concentrations of progesterone and testosterone in benign and cancer groups.

3.7.8 Statistical Analysis:

All clinical pathology, questionnaire and breast biopsy pesticide concentration data were statistically analysed using SPSS-17 computer software. Organochlorine pesticide concentrations and Organophosphate pesticide concentrations in the breast biopsy tissue and blood produced a skewed distribution when analysed for normality and were subsequently transformed logarithmically to mitigate the skewed effect. The data generated from the questionnaire, the clinical pathology and the analyses of breast tissue were analysed, firstly, by descriptive statistics, to determine scores, frequencies and the associated variance of each variable.

The logarithmically transformed pesticide data and other continuous variables were cross tabulated against the breast disease continuum, using one-way analysis of variance (ANOVA), to test for variance of means between groups and for any linear relationships throughout the disease continuum. The data derived from the questionnaire required coding for yes or no answers or for multiple response answers before analysis was cross-tabulated against the breast disease continuum using chi-square contingency tables. Significance was determined using Pearson’s statistic.

During the year 2008 to 2011, 300 women who approached for breast abnormalities and who underwent routine biopsy at the Guntur Government General Hospital, Bommidala Cancer Institute were recruited and met the criteria required for the study.
The chemical exposure and risk factor data from the questionnaire and transformed pesticide concentrations were analysed by factor analysis. After initial extraction, factors were rotated to aid interpretation and oblique rotation to identify the most correlated combination with the use of SPSS software. In simpler terms, rotation simplifies the factor patterns so that the variables in each factor with high factor loading scores are different for each factor, yielding distinct groupings of variables that were used to interpret the factors. To determine the number of factors to retain the following criteria was applied: there must be at least three variables in the factor with a high factor loading score (70.40 or greater); factors must have an eigenvalue greater than 1.00; and each factor must account for at least 5% of the total variance.

Ideally, the number of retained factors will be small, and will explain the majority of the variance in the observed data (Floyd and Widaman (1995). The number of pesticides was determined to assess if there was any synergy with more than one pesticide and its correlation with disease state.

3.8 Single Nucleotide Polymorphism of blood samples of breast cancer patients:

A single-nucleotide polymorphism (SNP, pronounced snip) is a DNA sequence variation occurring when a single nucleotide — A, T, C or G — in the genome (or other shared sequence) differs between members of a biological species. These studies have already shown that germ line polymorphisms play a significant role in disease initiation and response to therapy (Shastry (2007). However, what is less well studied is the role of germ line polymorphisms in cancer progression with relation to pesticide exposure.
Method for SNP analysis:

500 µl of blood was taken in a sterile 2ml eppendorf tubes. To this 1.5ml of red blood cell lysis buffer was added and mixed gently. The lysed sample was centrifuged at 6000 rpm for 5 minutes and the supernatant was discarded. To the pellet, 500µl of sodium EDTA was added and vortexed mild to hard to ensure that no R.B.C clumps remain. 5µl of proteinase-K and 100ul of SDS were added and kept for incubation at 50-55 °C for 3-4 hours. To this incubated sample, equal amount of phenol was added, and mixed using shaker for 20 minutes. The sample was centrifuged at 5000rpm for 20 minutes. After centrifugation the supernatant was carefully transferred into a fresh tube and to this equal volume of phenol-chloroform was added, and placed it in an overhead shaker for 20 minutes followed by centrifugation for 20minutes.

Then the supernatant was collected into a fresh tube and equal volume of chloroform isoamylalcohol (CIA) mixture was added. Again it was placed in an overhead shaker for 20 minutes and centrifuged for 20 minutes at 5000rpm. The supernatant was transferred into a fresh tube and 1/10th volume of 3M sodium acetate and equal volume of isopropanol or double the volume of absolute alcohol was added and mixed gently by inverting the tube to observe the DNA precipitate.

Precipitated DNA was taken in fresh sterile 1.5ml microfuge tube and centrifuged at 10000rpm for 30 minutes. After centrifugation the supernatant was decanted and 500µl of 70% ethyl alcohol was added and centrifuged at 10000rpm for 5 minutes. Once again the supernatant discarded and 500µl of ethyl alcohol was added and centrifuged at 10000rpm for 5 minutes and decanted the supernatant. The pellet was air dried at room temperature (or 37°C) for 20-30 minutes. Then the dried
pellet was dissolved in 10- 15µl of Tris-EDTA buffer. Later 1 to 2µl of sample solution was used to visualize DNA on 0.8% agarose gel.

3.8.1 Agarose Gel Electrophoresis:

Agarose 0.8% for visualization of genomic DNA (1.2% for the visualization of PCR amplification) was weighed and mixed with 25 ml of 0.5X TBE buffer. It was then boiled for 40 seconds in a microwave oven to dissolve the agarose in TBE buffer. 1µl of ethidium bromide was added to the agarose and was mixed well. Finally it was poured onto gel casting platform containing comb and allowed to solidify. The Agarose gel was then placed in horizontal gel electrophoresis apparatus containing 0.5X TBE buffer and the comb were carefully removed from the gel. 5 µl of the isolated genomic DNA sample was mixed with 5 µl of 6X gel loading dye and loaded onto the wells of the agarose gel and the electrophoresis was run at 40 mA and the samples were analysed for genomic DNA under the UV illumination.

3.8.2 Quantification of isolated genomic DNA:

Concentration of isolated genomic DNA was estimated spectrophotometrically as per Sambrooke et al, (2001) using formula OD 1@260 = 50 µg / ml DNA.

3.8.3 Designing of gene specific primer:

The sequence of all three genes was retrieved from NCBI and complete coding sequence and open reading frame was determined by ORF finder ExPAsy Tools. Primers for all these three genes were designed manually and confirmed by Invitrogen Primer Design Tool. After designing, the primer was sent to Sigma Aldrich for synthesis.
3.8.3.1  *h- RAS genes:*

NCBI Reference Sequence: NM_001130442.1

**Primer for PCR**

\begin{verbatim}
\textit{h-RAS FP 5'} ATG ACG GAA TAT AAG CTG GTG G
\textit{h-RAS RP 5'} GGA GAG CAC ACA CTT GCA GC
\end{verbatim}

3.8.3.2  *Hydroxysteroid (17-beta) dehydrogenase (ESD):*

NCBI Reference Sequence: NM_000413.2

**Primer for PCR**

\begin{verbatim}
\textit{ESD FP 5'} ATG GCC CGC ACC GTG GTG CTC
\textit{ESD RP 5'} TTA CTG CGG GGC GGC CGG AG
\end{verbatim}

3.8.3.3  *Vascular Endothelial Growth factor (VEGF):*

**GenBank:** AL136131.15

**Primer for PCR**

\begin{verbatim}
\textit{VEGF FP 5'} CTG ACG GAC AGA CAG ACA GAC
\textit{VEGF RP 5'} TCA CCG CCT CGG CTT GTC ACA
\end{verbatim}

3.8.3.4  *Re-suspension of primers:*

The primer was supplied in the lyophilized form and re-suspended in TE buffer (Tris EDTA). Two aliquots for both (forward and reverse) were made during the re-suspension of primer standard and working. Standard 100µm and working 10µm in TE buffer and stored in -20°C. For the PCR reaction working solution was used as per the volume of the reaction mixture. Each of oligos was used twice as excess freeze thaw may detach the hydroxyl group from the 3' position of the primer. All the enzymes were maintained in -2°C and christening was used in all DNA protocols.
3.8.4 Polymerase Chain Reaction (PCR):

PCR is a technique used to amplify the number of copies of a specific region of DNA in order to produce enough DNA to be adequately tested. PCR is based on thermal cyclic process in the sophisticated manner where the reaction mixture allows polymerase to amplify DNA. Reaction mixture contains all necessary requirements for DNA amplification while thermal cycling provides heating and cooling of reaction content needed for amplification of DNA. PCR has become one of the most widely used techniques in molecular biology. It is a rapid and simple means of producing a relatively large number of copies of DNA molecules from minute quantities of source DNA material.

3.8.5 PCR based amplification of genes:

The PCR analyser was programmed to carry out the initial denaturation for 4 minutes at 95°C, followed by 35 cycles of denaturation for 30 second at 95 °C, annealing for 45 second at 60°C (for h-RAS gene), 62°C (for ESD and VEGF gene) and extension for 1 minute at 72 °C. Then the final extension was continued for 10 minutes at 72°C and stored at 4°C for infinite time (Figure: 3.3).

**Figure: 3.3**
Diagrammatic illustration of PCR
After the completion of PCR reactions, 2 µl of the PCR samples was loaded on agarose gel 0.8% against the DNA ladder to confirm the amplification. The gel was kept in the buffer tank and run for 50 minutes at 50mV DC power supply. After completion of the electrophoresis, the gel was analysed in the Gel Doc system.

3.8.6 Purification of Amplified Gene:

The gel elution of the PCR product was carried out using the given Guanidine Thiocyanate Method (Chomczynski and Sacchi (2006)).

Procedure:

The amplified PCR mixture was run on 1.2% agarose gel against a 100 bp ladder and the desired PCR product was excised from gel under the UV fluorescence. The excised gel slices were transferred to a 2 ml micro centrifuge tube. Then 500 µl of bind mix was mixed with gel pieces and incubated at 45°C for 3 minutes and the mixer was centrifuged at 5000 rpm for 5 seconds. To this 500 µl of guanidine thiocyanate solution was added and mixed well. Then the sample was centrifuged at 5000 rpm for 3 seconds and 1 ml of propanol wash was added to the pellet. After addition the sample was centrifuged again at 5000 rpm for 3 seconds and 1 ml of ethanol was added to the pellet and centrifuged for 3 seconds at 5000 rpm.

The supernatant was discarded and the pellet was dried to the powder form to which 50 µl of TE buffer was added and kept in a water bath and maintained at 65°C for 3 minutes and again centrifuged at 5000 rpm for 10 sec to get the pure DNA from the supernatant. The supernatant was taken as the sample and loaded on 1.2% agarose gel against 100 bp ladder to analyse the DNA.
3.8.7 Sequencing of Purified Amplified Gene:

The purified PCR products were sent for sequencing with the PCR primers. The sequencing was performed by chain termination method (Dideoxy method/Sanger (1977)) by using forward primer of 10µm concentration. The sequencing results were analysed with the gene sequence retrieved from NCBI.

3.8.8 Analysis of Polymorphism:

After the sequential result for selected genes SNPs were analysed in 30 different patients by using “Sequencer 5.0” and further results were analysed by using other online tools (SNiPLAY). Thus the study was based on the comparative analysis in between the reference at NCBI SNP Database and sequences obtained after PCR amplification of h-RAS, ESD and VEGF. The purified PCR products were sequenced and loaded in the software (db SNP) and reference was taken from the NCBI SNP database. Here more specifically, checked restriction site analysis of both reference sequence and the sequence of PCR purified products of three genes. The variation in the sequence alters the recognition site for restriction enzyme. Here, the above three genes (h-RAS, ESD and VEGF) from 100 different samples were analysed and observed variable in a definite pattern.

3.9 Animal Studies:

Many pesticides are known endocrine disruptors, and several pesticides and their combinational effects in common use are known to cause mammary tumors in laboratory study of animals. The epidemiological data are inconsistent and difficult to evaluate because of limitations in the methods and data available to estimate exposures across a lifetime. In the light of present research, animal studies were
carried out to evaluate the effects of pesticides like endosulfan, aldrin, malathion and monocrotophos and their combinations.

3.9.1 Animals:

Female albino rats (Wistar strain) of average body weight 120 - 140 g was kept separately in individual polypropylene cages with stainless steel hopper in an air conditioned room at 24°C of the animal house under uniform animal husbandry conditions. The animals were fed a basal diet (Hindustan Lever Laboratory Animal Feed) and water ad libitum. The animals were acclimatized to temperature and lighting 12 h light/dark conditions of the animal house for one week.

3.9.2 Experimental Design:

Forty eight adult female albino rats were divided into eight groups- 6 each and treated with the organochlorine and organophosphate pesticides (endosulfan, aldrin, malathion and chlorpyrifos) through drinking water for 30 successive days as the following:

Group (1): Rats served as a control group (fed with food and drinking water).

Group (2): Rats treated with endosulfan (received 0.015 ml/kg b.w. /Day which represents the lower dose of acute poisoning) (1/10th of the reported value).

Group (3): Rats treated with aldrin (received 0.025 ml/kg b.w. /day, which represents the lower dose without inducing toxic effects) (1/10th of the reported value).

Group (4): Rats treated with aldrin malathion (received 17 ml/kg b.w. /day which represents the lower dose) (1/10th of the reported value).
Group (5): Rats treated with aldrin chlorpyrifos (received 5 ml/kg b.w. /Day which represents the lower dose) (1/10\text{th} of the reported value).

Group (6): Rats treated with endosulfan and aldrin in equal ratio (received 10 ml/kg b.w. / Day which represents the lower dose) (1/10\text{th} of the reported value).

Group (7): Rats treated with malathion and chlorpyrifos in equal ratio (received 10 ml/kg b.w./day which represents the lower dose) (1/10\text{th} of the reported value).

Group (8): Rats treated with Endosulfan, aldrin, malathion and chlorpyrifos (received 10\mu g/g b.w. /day which represents the lower dose) (1/10\text{th} of the reported value).

Pesticides were administered orally daily through drinking water in glass bottles, and the bottles were cleaned daily. All animals were observed at least once daily for behavioural observations; signs of intoxications, mortality, morbidity, and food and water consumption were monitored daily.

3.9.3 Sampling:

3.9.3.1 Blood samples:

At the end of each experimental period, (2, 3 & 4 weeks), blood samples were collected, from the orbital sinus vein of fasted rats (control and treat animals), using anaesthetic ether by heparinised capillary tubes in plain tubes, according to Schalm, and allowed to be clotted at room temperature to obtain serum for hormonal assay.

3.9.3.2 Steroid Hormones:

The IBL - AMERICA Unconjugated estradiol, progesterone and testosterone ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA).
3.9.3.3 Single Nucleotide Polymorphism:

For validation of polymorphism, analysis of chemical exposure to human being by an animal study was carried out with specific chemicals, and it has shown the maximum variant at the gene level. The albino rats were selected for polymorphism analysis in h-RAS gene, Vascular Endothelial Growth Factor gene (VEGF) and Estradiol gene.

The rats selected for study were examined for any natural variation in the gene, and further subjected to periodic chemical exposure. The rats were treated with the select chemicals for four weeks. The blood was collected in the second, third and fourth week from all the rats, preserved with an anticoagulant to avoid any coagulation, while storage and total genomic DNA from isolated blood sample was isolated as per standard protocol. The isolated genomic DNA was quantified and preserved in TE buffer at 4°C. For the isolation of genomic DNA and to run agarose gel electrophoresis, the same procedure was followed as described in the earlier part of the thesis. The gene sequence was retrieved from NCBI for selected rats for the following gene and primer, and were designed and synthesized at Sigma Aldrich Pvt. Ltd. The sequence of a gene and designed primer were found as under:

3.9.3.3.1 h-RAS genes:

Primer for PCR

\[ h\text{-RAS M FP 5'} ATGACCGAATATAAACTGGTGTTG \]
\[ h\text{-RAS M RP 5'} CTCAGCACGCATTGCAG \]

3.9.3.3.2 Vascular Endothelial Growth factor (VEGF):

Primer for PCR

\[ VEGF M FP 5'} A T G A A C T T T C T G C T G A G C T G G \]
\[ VEGF M RP 5'} G G A T C C T G C A C A A A C A G A T G T \]
3.9.3.3 Hydroxysteroid (17-beta) dehydrogenase (ESD):

Primer for PCR

\[
\begin{align*}
    &ESD\ M\ FP\ 5'\quad ATGAGCCCGTTTGCAGCGAAAG \\
    &ESD\ M\ RP\ 5'\quad ATGCACGCTTGCAGCGCGCG
\end{align*}
\]

3.9.3.4 PCR based amplification of genes:

The primer was suspended in fresh TE buffer in the different aliquots as stock 100µM and working 10µM and freeze in -80°C. The 10µM concentrations of primers were used for the setup of reaction mixture in ice trays. The genomic DNA was added at last during preparation of reaction mixture followed by Taq DNA Polymerase and the reaction was set up as given in the following (Table: 3.4)

**Table: 3.4**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Buffer (10x)</td>
<td>5 µl</td>
<td>1x</td>
</tr>
<tr>
<td>Deoxynucleotide mixture</td>
<td>1µl</td>
<td>200µM</td>
</tr>
<tr>
<td>Upstream Primer (10mM)</td>
<td>2µl</td>
<td>0.1µM</td>
</tr>
<tr>
<td>Downstream Primer (10mM)</td>
<td>2µl</td>
<td>0.1µM</td>
</tr>
<tr>
<td>Template DNA</td>
<td>2µl</td>
<td>8µg/ml</td>
</tr>
<tr>
<td>Tag DNA Polymerase</td>
<td>0.2 µl</td>
<td>0.2 unit/µl</td>
</tr>
<tr>
<td>Nuclease free Water</td>
<td>38.8µl to make a 50µl reaction volume</td>
<td></td>
</tr>
</tbody>
</table>

The PCR machine was programmed to carry out the following steps.

a) Initial denaturation for 4 minutes at 95°C.

b) 35 cycles of denaturation for 30 seconds at 95°C, annealing for 45 seconds at 62°C (For h-RAS gene, 60°C for ESD and 58°C VEGF gene) and extension for 1 minute at 72°C.

c) Final extension for 10 minutes at 72°C.

d) Storage at 4°C for infinite time.
After the completion PCR reaction 5 µl of the PCR samples were loaded on agarose gel 0.8% against DNA ladder to confirm the amplification. The gel was kept in the buffer tank and run for 50 minutes at 50mV DC power supply. After the electrophoresis completion, the gel was analysed in the Gel Doc system. The PCR products were purified by Guanidine Thiocyanate Method as described earlier.

The purified PCR products were sent for sequencing with the PCR primers. The sequencing was performed by Chain Termination Method (Dideoxy method) by using forward primer of 10µm concentration. The sequencing results were analysed with the retrieved gene sequence available at NCBI database.

3.9.3.4 Analysis of Polymorphism:

After the sequential result for selected genes SNPs were analysed by using “sequencer 5.0” and further results were analysed by using other online tools. Thus the study was based on the comparative analysis in between the reference at NCBI SNP Database and sequences obtained after PCR amplification of h-RAS, ESD and VEGF. The purified PCR products were sequenced and loaded in the software and reference was taken from the NCBI SNP database. Here, more specific restriction site analysis of both reference sequence and the sequence of PCR purified products of three genes were checked. The variation in the sequence alters the recognition site for restriction enzyme and found variations in definite patterns (Chapter IV).

3.9.3.5 Sacrifice and tissue preservation:

Animals were sacrificed on the 4th week of the study. Mammary tissue and liver were removed from sacrificed rats immediately, cleaned of adhering tissues and weighed. Then, the samples were taken for histopathological examination through the light microscope.
3.9.3.6 **Histopathological Changes:**

The liver and the mammary tissue of different groups were removed and fixed in 10% formal saline. Physiological saline solution (0.75% NaCl) was used to rinse and clean the tissue. They were fixed in aqueous Bouin's solution for 48 h, processed through a graded series of alcohol cleared in xylene and embedded in paraffin wax. Tissues alone were processed by double embedding technique. Sections were cut of 6µ thickness; stained with Ehrlich hamatoxillin and Eosin (dissolved in 70% alcohol) (Humason (1972) and were mounted in Canada balsam. Paraffin sections of 5 mm thick were stained with haematoxylin and eosin (Drury and Wallington (1980) and Masson trichrome stain to demonstrate the collagen fibers (Masson (1929). All sections were observed under the light microscope and the photo-monographs were taken and presented in chapter IV.