MATERIALS & METHODS
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Chemicals:
Phenobarbitone Sodium, Direct Red 80, Naphthol AS-MX Phosphate, Fast Red, Collagen Type I Standard, Ethidium Bromide were procured from Sigma Chemical (USA). Rest of chemicals (Ca Standard, Lanthanum Chloride, Ammonium Molybdate, Ammonium Metavanadate, Potassium Dihydrogen Phosphate, Pepsin, ALP Kit, Trizma, DMSO, NMA, LMPA etc.) were purchased locally.

Animals:
Female Wistar rats (90 days old and 210-220 gm body wt.) were obtained from animal facility of Jawaharlal Nehru University, New Delhi. They were randomly divided into three groups (Control, OVX and OVX+PEMF) and housed in an air conditioned room (temperature was maintained at 25°C). They were provided with standard food pellets (Hindustan lever ltd., India) and tap water ad libitum.

Experimental Setup

Induction of Osteoporosis:
Bilateral Ovariectomy in rats was performed as described by Bellino [200]. The animals were prepared for surgery using pre-operative and anesthetic procedures. Anesthesia was induced by intraperitoneal (IP) injection of 30 mg of Phenobarbitone Sodium per Kg body weight of rat. After the onset of anesthesia, the lumbar ventrum was shaved bilaterally and the exposed skin was prepared for aseptic surgery (10% povidone-iodine scrub followed by 70% alcohol wipe). For each ovary a 1-cm dorsal flank incision, penetrating the abdominal cavity was made. The periovaryan fatty tissue was identified and retracted. Using forceps, the periovaryan fat was gently grasped and exteriorized. Mosquito forceps were used to crush the fallopian tube and cranial-most part of the uterine horn. The ovary was dissected out by cutting above the clamped area. After removal of ovary, the uterine horn and other blood capillaries were ligated. The uterine horn was returned into the abdomen. Stitches were made on subcutaneous muscles and skin incision by Ethicon (absorbable) and nylon thread respectively. Similar process was followed to remove other ovary too. Postoperative care was taken by giving analgesic (Aspirin 30 mg/Kg) and antibiotic (Anthrocin 250; equivalent of 250 mg of Erythromycin
Estolate) twice a day intraperitonially (IP) to prevent against any infection. This was continued for a week till they recovered. After one month of surgery, induction of osteoporosis was taken place and then treatment of electromagnetic field exposure was done with bone stimulator.

**Bone Stimulator:** It is a device that is connected to the affected area, outside the body and requires no surgical intervention. The device, known as *Electrical Bone Stimulator* has already been in use for treating fractures for more than 20 years. It emits a pulsed electromagnetic field (PEMF) on the affected site of bone. The bone stimulator is connected to copper capacitor electrode which was tied to the affected site. It emits a low quantity of energy.

For the exposure, we used a bone stimulator with following specification.

- **Carrier Frequency:** 14.0 MHz
- **Modulated Frequency:** 16.0 Hz
- **Amplitude:** 10 V (peak to peak)
- **Output Wave Shape:** Square
- **Electrode diameter:** 1 cm
- **Average electric field between electrodes:** 7.8 Volt/m

Calibration was done with the help of Iwatsu Oscilloscope SS – 5711 C, Japan and output of bone stimulator was given to each rat separately by a pair of electrodes (Fig: 1 a. and b.) in one leg for 60 days for 2 hrs/day. Current density at the point of application was 80 μA/cm². Other leg was tied with same type of electrodes without any connection to stimulator (Sham-exposed). Rats were lightly anesthetized, before giving the exposure to avoid the disturbance in the exposure.
Fig. 2. Schematic diagram of exposure set up.

Fig. 3. Photograph shows exposure set up.
Mineralogical Analysis

**Bone Samples Preparation:**
Rats were then sacrificed with an intraperitoneal overdose of phenobarbital sodium, and tibia and femora of Control (Normal rats), OVX+PEMF (electrical stimulation to ovariectomised rats) and OVX (ovariectomised rats) groups were freed from soft tissues and stored at -20° C for various assays. Volume of all fresh bones were measured by submersion of bone in a water filled container with a scale sensitivity of 0.01 ml. After measuring the volume, bones were lyophilized for 10 hrs and powdered down into fine particles with the help of mortar and pestle. Powdered samples were kept at 60° C overnight in vacuum oven for determination of Total Dry Bone Weight (organic + inorganic contents).

**Mineral and organic content:**
Bone samples were kept at 700° C in a muffled furnace (Widsons Scientific Works, India) for 10 hrs. The ash weight was indicated as Bone Mineral Content. Difference in dry bone and ash weight was measured as organic content and following parameters were calculated:

Bone Mineral Content (BMC) = Ash weight (mg.)

Bone Organic Content (BOC) = Total Dry Bone Wt. - BMC

Bone Mineral Density \( BMD = \frac{\text{BMC}}{\text{BoneVolume}} \)

{The application of Archimedes principle is the standard method for determination of Bone Mineral Density (gm/cm³).}
Calcium Analysis:
Calcium was determined in rat bone samples by Atomic Absorption Spectroscopy (AAS). Before analysis, powdered bone samples were digested in acid and then calcium was quantified by flame atomic absorption spectrophotometry (SHIMADZU AA-6800). 50 mg of bone powder from each sample was digested in 5 ml of aquaregia and heated in Teflon bomb at 100°C for 10 hrs. Subsequently, the digested samples were diluted with milli Q water and 100 ml stock solution was made. 5 ml of stock solution was added with 95 ml of 0.5% lanthanum chloride and filtered through 0.45 μm pore size filter. The standards of different Ca concentrations (i.e., 1 PPM, 2 PPM, 4 PPM, 5 PPM and 10 PPM) were prepared from commercial standard solution (Ranbaxy, India). The standards and samples were read against the blank solution. The reading of samples, standards and blank were noted. The concentration of calcium in the samples were calculated by equation obtained from the standard curve (R²=0.944).

<table>
<thead>
<tr>
<th>Lanthanum Chloride (5% working stock)</th>
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<tbody>
<tr>
<td>58.64 g of lanthanum oxide (La₂O₃) was added to approximately 50 ml of distilled H₂O. Thereafter 250 ml of concentrated HCl was added slowly and diluted to 1 L with distilled H₂O.</td>
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</tbody>
</table>

<table>
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<th>Final ppm desired</th>
<th>Dilution</th>
<th>Amt. 1000 ppm stock</th>
<th>Amt distilled H₂O</th>
<th>Total volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 %</td>
<td>1/10</td>
<td>100 ml</td>
<td>900 ml</td>
<td>1000 ml</td>
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</table>

Calcium Standard

<table>
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<th>Final ppm Desired</th>
<th>Dilution</th>
<th>Amt. 1000 ppm Stock</th>
<th>Amt. 0.5% LaCl₃</th>
<th>Total volume</th>
</tr>
</thead>
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<tr>
<td>1 ppm</td>
<td>1/100</td>
<td>0.5 ml</td>
<td>49.5 ml</td>
<td>50 ml</td>
</tr>
<tr>
<td>2 ppm</td>
<td>1/50</td>
<td>1 ml</td>
<td>49 ml</td>
<td>50 ml</td>
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<td>4 ppm</td>
<td>1/25</td>
<td>2 ml</td>
<td>48 ml</td>
<td>50 ml</td>
</tr>
<tr>
<td>5 ppm</td>
<td>1/20</td>
<td>2.5 ml</td>
<td>47.5 ml</td>
<td>50 ml</td>
</tr>
<tr>
<td>10 ppm</td>
<td>1/10</td>
<td>5 ml.</td>
<td>45 ml.</td>
<td>50 ml</td>
</tr>
</tbody>
</table>
Phosphorus Analysis:
Phosphorus analysis of bone samples were performed by Vanado-Molybdo-phosphoric acid colorimetric method in UV-VIS spectrophotometer (Varion AA-20, Varion Analytical Instrument). 50 mg of dry bone powder from each sample was digested in a mixture of 1 ml H₂SO₄ and 5 ml of HNO₃ and then heated till the solution became 1 ml, for complete removal of HNO₃. Thereafter the solution was neutralized and 100 ml of sample stock solution was prepared with double distilled water. 5 ml of sample stock solution was added to 30 ml of double distilled water and 15 ml of Vanadate-molybdate reagent (VMR) and filtered through 0.45 μm pore size filter paper (Millipore). Phosphorus concentrations were evaluated with respect to standard solutions of 0.2 PPM, 0.4 PPM, 0.6 PPM, 0.8 PPM and 1 PPM concentration from phosphate (KH₂PO₄) standard stock solution. The standards and samples were read against the blank solution at 470 nm wavelength. The reading of samples, standards and blank were noted. The concentration of phosphorus in the samples were calculated by equation of standard curve (R²=0.9976).

Reagents
Vanadate-Molybdate Reagent (VMR):
Solution A: 25 g of ammonium molybdate was dissolved in 400 ml distilled water.
Solution B: 1.25 g of ammonium metavanadate was dissolved in 300 ml distilled water by heating up to boiling temperature and then cooled to room temperature and 330 ml of concentrated HCl was added to it. Solution A was poured into Solution B and diluted to 1L.

Standard phosphate solution (1.00 ml = 50 μg PO₄ - P): 219.5 mg anhydrous Potassium dihydrogen phosphate (KH₂PO₄) was dissolved in distilled water and diluted to 1 Liter.

Total Carbon Analysis:
Total carbon analysis of dry bone samples were performed by a carbon analyzer (ELTRA CS 500, Germany). 50 mg of sample was taken in a ceramic boat (pretreated at 800°C overnight and with HCl in a muffle furnace to avoid noise) and kept inside of the analyzer. The samples weighed in a combustion boat on an electronic balance, were to be interfaced to the CS-500. Analysis cycle was started after a ceramic boat with the sample was placed on the furnace platform. At the end of the cycle, the analysis results (percentage of carbon) were appeared on the monitor of instrument. Measurement of carbon percentage was taken at 1200°C.

**X-Ray Diffraction (XRD):**
The *X-Ray Diffraction* (XRD) of bone was carried out by heat treatment process. In brief, the procured bone samples were cleaned well to remove macroscopic adhering impurities. In order to avoid soot and crack formation during heat treatment, the bone samples were boiled in distilled water for 2 hrs. After boiling, the bone samples were immersed in 2% NaCl solution as a preservative for 2 hrs and then they were degreased by immersing in acetone-ether mixture (in the ratio of 3:2 for 2 hrs). Thereafter samples were stored at –20°C until assay. The bone samples were then preheated overnight at 700°C (Widsons Scientific Works, India). X-ray diffraction patterns were obtained with a Philips made X-ray diffractometer (PAN Analytical) with CuKa ($\lambda = 1.543 \text{ Å}$) incident radiation and operated at 45 KV and 35 mA. The XRD peaks were recorded in the $2\theta$ range of 10°–90°. International Center Diffraction Data (ICDD) or formerly known as Joint Committee on Powder Diffraction Standards (JCPDS) is the organization that maintains the database of inorganic and organic spectras. The crystalline phase gives sharp and narrow diffraction peaks whereas the amorphous component gives a broad peak (halo). The ratio between these intensities can be used to calculate the amount of crystallinity of the material.

**Densitometric Analysis**
Dual Energy X-ray Absorptiometry (DEXA) is a noninvasive and *in-vivo* method of measuring bone mineral density (BMD) and bone mineral content (BMC). Measurement of BMD and BMC in rat models has been accomplished using instruments designed for clinical and animal use. Validation studies of such instruments have indicated that DEXA is a useful measuring technique for the total body and regional bone BMC and BMD (such as the spine and hind limbs) [201–209]). DEXA has been used for measuring changes in BMC and BMD that are associated with a variety of experimental
manipulations such as ovariectomy, immobilization, and treatment with growth hormone and insulin like growth factor-I (IGF-1).[207–211]

The DEXA machine sends a thin, invisible beam of low-dose x-rays through bones via two energy streams. DEXA is an energy switching system where X-ray potential is rapidly switched between two energies, 43 and 110 Kev (70 and 140 KV respectively), synchronized with line frequency and resulting in 8.33-ms pulses in 60 Hz system and 10 ms pulses in 50 Hz system. It relies on two distinct energy peaks: one peak is absorbed mainly by soft tissue and the other by bone. The soft tissue amount can be subtracted from the total, and what remains is a bone mineral density.

DEXA analysis was conducted with (Hologic QDR-4500A, walthom, MA) at Apollo hospital, New Delhi and later in Delhi Osteoporosis Foundation. To facilitate the analysis, rats were anaesthetized with Phenobarbitone Sodium (IP) and were fixed on a hard transparent board in supine position with the help of surgical tape and placed on the bed of instrument. After the whole body scanning, region of interest (ROI in cm²) were selected and Bone Mineral Content (BMC) as well as Bone Mineral Density (BMD) were obtained. This procedure is painless and radiation exposure is minimal. BMD (gm/cm²) and BMC (gm) of femur and tibia were estimated in all groups of rats.

Histological Analysis

**SEM** (Scanning Electron Microscopy)

Femur and tibia of each group were dissected and removed all soft tissues. Transverse sections of 0.5 cm thickness of femur and tibia were made in each bone sample (CONTROL, OVX and OVX+PEMF). They were fixed in 2% Glutaraldehyde. Before doing any SEM characterization, the bone samples were dried and mounted on circular disc stubs with adhesive. Gold/Carbon coatings were applied at a thickness of about 20 nanometers, which is too thin to interfere with dimensions of surface features. Coating was done with the help of sputter coater. The samples were placed in a small vacuum chamber. After introducing argon gas in the chamber, electric field was applied to cause removal of electron from the argon atoms and make them positively charged. The Ar ions were then attracted to a negatively charged piece of coated material. The Ar ions were stricken with gold or carbon atoms from the surface of the foil. These gold atoms now settled onto the surface of the sample, produced a gold or carbon coat. SEM images were obtained on 'low vacuum SEM' **Leo 435 VP** (Cambridge, England) at National Facilities
of Electron Microscopy, AIIMS, New Delhi. The scale present in the SEM images was used to measure the cortical thickness of bone.

Histo-Chemistry:

**Bone Sample Preparation**

Wistar rats were sacrificed by cervical dislocation after mild anesthesia. Femur and tibia were dissected out with all soft tissues, and fixed with PLP fixative (2% paraformaldehyde containing 0.075 M lysine and 0.01 M sodium periodate solution, pH 7.4) and stored at 4° C for 24 hrs.

**Decalcification of Bone Samples**

After fixation, the samples were washed for 12 hrs at 4° C in each of the following series of 0.01 M PBS containing different concentration of glycerol (5%, 10% and 15%). The samples were then decalcified in 10% formic acid. However, it acts more slowly than mineral acid and faster than EDTA. Solution was taken 20 times of bone volume and replaced every alternate day till the progression of decalcification was completed. Using this protocol, rat bones were fully decalcified within 6-8 days. Decalcified Tissues were then washed to remove formic acid. They were washed at 4°C for 12 hrs in successive washes of 15% sucrose and 15% glycerol in PBS, 20% sucrose and 10% glycerol in PBS, 20% sucrose and 5% glycerol in PBS, 20% sucrose in PBS, 10% sucrose in PBS, 5% sucrose in PBS, and 100% PBS.

**Dehydration and Paraffin Embedding**

Femur and tibia were cut transversely in 3 parts: Proximal, Distal and Diaphysal. These bone samples were dehydrated in graded ethanols, cleared in chloroform, and embedded in paraffin block. Blocks were trimmed down to the tissue surface. The blocks were then placed in a microtome and 5 μm transverse sections were obtained from all the three parts (proximal, distal and diaphysal). Tissue sections were then floated in a water bath at 48° C and collected on glass slides pre-coated with poly-l-lysine. Sections were stained with Hematoxylin and Eosin (HE) for bone marrow cells, and Históchemical stain for collagen and Immuno-histochemical stain for alkaline phosphatase (ALP). Hematoxylin and Eosin (HE)
This is the standard reference stain used in the study of tissue pathology. After deparaffinizing, sections were immersed in the filtered Hematoxylin for 3 minute and then rinsed with running water until the water became clear. Sections were again immersed in Eosin stain for 1-2 minutes and rinsed until water became clear. Thereafter they were dehydrated in ascending graded alcohol solutions (50%, 70%, 80%, 95% X 2, 100% X 2 times) and cleared with xylene (3 times).

**Histochemistry of Collagen**
Decalcified paraffin bone sections were dewaxed and stained with Picro Sirius Red (a solution of 1 % Sirius Red or Direct Red 80 in saturated aqueous solution of picric acid). After that they were washed twice with of acidified water. Water was physically removed from the slides by blotting with filter paper and air drier. Then they were again dehydrated in three changes of 100% ethanol and cleared in xylene and mounted in a resinous medium. These slides were observed under bright-field microscopy.

**Immuno-Histochemical Demonstration of ALP Activity:**
ALP histochemistry was performed by a modified method of Yoshiki et al. [212]. The bone tissue sections were deparaffinized, hydrated through a xylene and graded alcohol series. Then they were preincubated overnight in 1% magnesium chloride in 100 mM Trismaleate buffer (pH 9.2). Thereafter they were again incubated for 2 hrs at room temperature with ALP substrate solution (freshly prepared 100 mM Tris-maleate buffer, pH 9.2, containing 0.2 mg/ml naphthol AS-MX phosphate and 0.4 mg/ml Fast Red TR). After washing with distilled water, the sections were counterstained with methyl green nuclear counter stain (0.5% Methyl Green Solution made in 0.1M Sodium Acetate Buffer at pH 4.2) and mounted with glycerol jelly.

**Biochemical Analysis**

**Collagen I Estimation in Bone Samples**
Bone samples were decalcified with EDTAG (0.5 M EDTA in 0.05 M Tris/HCl) and homogenized in 2 ml 0.05 M Tris/HCl with proteinase inhibitor. Homogenized samples were lyophilized in vacuum drier for 8 hrs. Lyophilized samples were extracted with 2 ml of 0.5 M acetic acid for 48 hrs. The acetic acid soluble collagen was separated by centrifugation at 15000g for 45 min. Supernatant of centrifuged material was transferred
in a microcentrifuge tube and the residue was subjected to subsequent digestion with pepsin (1 mg of pepsin/10 mg of lyophilized bone) in 2 ml of 0.5 M acetic acid (pH 2.0) for again 48 hrs. The pepsin-soluble collagens were separated by centrifugation at 15000g for 45 min. The pepsin-insoluble bone matrix was extracted with 0.05 M Tris/HCl (pH 7.5), containing 4.0 M guanidine hydrochloride for 24 hrs. All three separated supernatant were then fractionated by differential salt precipitation. Collagen type I molecules were precipitated with NaCl added to a final concentration of 2.6 M, pH 7.4, by stirring for 24 hrs and centrifuged at 15000g for 45 min. Collected collagen precipitates were dissolved in 2ml of 0.5 M acetic acid to make test sample stock solution. After preparation of sample stock solution, collagen type I standards were made with purified type I collagen from rat tail tendon (Sigma). 100 ml of 1 % Sirius Red (Direct Red 80) solution was prepared in 0.5 M acetic acid and was preserved for standard solution and test sample preparation.

<table>
<thead>
<tr>
<th>Collagen Type I standard</th>
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<td>Final ppm Desired</td>
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<td>20 ppm</td>
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<td>40 ppm</td>
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<tr>
<td>50 ppm</td>
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<table>
<thead>
<tr>
<th>Test Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amt. of extracted sample supernatant (µl)</td>
</tr>
<tr>
<td>200</td>
</tr>
</tbody>
</table>

The calibration graph was prepared using aliquots of collagen I standard solution. The concentration of collagen I was calculated in test samples with the help of equation obtained by the calibrated graph ($R^2=0.9973$). The sum of acid-soluble and enzyme-soluble collagen was defined as total collagen.

*ALP Activity Estimation in Bone Samples*
Phosphatases are enzymes which catalyse the splitting of a phosphate from monophosphoric esters. Alkaline phosphatase (ALP), a mixture of isoenzymes from liver, bone, intestine and placenta, has maximum enzyme activity at about pH 10.5. ALP measurements are of particular interest in the investigation of bone diseases. Paranitrophenyl phosphate, which is colourless, is hydrolysed by alkaline phosphatase at pH 10.5 and 37°C to form free paranitrophenol, which is yellow coloured. The addition of NaOH stops the enzyme activity and the final colour shows maximum absorbance at 410 nm. Process is same as in serum, but for bone ALP estimation a homogenate was prepared for the assay.

Femur and Tibia were removed and cleaned of all adherent soft tissues, stored at -20°C until assayed. Samples of the bone (Femur and Tibia) were washed with physiological saline (0.9% NaCl) and blotted on filter paper. Each bone was homogenized in 2 ml of glycine buffer (pH 10.4) using a high-performance homogenizer. Homogenized samples were centrifuged for 20 min at 8000g and 4°C. The activity of ALP in supernatant of the femur and tibia were determined using the same kit as for the total ALP in serum.

**Working solution:** Substrate powder (Disodium Paranitrophenyl phosphate) of each vial was dissolved in 10 ml buffer (ready to use and was supplied with kit). Solution was colourless and stored in brown glass bottle at 2-8°C. These working solutions were stable for 45 days.

1 ml of working solution and 200 µl of sample supernatant were taken in a microcentrifuge tube. Before proceeding further, temperature controlled spectrophotometer (Cary) was calibrated at 405 nm wavelength to zero with the blank. Absorbance reading was done at 1 minute time interval (0 min, 1 min, 2 min, 3 min, 4 min) at 37°C. ALP activity in IU/L is liberated milli moles of PNP per minute at 37°C incubation per liter sample. \( A_0 \) (absorbance at 0 min), \( A_1 \) (absorbance at 1 min), \( A_2 \) (absorbance at 2 min), \( A_3 \) (absorbance at 3 min), \( A_4 \) (absorbance at 4 min) were read against blank (distill water) and average change in absorbance per minute (Abs/min) was determined.

Calculation of PNP per minute in m.mol/L or ALP activity in IU/L in the test sample
\[ A = \frac{A_{\text{abs}}}{\text{min}} \times 275.7 \times tf \]

(Where the \(tf\) is temperature factor at 37°C, is equal to 1 and multiplication factor is 2757 when 20 μl sample is taken. But in this study 200 μl sample was taken, so multiplication factor in this case was 275.7)

**Test for Genotoxic Activity (DNA strand breaks):**

Proximal and distal end of Femur and Tibia were cut off and the bone marrow cells were flushed by injecting 1 ml PBS (PBS free from Ca\(^{++}\) and Mg\(^{++}\) was prepared and maintained at the pH 7.4, contained 10% DMSO and 20 mM EDTA) through diaphysis with the help of syringe. Flushed sample was collected in micro centrifuge tube and single cell suspension of bone marrow was made by mixing it vigorously with the help of pipette. 10 μl of sample was aliquoted in microcentrifuge tube. The sample was mixed with 90 μl of 0.5% LMPA in PBS and loaded on a precoated slide (first layer was of 80 μl 1% NMA in PBS). After loading the sample third layer of 80 μl 1% LMPA made and kept at 4°C for hardening. After hardening of LMPA, slides were dipped in Lysing solution (Lysing solution was prepared by mixing of 146.1 gm of NaCl, 37.2 gm of EDTA, 1.2 gm of Trizma base and 8 gm of NaOH in 1 liter dH\(_2\)O and adjusted at pH 10. Then 1% Triton X-100 and 10% DMSO was added just half an hour before use.) and left wet overnight. Slides were placed in electrophoresis chamber after lysing, chamber was filled with alkaline buffer (Electrophoresis buffer prepared by mixing of 30 ml 10 N NaOH and 5 ml of 200 mM EDTA in dH\(_2\)O and made final one liter volume). Power was not turned on for 30 min to allow unwinding of DNA. Thereafter power (24V and 300 mA) was turned on for 30 min. After electrophoresis, slides were lifted gently from the chamber and placed in washing tray. Slides were neutralized with neutralizing buffer (was prepared by mixing of 48.5 gm of Tris to dH\(_2\)O and maintained pH at 7.5) for 10 min and this step was repeated for 3 times. Slides were then stained with 80 μl of staining solution Ethidium Bromide (2μg/ml) and left for 5 min and overstaining was removed. Coverslip was then placed over and were observed in Reichert vertical fluorescent microscope equipped with a filter combination for fluorescent dye.