MATERIALS
AND
METHODS
Collection and storage of animals

The experimental animals snails *Lymnaea acuminata* and *Indoplanorbis exustus* were collected from the ponds and pools of Banda district, where they are found in the much more quantity. These species of the snails were found attached to the under surface of the leaves or moving around the green vegetation near the banks; they are also be found in the floating surface of the water.

The collected animals were stored in glass aquaria containing dechlorinated tap water for acclimatization to laboratory condition. Care was taken to remove the dead animals from the aquaria to avoid contamination of water. The size of adult *Lymnaea acuminata* (2.25 ± 0.2 cm in shell height) and *Indoplanorbis exustus* (0.8 ± 1.2 cm in shell height) were used for the experiment.

Collection of Plant material

The plant of the family Euphorbiaceae viz- *Euphorbia thymifolia*, *Euphorbia neriifolia* and *Euphorbia nivulia* were
collected locally from different places of Banda district. The white latex were obtained from these plants from their natural habitat. A short description of these three Euphorbiales are as follows:-

1. *Euphorbia thymifolia* Linn.

A small, more or less pubescent, much branched, prostrate, annual with opposite, oblong leaves, hairy capsules and quadrangular, wrinkled seeds. It occurs throughout India in plains and low hills, ascending to 5,500 ft. in Kashmir.

The dried leaves and seeds of the plant are slightly aromatic. They are considered stimulant, astringent, anthelmintic and laxative and given to children in bowel complaints. The plant juice is employed in south India as a cure for ringworm.

*Euphorbia thymifolia* yields a green essential oil with a peculiar pungent odour and irritating taste. Among its constituents, cymol, carvacrol, limonene, 2 sesquiterpenes and salicylic acid have been identified. The oil is used in medicinal soaps for the treatment of erysipelas, as a spray to keep off flies and mosquitoes, and as a vermifuge for dogs and farm foxes. The stem and leaves contain 5,7, 4-trihydroxy flavone-7-glycoside.

2. *Euphorbia neriifolia* Linn.

A large succulent shrub or a small tree, up to 20 ft. high, with jointed, cylindrical or obscurely 5-angled branches bearing
short, stipular thorns, more or less confluent in vertical or slightly spiral lines; leaves fleshy, deciduous, obovate-oblong, 6-12 in. long, terminal on the branches. The trunk is covered with reticulate bark. The plant closely resembles *Euphorbia nivulia* but can be distinguished from it by the position of the thorns, which in this species grow on warty knobs, while in the latter, they are borne on flat corky patches. The plant is common in rocky ground throughout the Deccan Peninsula and is often cultivated for hedges in villages throughout India.

The latex is acrid, rubefacient, purgative and expectorant. It is liable to cause dermatitis. It is used to remove warts and cutaneous eruptions. The juice is employed in ear-ache; mixed with soot it is applied in ophthalmia.

The latex contains; water and water-solubles, caoutchouc, 0.2-2.6%.


A shrub or a small tree, up to 30 ft. high, with green, cylindrical, joited, often whorled branches armed with spines; leaves fleshy, linear-oblanceolate or spatulate, up to 9 in. long; old plants bear a thick corky bark. The plant is found in the dry and rocky regions practically throughout India and is often grown for hedges.

The juice of the leaves is given as a purgative and diuretic. It is said to be used for relieving ear-ache. Mixed with neem oil it
is applied externally in rheumatis. The latex is liable to cause dermatitis.

The latex contains, water and water-solubles, caoutchouc, 0.7-1.1%.

Preparation of aqueous extract of latex

The white latex from these plants was drained in glass tubes by cutting their stem apices, this latex was lyophilized at 40°C and lyophilized powder was stored for further use. The freeze-dried powder was mixed with appropriate volume of distilled water to obtain the desired concentration. The wet weight of 1 ml latex of *Euphoriba thymifolia*, *Euphorbia neriifolia* and *Euphoria nivulia* were 810 mg, 805 mg and 800 mg respectively and dry weight was 305 mg, 307 mg and 300 mg respectively.

The mortality caused by latex of these plants of family Euphoribaceae was studied. Toxicity of aqueous extract of latex, of all the three euphorbiales against fresh water snails *Lymnaea acuminata* and *Indoplanorbis exustus* was studied.

Toxicity studies

Treatment of animals: Toxicity experiments were performed by the method of Singh and Agarwal (1988). Ten experimental animals *Lymnaea acuminata* or *Indoplanorbis exustus* were kept in glass aquaria containing 3 L of dechlorinated tap water for
24h, 48h, 72h or 96h. These were exposed to four different concentration of latex.

The toxic effects of the latex of the three plants were studied. In case of snails the contraction of the body within the shell and no response to a needle probe were taken as evidence of death. Dead animals were removed as soon as possible in order to prevent the decomposition of body in the aquarium, which was observed to cause rapid death in the remaining population. Snails mortality was recorded daily during the observation period of 96h at four different concentration.

Lethal concentration (LC_{10,50,90}) value, slope function, upper and lower confidence limits of the latex, of all the three plants to both the species of snails were calculated for all exposure periods. The values were calculated by probit log method of Russell et al., (1977).

Biochemical Estimation

**Treatment of snails for biochemical studies:** Adult snails were collected from fresh water ponds and stored in glass aquaria containing dechlorinated tap water. Group of 50 snails in dechlorinated tap were exposed to sublethal concentration (40% and 80% of 24h and 96h LC_{50}) of latex of different plants. Dead snails were removed as soon as possible from the aquaria in order to prevent the contamination of water. Treated snails were taken out from the glass aquaria every 24h and rinsed with water. The
control snails were not exposed to any treatment. Since *Lymnaea acuminata* is an active grazer the possibility existed that animals would not be able to eat during the treatment period because of immobility or incapacitation of redula musculature. No food was therefore given to the either the control or experimental snails.

In order to study the effect of withdrawal from treatment, the snails were first exposed to sub-lethal doses for 96h, following which these were transferred to freshwater. This water was changed every 24h for the next seven days, after which biochemical parameters were estimated in different tissues.

**Total protein**

Protein levels in the hepatopancreas and nervous tissues of snails *Lymnaea acuminata* was estimated according to Lowry et al., (1951). Animals were dissected, their hepatopancreas and nervous removed, weighed and homogenates (5mg/ml, w/v) were prepared in 10% trichloroacetic acid (TCA). Tissues were homogenized for 20 min. in an electrical homogenizer and centrifuged at 6000g for 20 min. The precipitate was washed with 5.0 ml of 5% TCA and centrifuged again at the same speed for another 20 min. The precipitate was dissolved in 4.0 ml of 1N NaOH. In 1.0 ml of the diluted supernatant 5.0 ml of freshly prepared alkaline copper solution (reagent C) was added. Reagent C was prepared by addition of 50.0 ml of reagent A (2% sodium potassium taratrate, 1% copper sulphate, mixed in 1:1 ratio at the time of the experiment). The reaction mixture was kept for
10 min, at room temperature following which 0.5 ml of FolinCiocalteu reagent (diluted 1:2 ratio with distilled water at the time of the experiment) was added and mixed thoroughly. Ten minutes after this the blue colour developed was measured at 600 nm. Standard curves were prepared with different concentration of bovine serum albumin. Values have been expressed as μg protein/mg hepatopancreas and nervous tissues.

**Total free amino acids**

Estimation of total free amino acid in the hepatopancreas and nervous tissue of *Lymanea acuminata* was made according to the method of Spices (1957). The hepatopancreas and nervous tissues were homogenized in 96% ethanol (10:1 w/v) in an electrical tissues homogenizer for 5 min and centrifuged at 8000 g for 20 min. In 0.1 ml of the supernatant, 0.1 ml of distilled water and 2.0 ml of ninhydrine reagent was added and mixed thoroughly. Ninhydrin reagent was prepared by mixing 1.0 g ninhydrin in 25.0 ml of absolute ethanol and 0.04 g of stannous chloride in 25.0 ml of citrate buffer pH 5.0. The reaction mixture was kept in boiling water bath for exactly 15 min 2.0 ml of 50% ethanol was added to the above after cooling. A violet colour developed which was measured at 575 nm. Standard curves using the same procedure were drawn with known amounts of glycine. Free amino acids have been expressed as μg/gm hepatopancreas and nervous tissue.
Glycogen was measured by the Anthrone method of Van der Vies (1954). Glycogen, present in submicroscopic form in tissue yields anamorphous product on reacting with KOH. This produces a green brown colour with anthrone reagent. 50 mg tissue was homogenized with 5 ml of TCA and filterated. To 1.0 ml of filterate, 1.0 ml of 10 N KOH was added and mixture was boiled for exactly 60 min. Excess alkali was neutralized with 0.5 ml volume of glacial acetic acid and distilled water added to make a fined volume of 10 ml. 1.0 ml of the above mixture was added to 2 ml of freshly prepared anthrone reagent (2 mg anthron/ml of 36 NH₂SO₄), shaken laterally and heated in a boiling water bath for 10 min. A green brown colour developed which was measured calorimetrically at 650 nm against blank, prepared simultaneously by using 1.0 ml of 5% TCA instead of 1.0 ml of tissue filterate. The optical density was compared with a set of glucose standard of varying concentrations (0.01 mg/ml to 0.04 mg/ml.). Appropriate calculations were made to complete glycogen.

Results have been expressed as mg/g fresh tissue.

Nucleic Acids

DNA and RNA in the ovotestis of Lymnaea acuminata were estimated according to Schneider (1957) using diphenyl amine and orcinol reagent respectively. Homogenates (1 mg/ml, w/v) were prepared in 5% TCA at 90° c centrifuged at 5000g for 20 min and supernatant used for estimation.
DNA

In 1.0 ml of supernatant, 1.0 ml of distilled water and 4.0 ml of freshly prepared diphenyl amine reagent (1 g diphenyl amine, 100 ml glacial acetic acid and 2.5 ml conc. H₂SO₄) was added. The reaction mixture was kept measured at 600 nm. Standard curves were drawn using different concentration of calf thymus DNA as standard.

RNA

In 1.0 ml of supernatant, 2.0 ml of distilled water and 3.0 ml of orcinal reagent (1 g orcinal, 100 ml conc. 0.5 ferric chloride) was added. The incubation mixture was kept in boiling water bath fro 20 min. The grenish blue colour which developed was measured at 660 nm standard curves were drawn using yeast RNA standard.

Both RNA and DNA have been experssed as g/mg tissue.

Statistical analysis

Result have been expressed as mean ± SE of six replicates. Student’s ’t’ test was applied between control and treated groups to locate significant (P<0.05) variation regression coefficient was determined between exposure time and LC 50 values (Sokal and Rohlf, 1973).
Cytogenetic toxicity (Genotoxicity)

For this study following procedure was adopted, and the experiments were performed in zoology department of D.D.U. Gorakhpur University, Gorakhpur.

Experimental animal

Four-6 week- old laboratory- bred Swiss albino mice Mus musculus (S-cdri, 2n=40, seed colony obtained form Central Drug Research Institute, Lucknow, India) of body weight (bw) 22 ±3g, matched for their sex, were used. There was a total of five groups of experimental animals, each comprising six mice (three males and three females). All the experimental animals were housed in stainless steel cages in a room maintained at 25 ± 2 °C with 12 hr day/ night cycle. "Gulmuhar" (Hindustan Lever, Bombay, India) diet was the basal food for all the experimental animals. Drinking water was made available ad libitum.

Treatment protocol

1. Treated group

Each time freshly prepared water soluble latex extract was administered to the animals (treated group) at the rate of (@) 0.5g (T₁), 1.0 g (T₂) or 2.0 g (T₃)/kg body weight (bw)/ day by dissolving the extract in 0.4 ml of glass double distilled (gdd) water. These doses are in fact equivalent to quarter, half and full maximum tolerated doses, respectively (Bateman, 1984). The treatment was given once daily through oral gavage (by
intubation) for seven consecutive days, and the duration from harvest of latex to the treatment of extract of *Euphorbia thymifolia*, *Euphorbia neriifolia* and *Euphorbia nivulia* were about 15 days.

2. Control group

1. Solvent control

The mice of the group were given 0.4 ml gdd water per day. Which is the amount equal to that used for dissolving the extract.

2. Positive control

The mice of this positive control group were dosed with aflatoxin-infested bread (Sinha and Dharmshila, 1994) that had 50 ±10 ppb of AFB₁, a well-established biomutagen of natural origin, elaborated as a secondary metabolite by the toxigenic strain (BG-185) of *Aspergillus flavus* (ascomycetes fungus). The amount of aflatoxin B₁ thus administered was 0.05 ug/kg/bw/day.

Experimental protocol

The experimental animals were injected colchicine (Sisco Laboratory, India) intraperitoneally (i.p.) @ 4 mg/kg bw, 24hr after the last treatment and 90 min prior to sacrifice by cervical dislocation. Bone marrow from both the disseeted-out femora was flushed in hypotonic KCl (0.075 m) solution and the slides were prepared by standard, fixative (1:3 aceto-methanol)- flame drying-Giemsa (1.20) staining technique (Preston et al., 1987).
About 600 well-spread metaphase plates (@ about 100 plates/animal) from each group were screened randomly from double-coded slides for detecting chromosome-related abnormalities, if any. The abnormalities detected were put either into two categories, viz., structural changes consisting of chromatid breaks (ct) and gaps (cg)- the later being lesion (s) in one or both the helices of DNA which failed to reconstitute and appeared as unstained attenuated regions having a diameter less than one chromatid, acentric fragment (af), centric fusion (cf) and stickiness (st) and mitosis-distruptive changes such as aneuploidy (a gain, hyperploidy or loss, hypoploidy of one or more chromosomes), polyploidy, c-mitosis and precocious separation.

Statistical analysis

The data from all the animals in each group were pooled together and the Equality of Proportion Test (z-test), using p<0.05 as the minimum acceptable criterion (level) of significance, was performed for statistical evaluation (Downie and Health, 1970).